A Tumor-intrinsic PD-L1-NLRP3 Inflammasome Signaling Pathway Drives Resistance to Anti-PD-1 Immunotherapy

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ABSTRACT

An in-depth understanding of immune escape mechanisms in cancer are likely to lead to innovative advances in immunotherapeutic strategies. However, much remains unknown regarding these mechanisms and how they impact immunotherapy resistance. Using several pre-clinical tumor models as well as clinical specimens, we report a newly identified mechanism whereby CD8+ T cell activation in response to PD-1 blockade induced a PD-L1-NLRP3 inflammasome signaling cascade that ultimately led to the recruitment of granulocytic myeloid-derived suppressor cells (PMN-MDSCs) into tumor tissues, thereby dampening the resulting anti-tumor immune response. The genetic and pharmacologic inhibition of NLRP3 suppressed PMN-MDSC tumor infiltration and significantly augmented the efficacy of anti-PD-1 antibody immunotherapy. This pathway therefore represents a tumor-intrinsic adaptive resistance mechanism to anti-PD-1 checkpoint inhibitor immunotherapy and is a promising target for future translational research.

KEYWORDS

NLRP3 inflammasome, PD-L1, Wnt5a, HSP70, myeloid-derived suppressor cells, adaptive resistance, anti-PD-1 immunotherapy, checkpoint inhibitor, melanoma, PKR
INTRODUCTION

Despite the significant impact that checkpoint inhibitor immunotherapies have generated in clinical oncology, the majority of cancer patients still do not benefit from this treatment modality (1). It is widely believed that a more intimate understanding of the underlying mechanisms driving cancer immunotherapy resistance will lead to the discovery and development of innovative strategies to augment the efficacy of immunotherapy while expanding the patient population capable of benefitting from these agents (2, 3). However, our understanding of the mechanisms driving both primary and secondary immunotherapy resistance remains incomplete.

There is an extensive body of literature describing the inhibitory role of myeloid-derived suppressor cells (MDSCs) in the generation of adaptive T cell immunity (4, 5). These data are consistent with additional studies that have correlated elevated circulating MDSC levels with poor clinical responses to both anti-cytotoxic T lymphocyte antigen-4 (CTLA-4) and anti-program death-1 (PD-1) antibody immunotherapy in advanced melanoma patients (6, 7). MDSCs have been shown to undergo chemotaxis toward tumor beds via chemokine gradients generated by the developing tumor. In particular, migration of the granulocytic subset of MDSCs (PMN-MDSCs) relies primarily on the chemokine receptor, CXCR2, and several of its cognate ligands, including CXCL5 (8). Additional work has shown that CXCR2 blockade enhances the efficacy of anti-PD-1 ab immunotherapy in models of both pancreatic cancer and sarcoma (8-10). These findings suggest that this immunosuppressive cell population plays a critical role in determining the outcome for those cancer patients undergoing anti-PD-1 ab therapy. However, the exact mechanism by which MDSCs interfere with the development of anti-tumor immunity in response to checkpoint inhibitor immunotherapy has remained unclear.
It is well known that the immune system is comprised of many negative feedback inhibitory pathways that serve to suppress the development of overzealous immune responses to avoid autoimmune pathology. Similar mechanisms are likely to serve as the molecular underpinnings for the development of adaptive resistance to anti-PD-1 antibody immunotherapy and represent key pathways of interest for the future development of improved combinatorial immunotherapy strategies (11). Indeed, recent studies have demonstrated both CD8\(^+\) T cell-dependent and interferon-dependent upregulation of colony-stimulating factor-1 in melanoma and CD38 in lung cancer promote adaptive resistance to anti-PD-1 checkpoint blockade (12, 13). These observations are reminiscent of interferon-dependent upregulation of the immunoregulatory enzyme, indoleamine 2,3-dioxygenase (IDO), which serves to re-establish immune tolerance in response to cytolytic T cell activity (14, 15). While a recent study has implicated tumor expression of CXCL1 and the recruitment of PMN-MDSCs as key factors that mediate against tumor T cell infiltration, a role for CXCR2-dependent chemokines in the generation of adaptive resistance to anti-PD-1 antibody immunotherapy has not been described (16).

