A20 regulates the therapeutic effect of anti-PD-1 immunotherapy in melanoma

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ABSTRACT

Background The therapeutic effect of immune checkpoint blockers, especially the neutralizing antibodies of programmed cell death (PD-1) and its ligand programmed death ligand 1 (PD-L1), has been well verified in melanoma. Nevertheless, the dissatisfactory response rate and the occurrence of resistance significantly hinder the treatment effect. Inflammation-related molecules like A20 are greatly implicated in cancer immune response, but the role of tumorous A20 in antitumor immunity and immunotherapy efficacy remains elusive.

Methods The association between tumorous A20 expression and the effect of anti-PD-1 immunotherapy was determined by immunoblotting, immunofluorescence staining and flow cytometry analysis of primary tumor specimens from melanoma patients. Preclinical mouse model, in vitro coculture system, immunohistochemical staining and flow cytometry analysis were employed to investigate the role of A20 in regulating the effect of anti-PD-1 immunotherapy. Bioinformatics, mass spectrum analysis and a set of biochemical analyses were used to figure out the underlying mechanism.

Results We first discovered that upregulated A20 was associated with impaired antitumor capacity of CD8+ T cells and poor response to anti-PD-1 immunotherapy in melanoma patients. Subsequent functional studies in preclinical mouse model and in vitro coculture system proved that targeting tumorous A20 prominently improved the effect of immunotherapy through the invagination of infiltrating CD8+ T cells via the regulation of PD-L1. Mechanistically, A20 facilitated the ubiquitination and degradation of prohibitin to potentiate STAT3 activation and PD-L1 expression. Moreover, tumorous A20 expression was highly associated with the ratio of Ki-67 percentage in circulating PD-1+ CD8+ T cells to tumor burden.

Conclusions Together, our findings uncover a novel crosstalk between inflammatory molecules and antitumor immunity in melanoma, and highlight that A20 can be exploited as a promising target to bring clinical benefit to melanomas refractory to immune checkpoint blockade.

INTRODUCTION

Eradication of immune destruction is a hallmark of cancer, which is primarily due to the abnormal activation of immune checkpoints and the termination of antitumor immune response. Programmed cell death (PD-1)/programmed death ligand 1 (PD-L1) have been demonstrated as a pair of major immune checkpoint molecules and valuable therapeutic targets for melanoma treatment.1 For instance, the binding of membrane PD-L1 on tumor cells to PD-1 on T cells evokes an immunosuppressive signal that results in the dysfunction and even the apoptosis of cytotoxic T cells, thereby impairing antitumor immunity.2 Therefore, PD-1/ PD-L1 blockade is in a position to disrupt the interaction between tumor cells and T cells, which restores tumor-specific immune response.3 For melanoma patients receiving anti-PD-1 antibody monotherapy, the median overall survival could be up to 32.7 months after almost 5 years of follow-up.4 However, a proportion of 40%–60% of patients does not achieve any significant therapeutic response, and some patients even show complete resistance to PD-1/PD-L1 blockade.5 Accumulative evidence has revealed multiple primary and acquired alterations leading to treatment resistance of immunotherapy, including low mutational burden, defective antigenicity and antigen presentation of tumor cell and genomic dysregulation of interferon-γ (IFN-γ) signaling pathway.6-8 These reports have prompted potential molecular profile for predicting the treatment response and effective synergized therapeutic strategy for cancer immunotherapy. Notably, tumor cells can adaptively increase the expression of PD-L1 or other immune checkpoints under the control of IFN-γ after the treatment with anti-PD-1 antibody, rendering melanomas refractory to immunotherapy.10-12 Hence, the molecular mechanism underlying aberrant tumorous PD-L1 expression associated with the resistance to immunotherapy in melanoma is in urgent need to be clarified to improve the therapeutic efficacy.

Inflammation is closely related to the development of various cancers including
melanoma, with its pivotal role in the regulation of cancer immune response demonstrated recently. To be specific, tumor necrosis factor-α (TNF-α) that is a common proinflammatory cytokine and its downstream pathway could induce tumor immune escape by elevating the expressions of immune checkpoint molecules. On the other, TNF-α is also one of the major cytokines secreted by activated CD8+ T cells in tumor microenvironment that potentiates antitumor immune response. These reports highlight the vital impact of inflammatory signaling on antitumor immunity, though the actual function remains controversial. A20 is a primary responsive gene of TNF-α and is documented as a crucial negative mediator of inflammation as well as immune response in multiple immune cells. As a ubiquitin-editing enzyme, it is composed of an N-terminal OTU domain with deubiquitinase activity and seven Cys2-Cys2 zinc finger C-terminal domains functioning as a ubiquitin ligase. The cooperative activity of these two ubiquitin-editing domains mediates the negative regulatory role of A20 in NF-κB signaling, so that the genetic deficiency of TNFAIP3 that encodes A20 protein can result in the onset and progression of multiple autoimmune diseases by amplifying the pro-inflammatory NF-κB signaling. For cancer pathogenesis, previous investigations emphasized on A20 expressed in tumor-infiltrating immune cells to clarify its effect on antitumor immunity. In B16 mouse melanoma tumor model, silencing of A20 in dendritic cells (DCs) enhanced NF-κB activity followed by elevated expression of interleukin 6 (IL-6), TNF-α and IL-12, leading to potentiated antitumor immune responses. In addition, tumor-infiltrating CD8+ T with the deletion of A20 had stronger antitumor capacity by relieving the brake on NF-κB signaling pathway. Of note, A20 is also abundantly expressed in tumor cells and can directly influence their biological behaviors. Therefore, A20 expressed in immune cells and tumor cells in tumor microenvironment are both greatly implicated in the pathogenesis of cancer. Previous studies regarding antitumor immunity mainly focused on the role of A20 that is expressed in immune cells. However, whether tumorous A20 can affect the function of tumor-infiltrating immune cells and the therapeutic effect of immunotherapy remains unknown.

In the present study, A20 was initially identified to be highly related with the effect of anti-PD-1 immunotherapy among melanoma patients. Preclinical melanoma mouse model and in vitro coculture system were then established to evaluate the therapeutic effect of anti-PD-1 immunotherapy with synergized suppression of tumorous A20 expression. Subsequently, the potential mechanism of A20-mediated resistance to immunotherapy was further investigated by bioinformatics, mass spectrum analysis and a set of biochemical analyzes, with a particular emphasis on the regulation of PD-L1 expression. Furthermore, the relationship between tumorous A20 expression and the invigoration of circulating exhausted-phenotype CD8+ T cells after anti-PD-1 antibody treatment was analyzed in melanoma patients.

METHODS

A detailed description of the methods used in this study is available in online supplemental methods.

Statistical analysis

All experiments were repeated at least three times unless otherwise indicated. Error bars represent SE of the mean. Student’s t-test was used to compare two groups of independent samples. Liner regression was used to confirm the correlation between two groups of dependent samples. P<0.05 was considered statistically significant. Kaplan-Meier analysis and log-rank (Mantel-Cox) test were used to evaluate the statistical significance for comparison of survival curves. All statistical analyzes were performed with GraphPad Prism (GraphPad software V.6.0, La Jolla, USA).

