USP15 stabilizes MDM2 to mediate cancer-cell survival and inhibit antitumor T cell responses

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Deubiquitinases (DUBs) are a new class of drug targets, although the physiological function of only few DUBs has been characterized. Here we identified the DUB USP15 as a crucial negative regulator of T cell activation. USP15 stabilized the E3 ubiquitin ligase MDM2, which in turn negatively regulated T cell activation by targeting the degradation of the transcription factor NFATc2. USP15 deficiency promoted T cell activation in vitro and enhanced T cell responses to bacterial infection and tumor challenge in vivo. USP15 also stabilized MDM2 in cancer cells and regulated p53 function and cancer-cell survival. Our results suggest that inhibition of USP15 may both induce tumor cell apoptosis and boost antitumor T cell responses.

Ubiquitination is an important protein modification that regulates diverse biological and pathological processes, including immune responses and oncogenesis1,2. A major function of ubiquitination is to target proteins to the proteasome for degradation, but many nondegradative functions have also been characterized3,4. Ubiquitin conjugation is mediated by the sequential action of three enzymes, E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzymes and E3 ubiquitin ligases5. Different types of polyubiquitin chains can be formed through connection of the C-terminal glycine of a ubiquitin to any one of the seven internal lysine residues of the preceding ubiquitin5. Lys48 (K48)-linked polyubiquitin chains are the best-studied ubiquitin chains that target proteins for proteasomal degradation, whereas other ubiquitin chains, such as the K63-linked polyubiquitin chains, can mediate nondegradative functions4. The specificity of protein ubiquitination is regulated by the E3 ubiquitin ligases, which exist in large numbers in mammalian cells and mediate substrate recognition3,6.

The E3 ubiquitin ligase MDM2 has been extensively studied as an oncogene product that mediates the ubiquitin-dependent degradation and functional inactivation of the tumor suppressor p53 (refs. 7,8). Elevated MDM2 protein expression has been documented in a large variety of human cancers and is thought to result from gene amplification as well as transcriptional and post-translational regulation. Although the best-characterized target of MDM2 is p53, MDM2 has additional substrates5. Small-molecule inhibitors of MDM2, which block MDM2-p53 interaction or inhibit the ubiquitin ligase activity of MDM2, have been actively investigated in preclinical studies and clinical trials for the treatment of cancer8,9. Although MDM2 has been extensively studied in cancer cells, its physiological role is relatively poorly understood. In particular, how MDM2 regulates immune responses is largely unclear.

Ubiquitination is a reversible reaction because the ubiquitin chains can be cleaved by a large family of ubiquitin-specific proteases, termed DUBs10,11. DUBs can have specificity for both protein substrates and ubiquitin chains and, thereby, enable multiple ways to regulate protein ubiquitination. Like the E3 ubiquitin ligases, DUBs regulate diverse biological processes and are a class of drug targets12–14. However, the biological function of only a small proportion of DUBs has so far been characterized using physiological models, which hampers the development of DUB-based therapies. USP15 is a DUB that belongs to the ubiquitin-specific protease family15. In vitro studies have implicated USP15 in the regulation of signaling by TGF-β and survival of cancer cells in cell line models16–18. However, the physiological role of USP15, particularly the regulation of immune functions, has remained unclear. The molecular mechanism underlying the signaling function of USP15 is also incompletely understood.

In this work, we studied the function of USP15 using a gene-targeting approach and identified USP15 as a negative regulator of T cell activation as well as a pivotal mediator of cancer-cell survival. We present biochemical and genetic evidence that USP15 functions by stabilizing the E3 ubiquitin ligase MDM2. In both activated T cells and cancer cells, loss of USP15 caused degradation of MDM2. MDM2 targeted a T cell transcription factor, NFATc2, and negatively regulated activation of T cells. USP15 deficiency promoted T cell responses to both bacterial infections and tumor-cell challenge. In cancer cells, USP15 stabilized MDM2 and regulated p53 responses. These results suggest that targeting USP15 may both induce...
tumor-cell apoptosis and boost antitumor T cell responses, and thus have important clinical applications.

RESULTS

USP15 is a negative regulator of T cell activation

Through analyses of the BioGPS database, we found that USP15 was abundantly expressed in immune cells (data not shown). We used a gene-targeting approach to investigate the physiological function of USP15 (Supplementary Fig. 1a-d). The USP15 homozygous knockout (Usp15−/−) mice were born at expected Mendelian ratios and had comparable survival rate as the wild-type control mice (Supplementary Fig. 1e). Usp15−/− mice also did not show obvious abnormalities in thymocyte development or peripheral T cell homeostasis (Supplementary Fig. 1f–i). However, the USP15 deficiency promoted the T cell antigen receptor (TCR) and CD28–stimulated production of cytokines, such as interleukin 2 (IL-2) and interferon-γ (IFN-γ), in naive CD4+ T cells, as assessed by real-time quantitative reverse transcription–PCR (qRT-PCR; Fig. 1a), intracellular cytokine staining (ICS; Fig. 1b) and enzyme-linked immunosorbent assay (ELISA; Fig. 1c). The loss of USP15 did not reduce the threshold of T cell responses but enhanced the cytokine induction at different doses of stimulation with anti-CD3 and anti-CD28 (Fig. 1d). Induction of the effector T cell marker CD69, although not of the early activation marker CD69, was also enhanced in the USP15-deficient T cells (Fig. 1e and data not shown). To examine whether USP15 has a T cell–intrinsic function in regulating activation of T cells, we reconstituted USP15-deficient T cells with USP15 by retroviral infection of T cell–depleted Usp15−/− bone marrow cells and adoptive transfer into Rag1−/− mice (Supplementary Fig. 2a). Naive CD4+ T cells derived from the USP15-reconstituted bone marrow cells displayed reduced induction of IFN-γ (Supplementary Fig. 2b). In contrast, reconstitution of the cells with a catalytically inactive USP15 mutant, C298A19, failed to suppress the induction of IFN-γ-producing cells (Supplementary Fig. 2b). Thus, USP15 negatively regulates T cell cytokine production via its DUB function.