Previous work, including our own, has demonstrated the Wnt5a ligand to be associated with tumor progression, immune evasion, and immunotherapy resistance (17-19). Interestingly, toll-like receptor-4 (TLR4) signaling regulates Wnt5a expression in myeloid cells and has also been associated with tumor progression in a variety of cancer types (20, 21).

Reports of tumor intrinsic signaling pathways induced by PD-L1 have emerged, linking PD-L1 with the promotion of epithelial-to-mesenchymal transition (EMT), the stimulation of the mTOR-Akt anti-apoptotic pathway, as well as with the inhibition of interferon-dependent
apoptosis (22-24). While each of these pathways may be pro-tumorigenic, there are no known associations between PD-L1 and the induction of adaptive resistance to anti-PD-1 antibody immunotherapy via the stimulation of tumor intrinsic signaling pathways. Many groups have described the role of NOD-, LRR- and pyrin domain-containing protein-3 (NLRP3) as a sensor for pathogen-derived danger signals by antigen-presenting cells in the innate immune system, however relatively little is known about the contribution of NLRP3 to tumorigenesis and its role in modulating tumor responses to immunotherapy has not been explored (25).

Herein, we describe a pathway mechanistically linking the upregulation of PD-L1 with the promotion of PMN-MDSC recruitment to the tumor bed in response to anti-PD-1 blockade and demonstrate that the inhibition of this process significantly enhances responses to checkpoint inhibitor immunotherapy (Figure 1A). Further evidence is provided supporting the existence of this pathway in cancer patients.
RESULTS

Anti-PD-1 Antibody Immunotherapy Induces the Recruitment of PMN-MDSCs.

We have found that the autochthonous BRAFV600E/PTEN-/- melanoma model exhibits a transient response to anti-PD-1 antibody (ab) immunotherapy followed by eventual escape and progression. After harvesting these melanoma tissues following anti-PD-1 ab escape as well as after IgG isotype control ab therapy, differential whole transcriptomic sequencing analysis was performed. This study revealed the upregulation of 51 genes in anti-PD-1 ab-treated tumor tissues using a fold-change cutoff of 2.0 ($P < 0.05$). Of these genes, two CXCR2 ligands, Cxcl5 (3.75-fold, $P = 8.88 \times 10^{-6}$) and Cxcl3 (3.49-fold, $P = 0.002$), were found in the top 7 upregulated genes while Cxcl2 was also noted to be upregulated by 3.63-fold ($P = 0.146$). These gene expression changes occurred concurrently with enhanced expression of the pro-inflammatory proteins, S100a8 (2.27-fold, $P = 1.61 \times 10^{-10}$) and S100a9 (2.27-fold, $P = 3.37 \times 10^{-11}$) as well as Arg1 (1.45-fold, $P = 1.95 \times 10^{-6}$) (Figure 1B). We repeated the above experiment using a serial tissue biopsy approach coupled with quantitative real-time polymerase chain reaction (qrt-PCR) gene expression analysis, confirming a time-dependent increase in the expression of Cxcl2, Cxcl5, Cxcr2, Ly6g, and the myeloid marker S100a8 during the course of anti-PD-1 ab therapy relative to those tumors undergoing treatment with an IgG isotype ab (Figures 1C, Supplementary Figure 1A). Together, these data suggest that immunosuppressive granulocytic myeloid-derived suppressor cell (PMN-MDSC) recruitment may correlate with a suppression of cytolytic T cell activity along with anti-PD-1 ab escape (Supplementary Figure 1A). To investigate this hypothesis, we evaluated resected melanoma tissues based on Gr-1 immunohistochemistry (IHC) as well as multi-parameter flow cytometry, both confirming a significant increase in an infiltrating
Gr-1+ and CD45+CD11b+Ly6G+Ly6CintF4/80− cell population (PMN-MDSCs), respectively, with progression through anti-PD-1 ab therapy (Figures 1D,E). These findings were recapitulated in the Lewis lung carcinoma lung cancer model, an orthotopic p53;Kras pancreatic cancer model, as well as a humanized autologous patient-derived xenograft model of renal cell carcinoma (Supplementary Figure 1B). However, we did not see any evidence of this effect following anti-CTLA-4 ab therapy (Supplementary Figure 1C). Qrt-PCR analysis of FACS sorted PMN-MDSCs from anti-PD-1 ab-treated BRAFV600EPTEN−/− melanoma tissue confirmed that these cells express high levels of Cxcr2, Tnfa, S100a8, and S100a9 (Supplementary Figure 1D). While we observed an increase in the expression of several CXCR2-dependent ligands following escape from anti-PD-1 ab therapy, CD8+ T cell ablation studies demonstrated the CXCL5 chemokine to be particularly responsive to the induction of CD8+ T cell activation (Figure 1F). In addition, CXCL5 has previously been implicated in melanoma pathogenesis (26). Thus, we genetically silenced CXCL5 expression in a BRAFV600EPTEN−/− melanoma cell line which effectively eliminated PMN-MDSC recruitment, enhanced tumor CD8+ T cell infiltration, and significantly increased the sensitivity of BRAFV600EPTEN−/− melanomas to anti-PD-1 ab immunotherapy (Figures 1G, Supplementary Figure 2). Further in vivo tumor studies utilizing a pharmacological CXCR2 inhibitor (AZD5069) also significantly suppressed PMN-MDSC recruitment in response to anti-PD-1 ab therapy, enhanced CD8+ T cell tumor infiltration, and suppressed tumor progression in the autochthonous BRAFV600EPTEN−/− melanoma model (Figure 1H). Notably, the impact of AZD5069 was noted to be more significant at later time points correlating with the period of PMN-MDSC influx into the tumor (Supplementary Figure 2D). Together, these data indicate that tumors exhibit an increase
in CXCR2 ligand-mediated PMN-MDSC recruitment to the tumor bed during their progression through anti-PD-1 ab immunotherapy.