RESULTS

A20 expression is correlated with the clinical response to anti-PD-1 antibody treatment in melanoma patients

Previously, several transcriptomic and proteomic researches have demonstrated that inflammation-related signals and molecules were highly correlated with the therapeutic effect of anti-PD-1 immunotherapy in melanoma. Of note, in a cohort of 54 melanoma patients receiving anti-PD-1 antibody monotherapy, we noticed that a high fraction of non-responding patients had abundant expressions of immune gene markers like TNFAIP3 (encoding A20) and TLR3 in tumor, which was associated with dampened immune response. A20 is a primary responsive gene of TNF-α and is documented as a negative mediator of inflammation. For cancer pathogenesis, previous investigations emphasized on A20 expressed in tumor-infiltrating immune cells and its effect on antitumor immunity. However, our immunofluorescence staining assay revealed that A20 was mainly distributed in infiltrating immune cells and its effect on anti-tumor immunity. However, our immunofluorescence staining assay revealed that A20 was mainly distributed in infiltrating immune cells (Melan A-positive) rather than lymphocytes (CD45-positive) in melanoma specimens (online supplemental figure 1A). We have recently demonstrated that upregulated A20 contributed to cell proliferation, metastasis and the treatment resistance to vemurafenib in melanoma (data not shown), whereas the role of tumorous A20 in the regulation of antitumor immunity and immunotherapy effect remains elusive. To this end, we collected baseline primary tumors from 11 patients who subsequently treated with anti-PD-1 antibody. According to the response status, these patients were separated into response group (responder, n=6, partial response) and resistance group (non-responder, n=5, progressive disease) after 6 months of follow-up visit (figure 1A; online supplemental table 1). The age of patients showed no prominent difference between both groups (online supplemental table 1). We digested and fractionated melanoma specimens with trypsin to obtain protein lysates and performed immunoblotting analysis. As was shown, the protein level of tumorous A20 was remarkably increased in resistance group compared with that in response.
group (Figure 1B). Moreover, the survival analysis of the patients revealed that high tumorous A20 expression was associated with poor prognosis after receiving anti-PD-1 antibody treatment (Figure 1C).

To further illustrate the relationship between the tumorous A20 expression and antitumor immunity, we employed H&E staining and found that the number of tumor-infiltrating lymphocytes was significantly reduced in tumors with high A20 expression (Figure 1D). Subsequent immunofluorescence staining analysis forwardly revealed that the number of infiltrated CD8+ T cells was negatively correlated with tumorous A20 expression in
these tumors (figure 1E). What’s more, we performed flow cytometry analysis on the cell suspensions derived from these tumors. It revealed that tumorous A20 expression was negatively correlated with the percentage of Ki-67-positive CD8+ T cells (figure 1F), so were the percentages of Perforin-positive and Granzyme B-positive CD8+ T cells (figure 1G,H). These results suggested that the loss of tumorous A20 might induce stronger antitumor immune response in melanoma, which was probably involved in rendering therapeutic resistance to anti-PD-1 immunotherapy.

A20 regulates the therapeutic response to anti-PD-1 antibody treatment in melanoma

Then, we established a preclinical xenograft mouse model to investigate whether tumorous A20 could regulate the treatment effect of anti-PD-1 immunotherapy in melanoma. C57BL/6 mice were subcutaneously injected with wild-type murine B16F10 cells, a cell line that is resistant to PD-1 antibody,30 or B16F10 cells with the knockout of A20 by CRISPR/Cas9 (online supplemental figure 1B), followed with or without the treatment with anti-PD-1 antibody. Compared with the control group, the implantation with A20-deficient tumors or the monotherapy with anti-PD-1 antibody slightly prolonged the survival of mice (figure 2A). However, mice implanted with A20-deficient B16F10 tumor that also received anti-PD-1 antibody treatment displayed superior survival rate (figure 2A). Moreover, we examined the alteration of tumor growth in another cohort of mice. The knockdown of A20 or the monotherapy with anti-PD-1 antibody significantly led to the reduction of tumor volume and tumor weight (figure 2B,C). More importantly, A20 knockout could sensitize melanoma to anti-PD-1 antibody treatment, leading to more prominent regression of xenograft tumors in mice (figure 2B,C). Together, tumorous A20 could impair the efficacy of anti-PD-1 immunotherapy in melanoma.

We then performed immunohistochemical staining analysis of xenograft tumors after anti-PD-1 antibody treatment and found that A20 deficiency led to significant enrichment of infiltrated lymphocytes in tumor (figure 2D). Subsequent flow cytometry analysis of the cell suspensions derived from these tumors displayed that the knockout of A20 induced significantly increased the number of Ki-67-positive CD8+ T cells and Granzyme B-positive CD8+ T cells (figure 2E,F), indicating that tumorous A20 suppressed the cytotoxicity and infiltration of CD8+ T cells in tumor microenvironment after anti-PD-1 antibody treatment. In order to investigate the effect of tumorous A20 deficiency on the function of infiltrated CD8+ T cells, we cocultured B16F10 melanoma cells with murine Peripheral blood mononuclear cells (PBMCs) extracted from C57BL/6 mice that were burdened with B16F10 cells and received anti-PD-1 antibody treatment (figure 2G). Flow cytometry analysis on PBMCs in the coculture system revealed that the knockout of A20 in melanoma cells increased the percentages of both Ki-67-positive CD8+ T cells and Granzyme B-positive CD8+ T cells (figure 2H,I). Moreover, the deficiency of A20 induced more apoptosis of melanoma cells in the coculture system (figure 2J), while the apoptosis rate in single culture of B16F10 cells was minimally influenced by A20 knockout (online supplemental figure 1C). These results reiterated that tumorous A20 affected the functional status of infiltrated CD8+ T cells and thereby participated in the regulation of anti-PD-1 immunotherapy efficacy. To further demonstrate that CD8+ T indeed mediated the effect, specific antibodies targeting CD8 were then injected intraperitoneally to block CD8+T cells systemically in xenograft mouse model (online supplemental figure 1D). CD8 antibody cotreatment could repotentiate the tumor growth with increased tumor growth and tumor weight when the mice received anti-PD-1 antibody treatment (figure 2K,L). Therefore, CD8+T cells were required for the observed synergistic effect of anti-PD-1 antibody treatment and the knockout of A20 on melanoma regression.

A20 modulates the expression of PD-L1

Accumulative evidence has revealed that the dysregulation of immune checkpoints is highly related with tumor immune escape and resistance to immunotherapy.3,31,32 In particular, IFN-γ-induced feedback up-regulation of PD-L1 in tumor microenvironment after anti-PD-1 antibody treatment has been regarded as a crucial mechanism for resistance to immunotherapy.3 Therefore, we proposed that A20 might manipulate the expression of PD-L1 or other immune checkpoints to affect the antitumor capacity of infiltrated lymphocytes, thereby affecting the treatment response to immunotherapy. To clarify this, we analyzed the relationship between TNFAIP3 mRNA level and the expressions of several immune checkpoints in The Cancer Genome Atlas (TCGA) Skin Cutaneous Melanoma (SKCM) database. TNFAIP3 mRNA level was in positive correlation with CD274 (encoding PD-L1) compared with PDCD1LG2 (encoding PD-L2), CTLA-4, CD80, CD86 or ICOSLG (figure 3A; online supplemental figure 1E). In addition, gene set enrichment analysis was performed in TCGA SKCM database to identify distinctively expressed genes and biological pathways in melanoma correlated with PD-L1 mRNA expression (figure 3B; online supplemental table 2). Inflammation-related pathways (including Toll pathway, TNFR1 pathway and TNFR2 pathway) were among the most significant pathways that positively correlated with PD-L1 expression (online supplemental table 2). We analyzed the relationship between TNFAIP3 mRNA level and several inflammation-related molecules, which revealed that PD-L1 mRNA level was in markedly positive correlation with the mRNA expressions of TNFAIP3 and other proinflammatory genes (figure 3B,C). In order to further confirm the relationship between tumorous A20 expression and PD-L1 expression, immunohistochemical staining analysis in tumor tissue microarray (TMA) that consists

of 82 melanoma cases was carried out. In line with the result from TCGA SKCM database, A20 expression was proportional to PD-L1 expression in melanoma tissues (figure 3D). Subsequent immunoblotting analysis of tumors from 11 patients reached a common conclusion (figures 3E and 1B).