We next examined the role of USP15 in the regulation of CD4+ T cell differentiation by stimulating naive CD4+ T cells under Th1 cell (10 μg/ml anti-IL-4 and 10 ng/ml IL-12), Th17 cell (10 μg/ml anti-IL-4, 10 μg/ml anti-IFN-γ, 15 ng/ml IL-6 and 2.5 ng/ml TGF-β) and inducible regulatory T (iTreg) cell (10 μg/ml anti-IL-4, 10 μg/ml anti–IFN-γ and 1.5 ng/ml TGF-β) conditions. Under these standard T cell differentiation conditions, USP15-deficient T cells had slightly enhanced apoptosis compared to wild-type T cells (Supplementary Fig. 2c–e). However, in the presence of suboptimal doses (0.1 and 1 ng/ml) of IL-12 in Th1 cell differentiation conditions, USP15−/− CD4+ T cells showed enhanced Th1 cell differentiation (Fig. 1f). We also examined whether USP15-deficient naive CD4+ T cells showed altered differentiation into iTreg cells. In the presence of a low concentration (0.5 ng/ml) of TGF-β and in the absence of an IFN-γ–blocking antibody, Usp15−/− naive CD4+ T cells did not efficiently differentiate into iTreg cells; however, the USP15-deficient naive CD4+ T cells differentiated into iTreg cells normally in the presence of the IFN-γ–blocking antibody or a higher dose (1.5 ng/ml) of TGF-β (Supplementary Fig. 2f). Moreover, wild-type and Usp15−/− mice had comparable frequencies of Threg cells in peripheral lymphoid organs and thymus (Supplementary Fig. 2g).

Collectively, these results suggest that USP15 negatively regulates naive CD4+ T cell activation and Th1 cell differentiation.

USP15 inhibits T cell responses to Listeria infection

To examine the in vivo role of USP15 in the regulation of T cell responses, we used a bacterial infection model known to induce strong T cell responses, particularly IFN-γ–producing CD4+ T cells20. In response to Listeria monocytogenes infection, both wild-type...
mice and Usp15<sup>−/−</sup> mice generated IFN-γ-producing CD4<sup>+</sup> T cells specific for the listeriolysin O (LLO) antigen (Fig. 2a). However, Usp15<sup>−/−</sup> mice had a higher frequency and absolute numbers of IFN-γ-producing CD4<sup>+</sup> T cells than the wild-type mice, although the IFN-γ-median fluorescence intensity (MFI) of the IFN-γ<sup>+</sup> T cells was comparable between the two genotypes (Fig. 2a and Supplementary Fig. 3a,b). Moreover, serum concentration of IFN-γ was significantly increased in L. monocytogenes–infected Usp15<sup>−/−</sup> mice compared to wild-type mice (Fig. 2b). Consistent with their heightened T cell responses, the Usp15<sup>−/−</sup> mice had a significantly lower bacterial load in the liver (Fig. 2c).

To examine the CD4<sup>+</sup> T cell–intrinsic function of USP15, we reconstituted T cell–deficient Tcr<sup>−/−</sup>Tcrd<sup>−/−</sup> mice with wild-type or Usp15<sup>−/−</sup> naive CD4<sup>+</sup> T cells along with an equal number of wild-type CD8<sup>+</sup> T cells. We did not detect differences in the spleen size or numbers of splenic CD4<sup>+</sup> T cells between hosts reconstituted with wild-type and Usp15<sup>−/−</sup> T cells under uninfected conditions after 4 d of T cell transfer (Supplementary Fig. 3c–e). However, after L. monocytogenes infection, hosts reconstituted with Usp15<sup>−/−</sup> T cells had stronger immune responses, as indicated by a more profound spleen enlargement (Supplementary Fig. 3f), increased numbers of splenic CD4<sup>+</sup> T cells (Fig. 2d) and elevated serum interferon-γ (Fig. 2e) compared to host reconstituted with wild-type T cells. The L. monocytogenes–infected recipients of Usp15<sup>−/−</sup> T cells also had significantly (two-tailed unpaired t-test; P < 0.05) higher numbers splenic CD11b<sup>+</sup> monocytes and CD11c<sup>+</sup> dendritic cells, consistent with increased activation and recruitment of myeloid cells by T cell–derived cytokines (Supplementary Fig. 3g). In correlation with stronger immune responses, hosts reconstituted with Usp15<sup>−/−</sup> T cells had reduced bacterial load in the liver (Fig. 2f) and increased survival rate (Fig. 2g).