We went on to observe whether A20 could regulate PD-L1 expression in melanoma. The immunoblotting
analysis of whole cell lysis in two human melanoma cell lines showed that the knockdown of A20 significantly suppressed PD-L1 expression (figure 3F), so was the transcriptional level of PD-L1 (online supplemental figure 1F). In addition, flow cytometry analysis revealed diminished membrane PD-L1 expression after the knockdown

**Figure 3**  A20 regulates the expression of PD-L1. (A) Correlation analysis between TNFAIP3 mRNA level and several immune checkpoints in TCGA SKCM database. (B, C) GSEA analysis of PD-L1-associated molecules in TCGA SKCM database, and the correlation analysis between PD-L1 and several inflammation-related molecules. (D) Representative immunohistochemical (IHC) staining images of A20 and PD-L1 expressions in TMA. The association between A20 and PD-L1 expression in melanoma tissues was analyzed. (E) Immunoblotting analysis of PD-L1 expression in melanomas derived from 11 patients related to figure 1A. The scatter diagram shows the linear correlation between A20 and PD-L1 protein expression. (F) Immunoblotting analysis of the expression of PD-L1 in melanoma cell lines after the intervention of A20. (G) Flow cytometry analysis of membrane PD-L1 expression in melanoma cells after the intervention of A20. (H) Immunohistochemical staining analysis of PD-L1 in xenograft tumors with the intervention of A20. Scale bars=100 µm. Data represent the mean±SEM of at least triplicates. P value was calculated by two-tailed Student’s t-test. GSEA, gene set enrichment analysis; PD-L1, programmed death ligand 1; SEM, SE of the mean; SKCM, Skin Cutaneous Melanoma.
of A20 (figure 3G). Moreover, PD-L1 staining intensity in previous B16F10 xenograft tumors was also decreased with A20 deficiency (figure 3H), which proved that A20 could promote PD-L1 expression in melanoma.

**Tumoral A20 induces therapeutic resistance to anti-PD-1 immunotherapy via PD-L1**

We continued to explore whether the increase of PD-L1 expression was responsible for A20-induced resistance to anti-PD-1 antibody treatment. To this end, C57BL/6 mice were subcutaneously injected with B16F10 melanoma cells with the interventions of both A20 and PD-L1, followed with anti-PD-1 antibody treatment. In response to anti-PD-1 antibody treatment, although the lack of A20 significantly inhibited melanoma progression, concurrent overexpression of PD-L1 reversed the inhibitory effect of A20 knockout (figure 4A,B). Through flow cytometry analysis of the cell suspension of implanted tumors, we discovered that the knockout of tumorous A20 could increase the number of CD8+ T cells in tumor microenvironment after anti-PD-1 antibody treatment, whereas the concurrent overexpression of PD-L1 reversed the infiltration of CD8+ T cells (figure 4C). Since that PD-L1 on tumor cells mainly affects the function of immune cells expressing PD-1,25 we examined the number of infiltrated CD8+PD-L1+T cells in tumor. PD-L1 overexpression inhibited the increase of CD8+PD-L1+ T cells caused by A20 deficiency as well (figure 4D). Moreover, the knockout of A20 in melanoma elevated the expressions of Ki-67 and Granzyme B in CD8+ T cells in tumor microenvironment, which were resuppressed after PD-L1 overexpression (figure 4E–F), indicating that the role of tumorous A20 in regulating the response to anti-PD-1 immunotherapy was dependent on PD-L1. For further verification, we cocultured B16F10 melanoma cells with murine PBMCs that were extracted from C57BL/6 mice burdened with implanted B16F10 cells and received anti-PD-1 antibody treatment (figure 2G). The knockout of A20 in B16F10 cells led to more apoptosis in the co-culture system, whereas concurrent overexpression of PD-L1 could repress cell death caused by cocultured PBMCs (online supplemental figure 2A). Subsequent flow cytometry analysis revealed that the deficiency of A20 in B16F10 cells increased the expressions of Ki-67 and Granzyme B in CD8+ T cells of co-culture system, whereas the current overexpression of PD-L1 eliminated the alteration (figure 4G,H), which was consistent with the results obtained in implanted tumors. Collectively, tumoral A20 could contribute to the resistance to anti-PD-1 antibody treatment via the regulation of PD-L1 expression.

**A20 promotes PD-L1 expression through STAT3 signal**

We then investigated the potential mechanism underlying the regulation of A20 on PD-L1 expression. Bioinformatics analysis on TCGA SKCM database suggested that the genes positively correlated with TNFAIP3 mRNA level were enriched in multiple signaling pathways (figure 5A,B; online supplemental table S3), among which we focused on JAK-STAT signaling pathway due to its canonical regulatory effect on PD-L1 expression.34 Although the analysis of TCGA SKCM database revealed that JAK2 mRNA expression was associated with TNFAIP3 mRNA expression, the alteration of neither JAK2 protein expression nor its downstream STAT3 protein expression was observed in melanoma with A20 knockdown (figure 5C; online supplemental figure 2B). Nonetheless, the phosphorylation of STAT3 at Y705 was significantly weakened in case of A20 deficiency and increased after A20 overexpression (figure 5C). The immunofluorescence staining analysis revealed that A20 knockdown induced the decrease of STAT3 phosphorylation as well (figure 5D). Consistently, the mRNA levels of the transcriptional targets of STAT3 including c-myc and Mcl-1 were remarkably reduced (figure 5E).35 Therefore, the knockdown of A20 suppressed the transcriptional function of STAT3.

We further examined whether STAT3 acted as an intermediate signal in the expression of PD-L1 regulated by A20. WP1066 that is a selective STAT3 inhibitor was used to inhibit STAT3 activity. Immunoblotting analysis showed that WP1066 treatment reversed the upregulation of PD-L1 caused by A20 overexpression (figure 5F). Flow cytometry analysis also displayed that WP1066 impeded membrane PD-L1 expression in melanoma cells with the overexpression of A20 (figure 5G). Chromatin immunoprecipitation assay showed impaired combination of STAT3 protein to PD-L1 promoter region after the knockdown of A20 (figure 5H). Together, A20 promoted PD-L1 transcription and expression in a STAT3-dependent way.