Because the L. monocytogenes strain used in our studies encodes chicken ovalbumin (LM-OVA), we crossed the Usp15<sup>−/−</sup> mice with OT-II mice, which express a transgenic TCR specific for an OVA peptide, to generate Usp15<sup>−/−</sup> OT-II mice and Usp15<sup>+/+</sup> OT-II littermate control mice. To test the effect of the USP15 deficiency on antigen-specific CD4<sup>+</sup> T cell responses, we adoptively transferred naive CD4<sup>+</sup> T cells isolated from Usp15<sup>−/−</sup> OT-II mice and Usp15<sup>−/−</sup> OT-II littermates into B6.SJL mice and tracked donor and host T cells based on expression of the congenic markers CD45.2 and CD45.1, respectively. In response to LM-OVA infection, recipients of Usp15<sup>−/−</sup> OT-II T cells produced a higher frequency (Fig. 2h,i) and larger absolute numbers (Fig. 2j) of OVA-specific IFN-γ–producing OT-II T cells compared to mice that received Usp15<sup>+/+</sup> OT-II T cells. Moreover, mice that received Usp15<sup>−/−</sup> OT-II T cells also had higher numbers of total splenic CD45.2<sup>+</sup> T cells compared to mice that received Usp15<sup>−/−</sup> OT-II T cells upon challenge with LM-OVA, which suggested increased expansion of the Usp15<sup>−/−</sup> OT-II T cells (Supplementary Fig. 3h). In contrast, the number of T<sub>H</sub> cells was similar in the recipients of Usp15<sup>−/−</sup> OT-II cells and Usp15<sup>−/−</sup> OT-II T cells (Supplementary Fig. 3i). Consistent with their T cell hyperactivation, mice transferred with Usp15<sup>−/−</sup> OT-II T cells had lower L. monocytogenes load in the liver, which suggested a better ability to clear the bacteria (Fig. 2k). These results suggest that USP15 is a negative regulator of T<sub>H</sub>1 cell responses.

USP15 deficiency enhances Nfatc2 activation in naive CD4<sup>+</sup> T cells T cell activation involves cascades of signaling events triggered by the TCR and CD28 (ref. 21). Upon stimulation with anti-CD3 plus anti-CD28, the Usp15<sup>−/−</sup> and wild-type naive CD4<sup>+</sup> T cells displayed similar TCR-proximal signaling events, including phosphorylation of the protein tyrosine kinases Lck and Zap70 (Supplementary Fig. 4a), and downstream signaling events, such as phosphorylation of the MAP kinase Erk, the kinase Akt or the ribosomal S6 protein (Supplementary Fig. 4b). Furthermore, although USP15 has been implicated as a mediator of TGF-β signaling in certain cancer cell lines, USP15-deficient T cells had normal TGF-β signaling, as evidenced by the comparable Smad phosphorylation, upregulation of Smad7 and downregulation of Myc in TGF-β–stimulated wild-type and Usp15<sup>−/−</sup> T cells (Supplementary Fig. 4c,d). Consistent
with that finding, wild-type and Usp15−/− T cells were both sensitive to TGF-β-mediated suppression of IL2 and Ifng mRNA induction by anti-CD3 plus anti-CD28 (Supplementary Fig. 4e-f). After stimulation with TCR and CD28, Usp15-deficient T cells showed increased nuclear expression of the transcription factor NFATc2 (Fig. 3a), which mediates the induction of T cell–specific cytokines22,23. The enhanced induction of NFATc2 nuclear expression in Usp15-deficient T cells was not inhibited by TGF-β (Supplementary Fig. 4g). Activation of NFATc2 and two major NF-kB members, c-Rel and p65, was similar in Usp15−/− and wild-type T cells after stimulation with TCR and CD28 (Fig. 3a). Overexpression of NFATc2 in EL4 T cells promoted the induction of IL-2 and IFN-γ (Supplementary Fig. 4h-j). To examine the function of NFATc2 in mediating cytokine induction in Usp15−/− naive CD4+ T cells, we used a bone marrow adoptive transfer approach. We transduced T cell–depleted Usp15−/− bone marrow cells with a green fluorescent protein (GFP)-expressing lentiviral vector encoding a control or Nfatc2-specific short hairpin RNA (shRNA) and adoptively transferred the transduced cells into Rag1−/− mice. Naive CD4+ T cells derived from bone marrow cells subjected to knockdown of NFATc2 expressed less IFN-γ and IL-2 than the naive CD4+ T cells derived from bone marrow cells subjected to knockdown using a control shRNA (Fig. 3b,c).

We tested whether the hyperactivation of NFATc2 in Usp15−/− T cells was due to enhanced nuclear translocation, expression or stabilization of NFATc2. Immunoblot (IB) assays revealed comparable cytoplasmic expression of NFATc2 in activated wild-type and Usp15−/− T cells, despite their increased nuclear expression of NFATc2 (Fig. 3a). Consistent with this result, the whole-cell level of NFATc2 was higher in activated Usp15-deficient T cells compared to activated wild-type T cells (Fig. 3d). Usp15 deficiency did not enhance induction of Nfatc2 mRNA, as revealed by a qRT-PCR assay (Supplementary Fig. 4k). These results suggested that Usp15 might regulate the stability of NFATc2. To examine this possibility, we stimulated T cells in the presence of a protein-synthesis inhibitor, cycloheximide (CHX). Treatment with CHX led to substantial loss of NFATc2 in wild-type T cells but not in the Usp15−/− T cells (Fig. 3e), which suggested that Usp15 regulates degradation of NFATc2. Blocking protein degradation with a proteasome inhibitor, MG132, resulted in accumulation of NFATc2 in wild-type T cells, similar to that observed in Usp15-deficient T cells (Fig. 3f). We also observed abundant ubiquitination of NFATc2 in activated wild-type T cells after treatment with MG132, but not in activated Usp15−/− T cells (Fig. 3g). Ubiquitination of NFATc2 was very low in both wild-type and the Usp15−/− T cells under resting conditions, and we could only detect it after extended immunoblot exposure (Fig. 3g and data not shown). These data suggest that Usp15 promotes the ubiquitination and degradation of activated NFATc2. Because NFATc2 is critical for mediating induction of T cell cytokines, including IL-2 and IFN-γ22,23, these results provide a mechanistic insight for the increased production of cytokines in Usp15−/− T cells.