**Prohibitin mediates the regulatory role of A20 in STAT3 phosphorylation**

Thereafter, the potential mechanism by which A20 regulated STAT3 phosphorylation was investigated. We first supposed that IL-6, a classic upstream cytokine of STAT3, may be regulated by A20 in melanoma cells. However, we failed to observe significant alteration of IL-6 secretion after the knockdown of A20 (online supplemental figure 2C), indicating that A20 was not capable of activating STAT3 by promoting autocrine IL-6 signal. Meanwhile, SOCS3, the crucial negative regulator of STAT3, was not significantly altered after A20 knockdown (online supplemental figure 2D), excluding the responsibility of SOCS3 in the phosphorylation of STAT3 induced by A20. Afterwards, mass spectrum analysis was used to identify differentially-expressed proteins after the knockdown of A20 in two melanoma cell lines A2058 and A375, respectively (figure 6A). As a result, the expressions of 1715 proteins were upregulated in both A375 and A2058 cells, while the expressions of 264 proteins were downregulated (figure 6A). Since that A20 could regulate protein expressions with its ubiquitin-editing enzymatic activity via direct interaction with its substrates,19 we performed coimmunoprecipitation (co-IP) assay, followed by mass spectrum analysis to uncover the proteins that directly interacted with A20, and examined whether the interacted proteins


were enlisted in upregulated or downregulated proteins after the intervention of A20. Conspicuously, 13 of the 1715 upregulated proteins and 3 of the 264 downregulated proteins were enlisted in A20-interacted proteins (figure 6A; online supplemental table 4), among which prohibitin (PHB) had been previously reported as a regulator of STAT3 phosphorylation.37 Afterwards, we performed co-IP assay by precipitating STAT3, A20 and
PHB in cell lysate respectively, and confirmed that PHB directly interacted with A20 and STAT3 (Figure 6B). Moreover, the knockdown of A20 led to upregulation of PHB expression, while the overexpression of A20 suppressed the expression of PHB (online supplemental figure 2E), which was consistent with the result of previous mass spectrum analysis. Subsequent immunoprecipitation assay denoted that elevated A20 expression inhibited the interaction between PHB and STAT3, along with the decline of PHB expression (Figure 6C). Furthermore, the knockdown of PHB in A2058 and A375 cell lines was established, which brought about prominent increase of STAT3 phosphorylation at Y705. Meanwhile, PD-L1 expression was upregulated (Figure 6D), implicating the negative regulatory role of PHB in STAT3 activation and PD-L1 expression. In summary, A20 was capable of alleviating the inhibitory effect of PHB on STAT3 phosphorylation and activation, thus activating PD-L1 transcription and expression.

We further explored how A20 regulated PHB expression. The intervention of A20 did not change the mRNA level of PHB (online supplemental figure 2F), suggesting that A20 regulated PHB at the post-translational level. A20 has been generally recognized as an ubiquitin-editing enzyme with integrated function of deubiquitinating and ubiquitinating its substrates. For this assessment, we then investigated whether A20 acted as a deubiquitinase or ubiquitin ligase of PHB. After the treatment with proteasome inhibitor MG132, A20-induced reduction of PHB expression was reversed in melanoma cell lines (Figure 6E), indicating that proteasomal pathway was involved in the degradation of PHB mediated by A20. Furthermore, cycloheximide pulse-chase analysis showed that the turnover of intracellular PHB was significantly delayed after the knockdown of A20 (Figure 6F,G). Moreover, immunoprecipitation assay stated that K48-linked ubiquitin chain bonded to PHB, which was markedly attenuated after the knockdown of A20 (Figure 6H).
implying that A20 may act as an E3 ubiquitin ligase of PHB and regulate its ubiquitination and degradation.

We continued to identify the ubiquitination site of PHB. Through the analysis in two websites that predict ubiquitination site, CKSAAP Ubsite and Ubpred (online supplemental table 5), 2 lysine residues (K186 and K202) with relative higher ubiquitination potential were suggested. We constructed two PHB mutants in which the K186 and K202 residues were replaced by arginine, respectively (named K186R and K202R), and then transfected vectors harboring WT PHB or the two PHB mutants into melanoma cells with the knockdown of endogenous PHB expression. Consequently, either K186R or K202R could give rise to significant reduction of K48-linkage ubiquitination of PHB (figure 6I). Moreover, the interaction between A20 and PHB was dampened after the transfection of either K186R or K202R mutant similarly (especially K202R) (figure 6I), indicating that K202 and K186 were both required for the ubiquitination of PHB by A20. Together, PHB was a direct ubiquitination substrate of A20, which at least partially mediated the proteasomal degradation of PHB. Additionally, A20 overexpression could promote PHB ubiquitination and proteasomal degradation, thereby restoring STAT3 activity to promote PD-L1 transcription.

**Tumorous A20 expression is associated with the invigoration of circulating exhausted-phenotype CD8+ T cells in response to anti-PD-1 antibody treatment**

After confirming the involvement of A20-PHB-STAT3-PD-L1 axis in the regulation of immunotherapy resistance, we performed immunohistochemical analysis in TMA to examine the association between A20 and PHB expression and the relationship between PHB and PD-L1 expression in melanomas. A20 knockdown was associated with a significant reduction in PD-L1 expression and a concomitant increase in CD8+ T cell infiltration, indicating that A20 may play a crucial role in the regulation of PD-L1 expression and immune checkpoint blockade resistance.
expression, so as to verify this axis further. PHB expression was significantly downregulated in melanoma tissues compared with nevus tissues (online supplemental figure 2G). Moreover, PHB expression was in negative correlation with PD-L1 expression, and the expression of A20 was also negatively correlated with PHB expression in melanoma in TMA (figure 7A,B). Our analysis on TCGA SKCM database showed that the level of PHB was negatively correlated with TNFAIP3 mRNA expression and CD274 mRNA level respectively (online supplemental figure 2H,I). These results provided in vivo evidence for the regulatory effect of A20 on PHB and PD-L1 expressions.

Of note, a previous study has proved that exhausted-phenotype CD8+ T cells (T<sub>ex</sub>), a subset of CD8+ T cells with restrained function because of inhibitory receptors like PD-1, is the major target of anti-PD-1 antibody treatment. The invigoration of circulating T<sub>ex</sub> characterized by increased ratio of Ki-67 percentage to tumor burden in particular the ratio of Ki-67 in circulating PD-1<sup>+</sup>CD8<sup>+</sup> T cells to tumor burden, is highly associated with the clinical response to anti-PD-1 antibody immunotherapy. By using flow cytometry analysis of PBMCs from the previously enrolled 11 melanoma patients receiving anti-PD-1 antibody treatment, we observed that the ratio of Ki-67 percentage in circulating PD-1<sup>+</sup>CD8<sup>+</sup> T cells to tumor burden was increased more prominently in patients with low tumorous A20 expression than those with high tumorous A20 expression (figure 7C). Evidently, the expression of tumorous A20 was in negative correlation with both Granzyme B and Perforin in circulating PD-1<sup>+</sup>CD8<sup>+</sup> T cells of 11 melanoma patients after anti-PD-1 antibody treatment (figure 7D,E). These results convey that tumorous A20 was significantly associated with the reactivation of circulating exhausted-phenotype CD8<sup>+</sup> T in response to anti-PD-1 antibody treatment, which is consistent with the biological effect of A20 on the therapeutic efficacy of anti-PD-1 immunotherapy.

**DISCUSSION**

So far, approved anti-PD-1 antibodies are widely applied in clinical practice, which significantly prolongs the survival of patients with advanced melanoma. However,
low response rate and the establishment of drug resistance significantly restrain the durable effect of anti-PD-1 antibodies. Some studies have signified that tumorous PD-L1 expression is closely associated with clinical response and therapeutic efficacy of immune checkpoint blockade in melanoma. Nevertheless, the role of tumorous PD-L1 in the efficacy of immunotherapy remains controversial. For one thing, high expression of PD-L1 in tumor cells before treatment is regarded as a biomarker of better response. For the other, in response to anti-PD-1 antibody treatment, the re-activation of CD8+ T cell is in position to promote the secretion of IFN-γ, which induces feedback upregulation of PD-L1 that limits the effect of PD-1/PD-L1 blockade. Hence, the role of tumorous PD-L1 in the regulation of therapeutic outcome of immunotherapy is equipped with a bearing on the time phase of treatment (before or during the treatment), and suppressing the sustained PD-L1 upregulation in tumor during treatment could be helpful for obtaining durable therapeutic effect and elevating the therapeutic efficacy. In the present study, the increased A20 expression contributed to the upregulation of PD-L1 expression in melanoma after anti-PD-1 antibody treatment, indicating that A20 is required for sustained PD-L1 expression that causes treatment resistance. Therefore, controlling A20 expression in tumor during anti-PD-1 antibody treatment is a valuable strategy to increase therapeutic efficacy and provide clinical benefits for patients with melanoma and other cancer types.

Inflammation-related signals can trigger the eradication of cancer immune escape and influence the outcome of immunotherapy, but the underlying mechanism needs to be further elucidated. A20 has always been recognized as an important modulator of inflammation and immune response. Through the ubiquitination of RIPK1 and TRAF6, A20 is capable of inhibiting proinflammatory NF-κB pathway and the expressions of downstream cytokines. Moreover, the loss of function of A20 could cause inflammatory response and even lead to the onset and development of many autoimmune diseases. Recently, the regulatory role of A20 in immune cells has been expanded to be associated with antitumor immunity. Precisely, the overexpression of A20 in CD8+ T cells can dampen the nuclear accumulation of NF-κB components and impose a brake on the antitumor activity of CD8+ T cells. In addition, in vivo silence of A20 expression in DCs could potentiate the antitumor immune response and delay tumor progression. It is necessary to note that A20 is also abundantly expressed in tumor cells that are dominant in the tumor microenvironment. Still, previous studies mainly paid attention to the role of tumorous A20 in the regulation of tumor cell behavior instead of surrounding immune cells. Extending to this, our present study demonstrated that A20 was proved as a novel upstream regulator of PD-L1 and was responsible for attenuated activity of infiltrated CD8+ T cell and thereby tumor progression and immunotherapy resistance. Therefore, A20 is capable of exerting its immunosuppressive effect against tumor immunity in various ways, not only by regulating the function of immune cells directly, but also by activating suppressive signals in immune cells indirectly through tumorous immune checkpoints like PD-L1.

Cumulating evidence has revealed the regulatory mechanisms of PD-L1 expression from the perspective of transcriptional, translational and post-translational modification, respectively, which can be connected to multiple hallmark characteristics of cancer, including oncogene, cell-cycle progression and lipid metabolism. Inflammation is also crucial for cancer pathogenesis owing to its contribution to tumor cell survival, angiogenesis, cancer metastasis and the eradication of antitumor immunity. A recent study demonstrated the proinflammatory cytokine TNF-α as a major factor triggering cancer immunosuppression against T cell surveillance via the stabilization of PD-L1. Our data forwardly emphasized the connection between inflammation and antitumor immunity by demonstrating that A20 promoted the expression of PD-L1 to impair the antitumor activity of CD8+ T cells. Mining the publicly available transcriptomic data in TCGA SKCM database also provided us with the notion that inflammation pathway was highly correlated with PD-L1 expression in melanoma. Therefore, targeting A20 or alternative inflammation-related signals could be adopted in a broad spectrum of therapeutic backgrounds in view of the interplay among inflammation, cancer pathogenesis and tumor immune response.

The pathogenic role of A20 has been verified in different kinds of cancers, which is associated with distinct ubiquitin-editing functions. In breast cancer, A20 facilitates TGF-β1-induced tumor metastasis through multifunctional ubiquitylation of Snail, whereas in gastric cancer, A20 mediates poly-ubiquitination of RIP1 and inhibits TRAIL-induced cell apoptosis. Supplementary to these, our study recognized PHB as a novel substrate of A20. A20 acted as a ubiquitin ligase of PHB by binding K48-linked ubiquitin chain and accelerating its proteosomal degradation, which extended the substrate network of A20 and the regulatory mechanism of PHB. In addition, PHB directly interacted with STAT3 and suppressed its phosphorylation and activation, which was attenuated after A20 overexpression. Our results indicated an A20-PHB-STAT3-PD-L1 regulatory axis in melanoma, which was supported by further bioinformatics analysis on TCGA SKCM database. It ought to be noted that previous studies have demonstrated that PHB mediated the resistance to chemotherapy and BRAF-targeted therapy in tumor. More than this, our study implicated that PHB also mediated the resistance to immunotherapy by transducing the signal from A20 to STAT3, suggesting PHB as a versatile regulator and a promising therapeutic target for the determination of the treatment outcomes of diverse therapeutic approaches in cancer.
inhibitory receptors like PD-1, is largely responsible for the effect of anti-PD-1 antibody. In consistent with this notion, the suppression of tumorous A20 could revive the antitumor capacity of infiltrated CD8+ T cells, synergizing with the effect of anti-PD-1 antibody treatment. In fact, several previous studies have demonstrated the crucial role of tumor intrinsic molecules in the regulation of surrounding immune cells and their antitumor immunity. Hence, to obtain the invigoration of dysfunctional immune cells by targeting tumor cells is promising for elevating the efficacy of immunotherapy. What’s more, T cell invigoration to tumor burden ratio is highly associated with the clinical response of anti-PD-1 antibody treatment. Since that the expression of tumorous A20 was in negative correlation with the T cell invigoration to tumor burden ratio, the application of tumorous A20 expression status to guiding appropriate use of anti-PD-1 antibody in treating advanced melanomas can be taken into consideration in the future.

Previous studies have expounded the excellent translational potential of targeting A20 in terms of treating various diseases. Precisely, A20 in DCs is able to suppress NF-kB signaling and constrain DCS-mediated T-cell stimulation, mitigating T-cell-dependent blood pressure elevation. In addition, the downregulation of A20 contributes to the development of childhood asthma, and acts as a possible biomarker for predicting asthma. Consequently, targeting A20 has the translational potential for the prevention and treatment of hypertension and asthma, and it is further expanded for cancer treatment with particular focus on PD-L1-dependent antitumor immunity according to our present investigation, providing a novel method for immunity-based cancer therapy for the crucial regulatory role of A20 in PD-L1 expression and inflammatory response as well. Conceivably, some clinical trials have enrolled the patients for the test of combined therapies of anti-PD-L1/PD-1 antibodies and the inhibitors of the upstream regulatory molecules of PD-L1 like Akt and STAT3 (NCT03421353, NCT03772561). Moreover, several studies demonstrated that the concurrent suppression of the upstream regulator of PD-L1 expression could increase the efficacy of immunotherapy in pre-clinical mouse model. Given the findings of the present study, it is necessary to testify the translational potential of targeting A20 in cancer immunotherapy in prospective trials.