**USP15 regulates the stability of the E3 ubiquitin ligase MDM2**

Because Usp15 is a DUB, we investigated whether Usp15 acts through regulating an E3 ubiquitin ligase of NFATc2. The E3 ubiquitin ligase MDM2 is known to induce NFATc2 ubiquitination in a breast cancer cell line24. Overexpression of MDM2 induced strong ubiquitination of NFATc2 in HEK293 cells (Fig. 4a). To assess the role of MDM2 in T cells, we examined whether T cell activation altered MDM2 expression and whether MDM2 is regulated by Usp15. Stimulation of naive CD4+ T cells by anti-CD3 plus anti-CD28 triggered induction of Mdm2 mRNA, which was similar in Usp15-deficient and wild-type T cells (Fig. 4b). Whereas stimulation with TCR and CD28 induced a transient loss of MDM2 protein in wild-type naive CD4+ T cells, this effect was enhanced and prolonged in Usp15−/− naive CD4+ T cells (Fig. 4c). Proteasome inhibitor MG132 prevented loss of MDM2 (Fig. 4d) and caused accumulation of ubiquitinated MDM2 with K48-linked polyubiquitin chains in Usp15−/− naive CD4+ T cells (Fig. 4e). These results suggest that Usp15 prevents the ubiquitin-dependent degradation of MDM2 in activated T cells.
We next examined whether USP15 directly targets MDM2. When we coexpressed USP15 and MDM2 in HEK293 cells, they formed a stable complex, as detected by co-immunoprecipitation (co-IP) assays (Fig. 4f). USP15 contains several structural domains: a large catalytic domain that covers about two-thirds of the molecule, a domain present in ubiquitin-specific protease (DUSP) and two ubiquitin-like (Ubl) domains, one of which is located within the catalytic domain55 (Fig. 4f). Domain-mapping studies revealed that two USP15 C-terminal truncation mutants, USP15 amino acids 1–941 and 1–583, lacking the catalytic activity of USP15 (C269A) isolated from transfected HEK293 cells (right). Ubiquitinated MDM2 was detected by IB using anti-MDM2 (Fig. 4f). A parallel HEK293 transfection experiment revealed that USP15 was important for its interaction with MDM2. Endogenous USP15 and MDM2 also formed a stable complex in naive CD4+ T cells, and this molecular interaction was not influenced by the TCR and CD28 signals (Fig. 4g). We also detected an USP15-DM2 association in p53-deficient T cells (Fig. 4h). Both USP15 and MDM2 were predominantly located in the cytoplasm of naive CD4+ T cells, where they formed a complex (Supplementary Fig. 5a). MDM2 was located in the cytoplasm in Usp15−/− T cells as well (Supplementary Fig. 5b). When we examined the subcellular localization of p53, this protein was barely detectable by IB in untreated naive CD4+ T cells (data not shown). However, we detected it in the cytoplasm and, to a lesser extent, in the nucleus, after treatment with MG132 (Supplementary Fig. 5c). Stimulation with TCR and CD28 enhanced the amount of nuclear p53. These results suggest that USP15 physically interacts with MDM2 in a p53-independent manner.

Considering the USP15-DM2 direct association and the role of USP15 in stabilizing MDM2 and inhibiting MDM2 ubiquitination in activated T cells (Fig. 4c–e), we examined whether USP15 directly deubiquitinates MDM2. MDM2 was abundantly ubiquitinated when overexpressed in HEK293 cells, likely through autoubiquitination (Fig. 4i). When we expressed MDM2 together with wild-type USP15, but not with the catalytically inactive USP15(C298A) variant, ubiquitination of MDM2 was potently inhibited (Fig. 4i). A parallel HEK293 transfection experiment revealed that USP15 did not inhibit the ubiquitination of NFATc2 (Supplementary Fig. 5d). In addition, USP15 directly deubiquitinates MDM2 in vitro, and this required the catalytic activity of USP15 (Fig. 4i). Thus, USP15 appears to be a deubiquitinase of MDM2 that prevents ubiquitin-dependent degradation of MDM2.

**MDM2 negatively regulates NFATc2 and T cell activation**

Because MDM2 induced NFATc2 ubiquitination in transfected cells (Fig. 4a), we tested whether endogenous MDM2 regulated the activation or the expression of NFATc2. We found that activation of wild-type T cells in the presence of a small-molecule inhibitor (HLI373), which blocks the ubiquitin ligase activity of MDM2 (ref. 26), enhanced the accumulation of NFATc2 in the nucleus (Fig. 5a). The MDM2 inhibitor did not affect the activation of several other transcription factors. Furthermore, HLI373 increased the amount of NFATc2 in the cell (Fig. 5b) and caused hyperinduction of IL-2 and IFN-γ in wild-type T cells (Fig. 5c). In contrast, HLI373 did not appreciably enhance NFATc2
activation or cytokine induction in Usp15−/− T cells (Supplementary Fig. 5e,f), which suggested that the USP15 deficiency caused T cell hyperactivation via downregulation of MDM2 (Fig. 4c).

To directly examine whether the lower MDM2 expression in USP15-deficient T cells contributed to the enhanced activation of NFATc2 and induction of cytokines, we used bone marrow adoptive transfer to express MDM2 in Usp15−/− T cells. We transduced T cell–depleted Usp15−/− bone marrow cells with a GFP-expressing retroviral vector or a vector encoding MDM2 and adoptively transferred the cells into Rag1−/− mice. Expression of the exogenous MDM2 substantially reduced TCR and CD28–stimulated nuclear accumulation of NFATc2 and production of IFN-γ (Supplementary Fig. 5g–i). Using the same approach, we found that reconstitution of Usp15−/− naïve CD4+ T cells with wild-type USP15, but not its variant with a C298A substitution, prevented TCR and CD28–induced loss of MDM2 and reduced activation of NFATc2 (Supplementary Fig. 5i), which emphasized a cell-intrinsic role for USP15 in regulating MDM2 and NFATc2.

Although MDM2 is known as an inhibitor of p53 (ref. 27), MDM2 deficiency promoted NFATc2 accumulation and cytokine induction even in p53-deficient T cells, which suggested that the role of MDM2 in T cell regulation was independent of p53 (Fig. 5d–f). Another MDM2 inhibitor, Nutlin3, known to block the MDM2-p53 interaction without inhibiting the overall ubiquitin ligase activity of MDM2 (refs. 7,28) did not promote the activation of NFATc2 or the induction of cytokines in wild-type naïve CD4+ T cells, although Nutlin-3 did enhance the induction of the p53 target gene Cdkn1a (Supplementary Fig. 5k,l). These results suggest that MDM2 negatively regulates NFATc2 activation and cytokine induction in naïve CD4+ T cells in a p53-independent manner.

We also noticed that wild-type CD8+ T cells had markedly lower expression of MDM2 than CD4+ T cells, which was due to reduced Mdm2 mRNA expression (Supplementary Fig. 6a,b). Consistently, unlike the effect seen in naïve CD4+ T cells, USP15 deficiency did not appreciably affect activation of NFATc2 or production of cytokines in naïve CD8+ T cells (Supplementary Fig. 6c–e), which emphasizes the role of MDM2 in the negative regulation of NFATc2 activation and cytokine induction in T cells. Together, these results suggest that MDM2 functions as a negative regulator of NFATc2 in naïve CD4+ T cells.

USP15 regulates MDM2 in cancer cells

USP15 is overexpressed in cancer cell lines, although its role in MDM2 regulation in these malignant cells has not been investigated. We found that USP15 was abundantly expressed in most of the melanoma and colorectal cancer cell lines analyzed (Supplementary Fig. 7a). We examined the role of USP15 in the regulation of MDM2 stability and cancer-cell survival using two different cancer cell lines, the melanoma cell line A375 and the colorectal cancer cell line HCT116, both of which express wild-type p53 (refs. 30,31). Knockdown of USP15 in A375 cells using two different shRNAs caused spontaneous reduction in MDM2 protein expression (Fig. 6a) without reducing the level of Mdm2 mRNA (Fig. 6b). This phenotype differed from the degradation of MDM2 in primary T cells, which required the TCR and CD28 signals (Fig. 4c). The spontaneous degradation of MDM2 appears to be specific for tumor cells because MDM2 protein was only slightly reduced in human primary fibroblasts subjected to USP15 knockdown (Supplementary Fig. 7b) and not changed in Usp15−/− mouse B cells or thymocytes (Supplementary Fig. 7c).

In the melanoma and colorectal tumor cell lines analyzed, expression of NFATc2 was very low (data not shown). However, MDM2 downregulation in cells in which USP15 was knocked down resulted in upregulation of p53 and its target genes Cdkn1a and Bbc3 (Fig. 6a,c), coupled with enhanced apoptosis (Fig. 6d,e). These phenotypes of the cells that we subjected to USP15 knockdown were efficiently reversed upon reconstitution with a USP15 expression vector encoding an shRNA-resistant transcript (Supplementary Fig. 7d,e). Knocking down USP15 in the colon cancer cell line HCT116 also caused downregulation of MDM2 and concomitant upregulation of p53 and p53 target genes as well as enhanced apoptosis (Fig. 6f,g). These effects of USP15 knockdown were dependent on p53, because we detected them in wild-type, but not in p53-deficient, HCT116 cells (Supplementary Fig. 7f,g).

Incubation of A375 cells that we subjected to USP15 knockdown with a proteasome inhibitor (MG132) prevented the loss of MDM2 and caused accumulation of ubiquitinated MDM2 (Fig. 6b and Supplementary Fig. 8a), which suggested that, similar to the case in activated T cells, USP15 was required to prevent ubiquitin-dependent MDM2 degradation in cancer cells. Conversely, when we subjected HCT116 and A375 cells to USP15 knockdown and incubated them with CHX resulted in accelerated degradation of MDM2 and delayed degradation of p53 (Supplementary Fig. 8b).

Recent studies suggest a role for USP15 in the regulation of TGF-β signaling in some cancer cell lines17,18. However, knockdown of USP15 in A375 or HCT116 cells did not interfere with TGF-β–stimulated phosphorylation of Smad or induction of two TGF-β target genes.
Figure 6  USP15 stabilized MDM2 and negatively regulated p53-dependent gene expression and apoptosis in cancer cells. (a) IB analysis of A375 melanoma cells stably infected with a nonsilencing control shRNA (C) or two different USP15 shRNAs. (b-c) qRT-PCR analysis of the indicated mRNAs relative to Actb in the control and USP15-knockdown A375 and HCT116 cells. (d-e) Analysis of spontaneous apoptosis of the control (Ctrl) cells and A375 cells subjected to USP15 knockdown (shUSP15) based on annexin V staining (d) and content of nuclear PI staining (e). Numbers in plots indicate percentage of apoptotic cells. (f-g) IB (f) and apoptosis (g) analyses of the control (C) and HCT116 cells subjected to USP15 knockdown. (h) MDM2 ubiquitination in the control (C) and A375 cells subjected to USP15 knockdown, treated with MG132 for 2 h. Error bars, s.e.m.; *P < 0.05; **P < 0.01 (two-tailed unpaired t-test). Data are representative of five (a-d,g) or three (b,h) independent experiments.