CONCLUSIONS

In summary, our findings delineate the interplay between inflammation-related signal and antitumor immunity by expounding that A20 controls PD-L1 expression via PHB-STAT3 axis so as to confer therapeutic resistance to anti-PD-1 immunotherapy. Targeting tumorous A20 is able to potentiate the antitumor capacity of cytotoxic CD8+ T cells and promote the therapeutic response to PD-1/PD-L1 blockade in melanoma. Given the fact that dysregulated A20 contributes to the pathogenesis of various cancers and A20 is a crucial regulator of inflammation and immunity, our study provides the proof of a principle for the development of immunotherapeutic strategy in cancers that are associated with inflammation.

References


28 Zou W, Wolchok JD, Chen L. PD-L1 (B7-H1) and PD-1 pathway blockade for cancer therapy; mechanisms, response biomarkers, and combinations. Sci Transl Med 2016;8:328rv324.


Supplementary information

Supplementary Methods

Supplementary Figure Legends
Supplementary Methods

Cell culture, reagents and clinical samples

Human melanoma cell lines A375, A2058 and WM35 were grown in Dulbecco’s modified Eagle’s medium (Gibco, Grand Island, USA). B16F10 melanoma cells (TCM2, Chinese Academy of Sciences, Shanghai, China) and PBMCs derived from C57BL/6 mouse (Jackson Lab, Bar Harbor, USA) were grown in RPMI 1640 Medium (Gibco). All the cultured cells were supplemented with 10% fetal bovine serum (Gibco) and 1% Pen Strep Glutamine (Gibco). Cells were grown at 37 °C in 5% CO₂ and lifted with 0.05% Trypsin-EDTA (Gibco). In co-culture system, B16F10 melanoma cells and PBMCs derived from C57BL/6 mouse were incubated in 12-well plates, with melanoma cells on the bottom layer and PBMCs suspended in the medium for 24 hours. All these melanoma cell lines were authenticated by short-tandem repeat (STR) fingerprinting in center of DNA typing in Fourth Military Medical University and tested for mycoplasma contamination. The phosphorylated JAK2 inhibitors WP1066 (S2796, Selleck, Houston, USA) was dissolved in DMSO and used at the concentration of 2.5 μM for 24 hours. Protein synthesis inhibitor Cycloheximide (HY-12320, MCE, South Brunswick, USA) was dissolved in DMSO and used at the concentration of 5 nM for 0, 4 hours and 8 hours, respectively. Proteasome inhibitor MG132 (474790, Calbiochem, Darmstadt, Germany) was dissolved in DMSO and used at the concentration of 5 μM for 24 hours.

11 melanoma patients treated with anti-PD-1 antibody were included in this study. Five patients were treated with toripalimab and the other six were treated with pembrolizumab. Patients were categorized into 6 responders (CR, PR, or SD of greater than 6 months with no progression) and 5 non-responders (PD or SD for less than or equal to 6 months before disease progression) as
evaluated by standard Response Evaluation Criteria In Solid Tumors (RECIST v1.1) guidelines.\(^1\) The tumor tissues and peripheral blood were obtained before the initiation of the indicated treatment from melanoma patients who were diagnosed as melanomas at AJCC stage III or stage IV. Informed consent was obtained from all the enrolled patients and the study was approved by the ethics committee of Xijing Hospital, Fourth Military Medical University. Clinical information of each patient is displayed in Figure 1A and Supplementary Table S1. Moreover, peripheral blood was extracted after 6 weeks of the treatment with anti-PD-1 antibodies and PBMCs were separated by lymphosep (DKW) according to the manufacturer’s recommended procedures. Flow cytometry was used for analyzing the characteristics of these cells with indicated interventions.

**ChIP assays**

ChIP assays were performed as previously described.\(^2\) Briefly, 3-5 \(\times 10^7\) cells were crosslinked with 1% paraformaldehyde at room temperature for 15 minutes and sonicated to generate chromatin fragments of 200-600 bp. Fragmented chromatin was then immunoprecipitated overnight with STAT3 antibody (ab119352, abcam), followed by washes and elution. ChIP DNA from A375 and A2058 cells transfected with A20 siRNA or control siRNA was quantified by qRT-PCR. The \textit{PD-L1} primers used were: Forward: 5’-GAGGAAGTCACAGAATCCACGA-3’, Reverse: 5’-AAAGTCAGCAGCAGACCCAT-3’.

**Mass spectrum analysis**

As previously described,\(^3\) the protein bands of interest were excised from Coomassie blue-stained gel. Each gel slice was diced into small pieces (1 mm \(\times\) 1 mm) and placed into a 1.5-ml tube. A gel
piece that was removed from a protein-free region of the gel was used as a parallel control. Sample preparation used for Q-Exactive mass spectrometry was performed according to the standard protocol as described previously. Gel slices were de-stained and digested in 20 μl of sequencing grade trypsin at 37 °C overnight. The protein digests were later desalted for MS and MS/MS analysis, which were performed with the use of the QExact system (Thermo Fisher Scientific, USA). Afterward, Proteome Discoverer software (version 1.4; Thermo Fisher Scientific, USA) was applied for protein identification and quantitation.

**Plasmid vectors and siRNA transfection**

Small interfering RNA (GenePhama, Shanghai, China) was used to knockdown A20 and PHB expression. Lipofectamine 3000 transfection Reagent kit (L3000-015, Invitrogen, Carlsbad, USA) was used for transfection according to the manufacturer’s recommended procedures. Mouse CRISPR/Cas9 plasmid (sc-423436, Santa Cruz, Dallas, USA) was used to obtain the knockout of A20 in B16F10 melanoma cells with the use of Lipofectamine 3000 transfection Reagent kit. After 72 hours of transfection, puromycin (ab141453, abcam, Cambridge, UK) was used to remove the cells with no transfection efficiency. Then the screened cells were separated in 96 wells plate as 1 cell/well to incubate. Gene sequencing was used to obtain the clone with A20 knockout. The PD-L1 overexpression plasmid (CD274-pEX-4 plasmid), A20 overexpression plasmid (A20-pHB plasmid), wild type PHB (PHB WT-pEX-3) and mutant PHB plasmids (PHB mut186-pEX-3 and PHB mut202-pEX-3) as well as empty plasmid vectors were designed and made by GenePharma, and transferred into melanoma cells with Lipofectamine 3000 transfection Reagent kit according to the manufacturer’s recommended procedures. Neomycin (S2568, Selleck) and puromycin (abcam) was used to remove the non-transfected cells. The sequences of used siRNAs against A20 and PHB were
as follows: siRNA A20 seed sequence-1: 5′-CAUGCACCGAUACACACUUTT-3′; siRNA A20 seed sequence-2: 5′-CUGGAAGAAAUACACAUAUTT-3′; siRNA PHB seed sequence-1: 5′-AGCCAGCUCUCCUCGCAUCUdTdT-3; siRNA PHB seed sequence-2: 5′-CCCAGAAAUCAACUGUGAAAdTdT-3′.

**Immunoprecipitation**

For immunoprecipitation (IP) analysis, cultured cells were solubilized and protein extracts (500 μg) were used for immunoprecipitation with antibodies against A20, PHB or STAT3. Cells with indicated treatment were collected and put into immunoprecipitation assay using Pierce Co-IP kit (Thermo Scientific) according to manufacturer’s instructions. In brief, 20 μg rabbit polyclonal anti-A20 antibody (ab93868, Abcam), rabbit monoclonal anti-PHB antibody (ab75766, Abcam) or mouse monoclonal anti-STAT3 antibody (9139, Cell Signaling Technology) was added directly to the resin in the spin column. The column was capped and incubated at room temperature for 90-120 mins using a rotating body or mixer. After the antibody was immobilized, the protein extracts (500 μg) was added to the resin, followed by being evenly turned over at 4 °C overnight. Immune complexes were washed extensively 4 times with Tris-buffered saline containing 0.05% Tween-20 detergent and subjected to western blot.

**Western blot**

Western blot was performed as described previously. In brief, samples were separated with SDS-PAGE, transferred to polyvinylidene difluoride membrane and probed with the corresponding antibodies. The dilution of antibodies were as follows: anti-human A20 monoclonal rabbit (1:1000, ab92324, abcam), anti-mouse A20 monoclonal mouse (1:1000, ab13597, abcam),
anti-phospho-STAT3 (Y705) monoclonal rabbit (1:1000, ab76315, abcam), anti-STAT3 monoclonal mouse (1:1000, 9139, Cell Signaling Technology, Cambridge, UK), anti-phospho-STAT3 (S727) polyclonal rabbit (1:1000, 9134, Cell Signaling Technology), anti-JAK2 rabbit polyclonal rabbit (1:1000, ab39636, abcam), anti-PHB monoclonal rabbit (1:1000, ab75766, abcam), anti-human PD-L1 monoclonal rabbit (1:1000, ab205921, abcam), anti-mouse PD-L1 monoclonal rabbit (1:1000, ab213480, abcam), anti-Ubiquitin (linkage-specific K48) monoclonal rabbit (1:1000, ab140601, abcam), anti-c-myc monoclonal rabbit (1:1000, ab32072, abcam), anti-mcl-1 monoclonal rabbit (1:1000, ab32087, abcam), anti-mouse and anti-rabbit secondary antibodies (1:3000, 115-035-003, Jackson ImmunoResearch, West Grove, USA). Signals were detected using Western ECL Substrate (Thermo Scientific).

**Real-Time PCR**

Total RNA was extracted by RNAiso Plus (Takara, Kyoto, Japan). Isolated RNA was reversely transcripted into cDNA using the First Strand cDNA Synthesis Kit (Takara) according to the manufacturer’s instruction. Subsequent gene expression was then analyzed using SYBR Select Master Mix (Takara). We designed and used the following gene-specific primers: 5’-GGTGCCGACTACAAGCGAAT-3’ (Forward) and 5’-AGCCCTCAGCCTGACATGTC-3’ (Reverse) for PD-L1, 5’-GCAGATAGCCAAGGGTATGAGTTACC-3’ (Forward) and 5’-TTTTGCCAGCCCAAAATCTGT-3’ (Reverse) for PHB, and designed forward primers span exon-exon junctions where possible.

**Tumor tissue microarray**
Melanoma tissue microarray (M1004e) was purchased from US Biomax Inc. (Rockville, USA).

Detailed information can be accessed via http://www.biomax.us/tissue-arrays/Melanoma/ME1004e.

**Mouse xenograft tumor model**

All mice were maintained under pathogen-free conditions at the Fourth Military Medical University, Xi’an, China, and were at the age of 4-6 weeks at the time of cell implantation. Mice were kept at 12 hours/12 hours light cycle and received standard food and water. All animal studies and experimental procedures were approved by the Animal Care and Use Committee of the animal facility at the Fourth Military Medical University. The experimental design and number of mice assigned to each treatment were based on prior experience with similar models and provided sufficient statistical power to discern significant differences.

In C57BL/6 mice, the xenograft mice model was established with the subcutaneous implantation with wild type-B16F10 melanoma cells or B16F10 melanoma cells transfected with either CRISPR/Cas9 A20 KO plasmid (sc-423436, Santa) or CD274-pEX-4 plasmid (GenePhama) which encodes PD-L1 protein. $5 \times 10^5$ cells were injected subcutaneously per mouse. In the case of anti-PD-1 antibody treatment, the mice were randomly divided into 4 groups and, once the tumor volume reached around 100mm,$^3$ the mice were intraperitoneally injected with anti-PD-1 antibodies (100μg/mouse) (Clone J43, Bioxcell, West Lebanon, USA) every second day. For the depletion of immune cells, mice were injected i.p. with 200μg monoclonal antibodies against CD8$^+$T cells with anti-CD8 antibody (clone 2.43, Bioxcell) on the first day of treatment and followed by every 2 days throughout the experiment. For mouse blood samples, up to 2ml blood per mouse was extracted and PBMCs were separated according to the manufacturer’s recommended procedures of lymphosep (DKW). Lymphocytes were calculated and incubated in co-cultured system with B16F10 cells as
human cells co-culture system mentioned above. Flow cytometry was used for analyzing the characteristics of these cells with indicated interventions.

**Flow cytometry analysis**

For the analysis of the characteristics of infiltrating CD8\(^+\)T cells in tumor, flow cytometry assay was immediately performed at the time of specimen collection on freshly obtained melanoma patients or mouse tumor specimens after tumor dissociation. Tumors were collected, cut and minced in HBSS containing collagenase IV (sigma), and then incubated in 37°C incubator for 45mins with shake. PBS with trypsin and DNase (sigma) were used for another 15 mins incubation in shaking-37°C incubator. The digested tissues were washed with PBS twice and then filtered with 80μM filter. Then single cell suspensions were incubated with ACK lysis buffer (Invitrogen) for 5 mins in room temperature to remove red blood cells as previously described.\(^6\) Cells were then re-suspended and analyzed on a Beckman Coulters Gallios flow cytometer. Antibodies were used as follows: anti-human CD3 (317308, biolegend, San Diego, USA), anti-human PD-L1 (329708, biolegend), anti-mouse PD-L1 (329718, biolegend), anti-human CD8 (344704, biolegend), anti-mouse CD8a (100706, biolegend), anti-human Ki67 (350526, biolegend), anti-mouse Ki67 (652406, biolegend), anti-human PD-1 (329908, biolegend), anti-mouse PD-1 (135216, biolegend), anti-human/mouse Granzyme B (515403, biolegend), anti-mouse Granzyme B (372220, biolegend), anti-human Perforin (308105, biolegend), anti-mouse Perforin (154306, biolegend). Samples were analyzed with Cytexpert software.

For the detection of cell death in melanoma with or without the knockout of A20, melanoma cells with indicated treatment were harvested by trypsinization, washed twice with 4 °C PBS and re-suspended in binding buffer. Annexin V-PE and 7AAD solution (4A Biotech, Beijing, China)
were then added to stain the cells before analysis by flow cytometry (Beckman Coulter). The apoptotic rate was assessed and calculated after at least three independent experiments, with at least three biological replicates each time.

**Gene set enrichment analysis**

To perform the gene set enrichment analysis, GSEA software was used to derive the absolute enrichment scores using C2 curated subset (experimentally validated gene sets) of the Molecular Signature Database version 6.0,\(^7,8\) based on KEGG pathways.\(^9\)

**Cell co-culture system**

For the construction of the *in vitro* co-culture system, PBMCs were isolated from peripheral whole blood of C57BL/6 mouse that were burdened with B16F10 tumor using Ficoll (DKW, China). The PBMCs were seeded at 1×10\(^6\) cells/well and cultured with B16F10 melanoma cells (2.5×10\(^5\) cells/well) in the presence of 100 ng/ml anti-CD3\(\varepsilon\) mAb-coated co-culture system. The PBMCs and B16F10 cells were cultured in RPMI 1640 supplemented with 10% FBS, L-glutamine, antibiotic and IL-2 (100 U/mL) at 37°C in a 5%-CO\(_2\) incubator. After 72h, cells were harvested and go through FACS analysis. For the detection of apoptotic melanoma cells, group of CD45\(^-\) cells from the co-cultured system were gated, and PI and Annexin V staining were used for the detection of cell apoptosis. For the analysis of CD8\(^+\) T cells, group of CD45\(^+\)CD3\(^+\)CD8\(^+\) cells were gated, and the percentage of Ki-67\(^+\) and Granzyme B\(^+\) cells were analyzed with the use of the corresponding antibodies described in *Flow cytometry analysis* part.