USP15 regulates both tumor growth and antitumor immunity

The findings that USP15 deficiency promotes T cell activation and cancer cell apoptosis indicated that inhibition of USP15 might suppress tumor growth both via a tumor cell–intrinsic mechanism and by enhancing the anti-tumor host defense. Knockdown of USP15 in A375 and HCT116 cells attenuated the growth of xenograft tumors in nude mice (Fig. 7a and Supplementary Fig. 9a,b). We did not see this effect in p53-deficient HCT116 cells (Supplementary Fig. 9c). Furthermore, introduction of exogenous MDM2 in A375 cells in which USP15 was knocked down reduced the level of p53, promoted tumor growth and inhibited apoptosis (Fig. 7b–d). We next examined the role of USP15 in regulating antitumor host defenses in a B16 melanoma model32. Compared to wild-type mice, Usp15−/− mice had a profound reduction in size of B16 tumors and tumor-induced lethality (Fig. 7e,f). Knocking down USP15 in the B16 tumor cells revealed a combinatorial effect on tumor inhibition (Supplementary Fig. 9d–f). Compared to wild-type hosts, B16 cell–challenged Usp15−/− mice had an increased frequency of IFN-γ CD4+ T cells infiltrating to the tumors (Fig. 7g and Supplementary Fig. 9g). Despite the lack of a direct effect of USP15 deficiency on CD8+ T cell activation (Supplementary Fig. 6), B16 cell–challenged Usp15−/− mice also had an increased frequency of tumor-infiltrating CD8+ effector T cells. This is probably due to increased activation and recruitment of CD8+ T cells to tumor site by the CD4+ T cells via different mechanisms, including the production of IFN-γ35. We also considered the potential involvement of Treg cells. Wild-type and Usp15−/− mice had similar frequencies of Treg cells in both the lymphoid organs and the tumors (Supplementary Fig. 10a,b).

We next examined the role of IFN-γ-producing T cells in mediating tumor suppression. Injection of an IFN-γ neutralizing antibody in the Usp15−/− mice promoted tumor growth, which suggested that IFN-γ contributes to the stronger anti-tumor immunity in Usp15−/− mice (Supplementary Fig. 10c). To examine the T cell–intrinsic function of USP15 in regulating antitumor immunity, we reconstituted T cell–deficient Terb−/− Terd−/− mice with wild-type or Usp15−/− naive CD4+ T cells along with wild-type CD8+ T cells and then challenged the recipient mice with B16 melanoma cells. Compared to hosts receiving wild-type T cells, mice receiving Usp15−/− T cells were much more efficient in controlling melanoma growth (Fig. 7h). When not challenged with the B16 melanoma cells, the mice receiving wild-type and Usp15−/− T cells had similar numbers of splenic CD4+ T cells in the spleen (Supplementary Fig. 10d). These findings suggest that USP15 inhibition may not only promote cancer cell apoptosis but also boost T cell–mediated antitumor immunity.

DISCUSSION

We identified USP15 as a negative regulator of T cell activation. USP15 deficiency promoted T cell activation in vitro and enhanced T cell responses in vivo to L. monocytogenes infection and tumor challenge. USP15 stabilized MDM2, which in turn mediated ubiquitin-dependent degradation of NFATc2 and negatively regulated T cell signaling. However, we did not detect an obvious negative role of USP15 in regulating T cell activation; the former may be ideal for use together with USP15 inhibition to promote antitumor immunity.

MDM2 is known as an inhibitor of p53 and a mediator of cancer-cell survival; however, the role of MDM2 in the regulation of immune responses has remained poorly understood. Our data suggested a p53-independent function of MDM2 in the regulation of T cell activation. Notably, there are two different types of MDM2 inhibitors, which function by inhibiting the E3 ligase activity of MDM2 and interfering with the MDM2-p53 interaction, respectively. These two different types of MDM2 inhibitors had very different effects on T cell activation, which suggests possible differences in their therapeutic efficacies when used in combination with immunotherapeutic approaches. Because HLI373, but not Nutlin-3, promotes activation of T cells, the former may be ideal for use together with cancer immunotherapy.

Prior studies suggest that USP15 also regulates TGF-β signaling in certain cancer cell lines17,18. Because TGF-β is an immunosuppressive cytokine, it raises the intriguing question of whether the negative role of USP15 in regulating T cell activation also involves altered TGF-β signaling. However, we did not detect an obvious...
defect of the USP15-deficient CD4+ T cells in TGF-β–stimulated phosphorylation of Smad or expression of target genes. These data suggest that the function of USP15 in regulating TGF-β signaling may be dependent on cell types and/or activation conditions. Future studies will examine this potential role of USP15 in different types of immune system cells.

We found that USP15 mediates the stability of MDM2 in both T cells and cancer cells. USP15 functioned as a deubiquitinase of MDM2 because it bound to MDM2 and directly cleaved the ubiquitin chains from MDM2. Loss of USP15 in melanoma and colorectal cell lines caused spontaneous ubiquitination and degradation of MDM2, which suggested that the amount of MDM2 in cancer cells is maintained by a dynamic balance between ubiquitination and deubiquitination. In resting T cells, MDM2 was stable even in the absence of USP15; however, the TCR and CD28 signals stimulated ubiquitin-dependent degradation of MDM2, which was negatively regulated by USP15. How T cell activation signals trigger the degradation of MDM2 remains to be studied, but one possibility is that the TCR and CD28 signals stimulate the E3 ligase activity of MDM2, which causes both MDM2 self-ubiquitination and the ubiquitination of its targets. This hypothesis also explains why NFATc2 undergoes ubiquitination in activated T cells but not in resting T cells. In addition to USP15, USP7 has been implicated in the regulation of MDM2 in cancer cells. USP7 originally had been identified as a DUB that stabilizes p53, but subsequent studies suggest that USP7 also regulates MDM2 (refs. 34,35). It is unknown whether USP7 also regulates functions of the immune system, particularly activation of T cells. Another intriguing question is whether USP15 and USP7 functionally cooperate in the regulation of MDM2. A protein-protein interaction screen study has revealed the physical interaction between USP15 and USP7, although the functional importance remains to be studied.36

Our findings have important implications for cancer therapy. Immunotherapy and targeted therapy are two promising approaches in the treatment of cancer.37 It has been suggested that the combination of these approaches may represent a paradigm shift to greatly improve the clinical efficiencies. However, for the successful partnering of targeted therapy with immunotherapy, it is important that the drug targets are not required for immune system functions. Ideally, the anticancer drugs should also promote antitumor immunity. Our current findings suggest that USP15 may be an excellent drug target for partnering with immunotherapy. Future studies will directly examine this possibility using animal models of cancer therapy.

METHODS
Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS
Q.Z. designed and performed the experiments, prepared the figures and wrote part of the manuscript; J.J., H.H., H.S.L., S.R., Y.X., M.N., X.Z., X.C., P.Y. and C.Z. contributed to the performance of the experiments; S.S.W. and G.L. were involved in supervision of H.S.L. and P.Y., respectively; S.E.U. was involved in collaboration on cancer model studies, and S.-C.S. supervised the work and wrote the manuscript.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.
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ONLINE METHODS

Mice. Usp15−/− mice were generated using the OmniBank retroviral gene-trapping technique (Taconic). The mice were originally in B6BL/6-129 mixed background and subsequently backcrossed for four generations to the C57BL/6 background. Usp15−/− heterozygous mice were bred to generate wild-type (+/+) and knockout (KO) (−/−) littermates for experiments. Complete ablation of Usp15 was confirmed by both IB assays and RT-PCR to amplify different regions of the Usp15 mRNA (primers used are listed in Supplementary Table 1). Mice harboring double deletions of the genes encoding TCR beta and delta (Tcrβ−/− Tcrδ−/−) and thus completely lacking T cells were obtained from Jackson Lab (in C57BL/6 background). B6.5JL mice (expressing the CD45.1 congenic marker), Rag1−/− mice, and the OT-II TCR–transgenic mice (all on C57BL/6 background) were from The Jackson Laboratory. OT-II mice were crossed with the Usp15−/− mice to generate Usp15−/− OT-II and wild-type OT-II control mice. The Tpr53−/− mice were from L.A. Donohoe (Baylor College of Medicine) and bred in C57BL/6-129 mixed background, and the Tpr53−/− Mdm2−/− mice were generated as described38. Mice were maintained a specific pathogen–free facility, and all animal experiments were conducted in accordance with protocols approved by the Institutional Animal Care and Use Committee of the University of Texas MD Anderson Cancer Center. Experiments were performed using age- and sex-matched (1–20 months of age; female and male) mice.

Plasmids. The retroviral expression vector for human USP15 (double-tagged with hemagglutinin (HA) and Flag) was purchased from Addgene (plasmid #22570) and used as template for creating the catalytically inactive USP15 variant, C269A, was named based on the sequence of USP15 isoform 2 cDNA19. This corresponding residue in USP15 isoform 1 is Cys298. Sequences encoding USP15 and mouse MDM2, respectively. For ubiquitination assays, MDM2 and NFATc2 were detected by IB using a ubiquitin antibody specifically

Flow cytometry, cell sorting and intracellular cytokine staining. Suspensions of splenic and lymph node cells were subjected to flow cytometry and cell sorting as described40 using LSR II (BD Bioscience) and FACSAria (BD Bioscience) flow cytometers, respectively. The data were analyzed using FlowJo software. For ICS, the anti-CDS3/anti-CDS28-activated T cells were further stimulated for 4 h with PMA plus ionomycin in the presence of monensin and then subjected to intracellular IFN-γ-staining and flow cytometry. For analyzing in vivo primed antigen-specific T cells, the T cells were stimulated in vitro with the indicated antigenic peptides in the presence of monensin and then subjected to ICS.

ELISA and quantitative reverse transcriptase–PCR. Supernatants of in vitro cell cultures or sera of mice were analyzed by ELISA using a commercial assay system (eBioscience). Total RNA was prepared for the indicated cells and subjected to qRT-PCR39 using gene-specific primers (Supplementary Table 5).

Immunoblot and ubiquitination assays. Total cell lysates or subcellular extracts were prepared and subjected to IB assays as described41. The Santa Cruz anti-Usp15 (2DS) and the Proteintech anti-USP15 antibodies were used for detecting human and mouse Usp15, respectively. The Millipore anti-MDM2 and the Sigma anti-MDM2 antibodies were used for detecting human and mouse MDM2, respectively. For ubiquitination assays, MDM2 and NFAT2 were detected by IB using a ubiquitin antibody specifically detecting K48-linked ubiquitin chains (Millipore). For transfection models, the expression vector encoding MDM2 or NFAT2 was transfected into HEK293 cells along with the HA-ubiquitin expression vector and the ubiquitinated MDM2 and NFAT2 were isolated by denaturing IP and detected by anti-HA IB. For deubiquitination assays, the isolated MDM2–ubiquitin conjugates were incubated with purified Usp15 or its catalytic mutant in a deubiquitination buffer42 for 4 h and then subjected to IB using anti-ubiquitin.

Cell culture, gene silencing and overexpression. Human melanoma cell line A375 and colon cancer cell line HCT116 were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS (FBS) and 1% penicillin/streptomycin. HCT116 p53 null (Trp53−/−) and wild-type control (Trp53+/−) cells were provided by B. Vogelstein (Johns Hopkins University), and the normal human dermal fibroblasts were from PromoCell. Murine B16 melanoma cells stably transfected with OVA were provided by Q. Yi (Cleveland Clinic Foundation)43. For gene silencing, lentiviral vectors were prepared as described39 by transfecting HEK 293T cells with lentiviral vectors encoding specific shRNAs along with packaging plasmids. The packaged viruses were then used to infect the indicated cells, followed by selection of the infected cells by puromycin (1 μg/ml) for 7 d or by sorting GFP+ cells (for the pGIPZ vector). For overexpression studies, lentiviral transduction was as described above, and retroviral packaging and transduction were as described previously44.