**Histology and immunohistochemical staining analysis**
Melanoma tissues were fixed in 10% neutral buffered formalin and paraffin-embedded (FFPE). 2μm FFPE consecutive tumor sections were stained with Hematoxylin (Dako, Copenhagen, Denmark) and Eosin G (Dako). For immunohistochemical staining analysis, paraffin-embedded melanoma tissues of tumor tissue microarray (TMA) or implanted tumor in C57BL/6 mice were de-paraffinized and rehydrated with graded ethanol dilutions. After antigen retrieval in Tris-EDTA Buffer (10 mM Tris Base, 1 mM EDTA Solution, 0.05% Tween 20, pH 9.0), goat serum was added to block nonspecific binding for 30 mins. Tissue sections were then incubated with anti-mouse/human A20 (1:50, ab92324, abcam), anti-human PHB (1:100, ab 75766, abcam), anti-human PD-L1 (1:250, ab213524, abcam) or anti-mouse PD-L1 (1:100, ab238697, abcam) at 4°C overnight, followed by anti-rabbit alkaline phosphatase secondary antibody. The section was then incubated in Fast Red solution, and subsequently counterstained with hematoxylin and mounted with glycerol. The staining intensity was calculated as integrated optical density (IOD) using Image-Pro® Plus software (version 5.1; Media Cybernetics, Inc., Rockville, MD, USA) as previously described.10

Immunofluorescence staining analysis

Melanoma cells for immunofluorescence staining analysis were first washed with PBS, fixed with 4% paraformaldehyde for 10–20 mins and permeabilized with 0.5% Triton for 15 mins. After washing the slides with PBS, cells were blocked with normal goat sera at room temperature for 1 hour. Then, cells were incubated with primary antibody diluted in the blocking solution at 4°C overnight. Paraffin-embedded tissue sections were de-paraffinized and rehydrated with graded ethanol dilutions. After antigen retrieval in Tris-EDTA Buffer (10 mM Tris Base, 1 mM EDTA Solution, 0.05% Tween 20, pH 9.0), goat serum was added to block nonspecific binding for 30 mins,
followed by the incubation with primary antibody. The primary antibodies were as follows:
anti-human/mouse A20, 1:200, ab13597, abcam; anti-Melan-A, 1:200, ab51061, abcam;
anti-phospho-STAT3 Y705, 1:100, 9145, Cell Signaling Technology; anti-STAT3, 1:1500, #9139,
Cell Signaling Technology; anti-PHB, 1:100, ab75766, abcam; anti-CD8, 1:100, ab237709, abcam;
anti-CD45, 1:100, 60287-1-Ig, Proteintech. Cells or tissue sections were then washed with PBS and
probed with the secondary antibodies conjugated to Alexa Fluor 488 (Invitrogen, Carlsbad, USA)
and Alexa Fluor 568 (Invitrogen) for 1 hour and were stained with DAPI (Roche, Basel,
Switzerland). Images were obtained using an inverted confocal laser scanning microscope (Zeiss).
The staining intensity was calculated as integrated optical density (IOD) using Image-Pro® Plus
software (version 5.1; Media Cybernetics, Inc., Rockville, MD, USA) as previously described ⁶⁸.

**ELISA assay**

ELISA analysis on culture medium of melanoma cells with/without A20 knockdown was performed
using the Human IL-6 ELISA Kit (Neobioscience) according to the manufacturer’s instructions. The
absorbance (A450) was measured with a plate reader (Bio-Rad).
Reference


**Supplementary Figure Legends**

**Figure S1.** (A) Immunofluorescence staining analysis of the subcellular distribution of A20 in melanoma tissues (n=5). Scale bar = 50μm. (B) Immunoblotting analysis shows expression of A20 in B16F10 melanoma cells with or without the knockout of A20 expression. (C) Apoptotic tumor cells were analyzed by flow cytometry analysis in B16F10 cells with or without the knockout of A20. (D) CD8 expression in peripheral blood lymphocytes of C57BL/6 mice intraperitoneally injected with anti-CD8 antibody was determined by flow cytometry at day 10 after the first injection. (E) Analysis in TCGA SKCM database shows the association between CD274 level and TNFAIP3 level. *P* value was calculated by linear regression analysis. (F) The histogram illustrates relative mRNA expression of PD-L1 in A375 and A2058 cells with or without the knockdown of A20. *P* values were calculated by paired Student’s *t*-test.

**Figure S2.** (A) The apoptosis of melanoma cells with indicated interventions in the co-culture system. *P* values were calculated by paired Student’s *t*-test. (B) Western blots shows expression of JAK2 in melanoma cells with indicated interventions of A20. (C) ELISA assay revealed changes of IL-6 level in supernatant of cultured melanoma cells. (D) Western blot analysis shows expression of SOCS3 in melanoma cells with indicated interventions of A20. (E) Western blot analysis shows the expression of PHB after the indicated interventions of A20. (F) Relative mRNA expression of PHB in A375 and A2058 cells after the indicated interventions of A20. (G) IHC staining of the PHB proteins in TMAs consisting of 18 nevus, 62 primary melanomas and 20 metastatic melanomas. Scale bar = 100μm. (H) The correlation between CD274 and PHB mRNA expressions in TCGA SKCM database. *r* value was calculated by Spearman correlation, and *P* value was calculated by two-tailed Student’s *t*-test. (I) The correlation between TNFAIP3 and PHB mRNA expressions in
TCGA SKCM database. $r$ value was calculated by Spearman correlation, and $P$ value was calculated by two-tailed Student’s $t$-test.
Figure S1

A

![Images of fluorescent staining and western blots for melan-A, A20, DAPI, and Merge]

B

![Western blots for A20 and Tubulin with annotations]

C

![Flow cytometry plots for 7AAD and Annexin V-PE for NC and KO A20]

D

![Flow cytometry plot for CD8 and CD3+ gate]

E

![Scatter plot showing Log2(CD274) vs Log2(TNFAIP3) with correlation r=0.2133, P<0.0001]

F

![Bar graphs comparing PD-L1 and A375/A2058 with annotations]

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Figure S2

A

Apoptosis (% in tumor cells)

B

A375

A2058

E

A2058

A375

E

A2058

C

A375

A2058

P<0.0002

P<0.0001

P<0.0001

P<0.0001

JAK2

Tubulin

Tubulin

P=0.09639

P=0.0456

Phb

Phb

D

A375

A2058

E

A2058

F

A375

A2058

P=0.0001

P=0.0001

P<0.0001

P<0.0001

PHB

PHB

G

NV

PM

MM

H

Spearman r = 0.2651

Spearman r = 0.4311

P<0.0001

P=0.0035

I

Log2(PHB)

Log2(TNFAlpha)