Bone marrow transduction and adoptive transfer. Bone marrow cells were taken from Usp15−/− or wild-type control mice that were treated with 5-fluorouracil (150 mg/kg) for 48 h. After removal of RBCs and CD90.2+ cells,
they were cultured in IMDM with 15% FBS in the presence of IL-3 (20 ng/ml), IL-6 (20 ng/ml) and stem cell factor (50 ng/ml) for 3 d. Bone marrow cells were mixed with viral supernatant in the presence of polybrene (8 ng/ml), cultured in the presence of IL-3, IL-6 and stem cell factor for an additional 24 h, and then mixed with viral supernatant again. The efficiency of viral transduction of bone marrow cells was examined by monitoring GFP expression using fluorescence microscopy. Before adoptive transfer, transduced bone marrow cells were harvested and washed once with IMDM without FBS. A total of 10^6 cells were injected intravenously (i.v.) via a tail vein into lethally irradiated (950 rad) Rag1−/− mice.

**T cell adoptive transfer.** Sorted wild-type or Usp15−/− naive CD4+ T cells (CD4+CD44+CD62L+CD25−) (5 × 10^5) were adoptively transferred, via i.v. injection, into age- and sex-matched Tcrb−/− Tcrd−/− mice along with the same number of wild-type CD8+ T cells. After 8 h, the recipient mice were challenged with L. monocytogenes or B16 melanocytes. Wild-type or Usp15−/− OT-II naive CD4+ T cells (5 × 10^6) were adoptively transferred, via i.v. injection, into age- and sex-matched B6.SJL mice. After 8 h, the recipient mice were challenged with OVA-expressing L. monocytogenes.

**L. monocytogenes infection.** Recombinant L. monocytogenes expressing a truncated OVA protein (LM-OVA) was provided by H. Shen via DMX Incorporated. Age- and sex-matched wild-type and Usp15−/− mice (8–12-week-old) were infected i.v. with LM-OVA (5 × 10^7 CFU per mouse) and sacrificed after 6 d of infection to analyze the primary host response. Livers were homogenized in 10 ml 0.2% (vol/vol) Nonidet P-40 in PBS, and the organ homogenates were serially diluted and plated on streptomycin agar plates to determine the CFU of L. monocytogenes. Splenocytes were collected for flow-cytometric analysis of listeriolysin O (LLO)-specific CD4+ T lymphocytes. In brief, the splenocytes were stimulated with 5 μg/ml of LLO190–201 peptide (NEKYAQAYPNVS) in the presence of monesin, followed by intracellular IFN-γ staining and flow cytometry analysis. LM-OVA infection was also carried out using the T cell-transferred Tcrb−/− Tcrd−/− mice, followed by analysis of immune cell activation as described above. We found that these mice (especially those transferred with wild-type CD4+ T cells) had a high rate of lethality when infected at the regularly used dose (5 × 10^6 CFU per mouse) and therefore used a lower dose (5 × 10^5 CFU per mouse) for analyzing the liver bacterial loads.

The B6.SJL mice, adoptively transferred with wild-type or Usp15−/− OT-II naive CD4+ T cells, were infected i.v. with LM-OVA (5 × 10^6 CFU per mouse) as described above. Splenocytes were stimulated with 5 μg/ml of OVA253–339 peptide, and the IFN-γ–producing OT-II T cells were detected by ICS and flow cytometry based on their expression of the CD45.2 congenital marker.

**Analysis of apoptosis.** Apoptotic cells were measured based on nuclear staining with PI46. Briefly, the cells were incubated for 0.5 h in a hypotonic PI-staining buffer (0.1% sodium citrate, 0.1% Triton X-100 and 50 μg/ml PI) and then subjected to flow cytometry to quantify the apoptotic/necrotic cell population (sub-G1/G0 cell fraction). Early-stage apoptosis was determined by FITC-annexin V staining (BD Biosciences), based on the translocation of phosphatidylserine to the extracellular membrane leaflet in apoptotic cells.

**B16 model of melanoma and human cancer xenograft studies.** Wild-type and Usp15−/− mice were injected s.c. with 5 × 10^5 B16 melanoma cells expressing a surrogate tumor antigen, OVA. The challenged mice were monitored for tumor growth, and the mice were sacrificed when their tumor size reached 225 mm^2 based on protocols approved by the Institutional Animal Care and Use Committee of the University of Texas MD Anderson. Thus, lethality was defined as tumor size reaching 225 mm^2. To minimize individual variations, littermate wild-type and Usp15−/− mice were used. For the human cancer xenograft study, female nude (nu/nu) mice (8-week-old) were injected s.c. with 1 × 10^7 A375 human melanoma cells or HCT116 human colorectal cell line and monitored for tumor growth. Mice were randomly selected for tumor injection. Analysis of tumor size was done in a blinded fashion by one examiner with the results confirmed by a second examiner, also in a blinded fashion.

**Statistical analysis.** Statistical analysis was performed using Prism software. Two-tailed unpaired t-tests were performed and P values less than 0.05 were considered significant, and the level of significance was indicated as *P < 0.05 and **P < 0.01. In the animal studies, four mice are required for each group based on the calculation to achieve a 2.3 fold change (effect size) in two-tailed t-test with 90% power and a significance level of 5%. All statistical tests are justified as appropriate, and data meet the assumptions of the tests. The variance is similar between the groups being statistically compared.

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