**Wnt5a Promotes CXCR2 Ligand Expression in Response to Anti-PD-1 Immunotherapy.**

The differential whole transcriptomic analysis of the autochthonous BRAF<sup>V600E</sup>PTEN<sup>−/−</sup> melanoma model undergoing anti-PD-1 ab versus IgG isotype control ab therapy demonstrated several genetic changes suggestive of enhanced Wnt ligand signaling during anti-PD-1 ab escape (Supplementary Figure 3A). Additional analysis of transcriptomic data from patients with metastatic melanoma in the TCGA database (SKCM) also revealed a statistically significant association between WNT5A and CXCL2/CXCL5/CXCR2 gene expression (Figure 2A). Previous studies have shown non-canonical Wnt ligands to activate Yes-associated protein (YAP)-dependent signaling pathways while YAP signaling has also been implicated in the migration of PMN-MDSCs into tumor tissues (27, 28). Consistent with these data, using whole tissue Western blot analysis, we found that resected melanoma tissues derived from the autochthonous BRAF<sup>V600E</sup>PTEN<sup>−/−</sup> model exhibit enhanced Wnt5a expression, YAP stabilization, and increased CXCL5 expression in response to anti-PD-1 ab therapy (Figure 2B). This enhanced level of CXCL5 expression by the tumor was also reflected by increased circulating plasma CXCL5 levels based on an enzyme-linked immunosorbent assay (ELISA) (Figure 2C). Further in vitro studies confirmed that recombinant Wnt5a (rWnt5a) induces Cxcl2, Cxcl5, and Cxcl1 gene expression in the BRAF<sup>V600E</sup>PTEN<sup>−/−</sup> melanoma cell line based on qrt-PCR analysis (Figure 2D). Given these findings, we hypothesized that the upregulation of Wnt5a results in enhanced CXCR2-dependent chemokine expression by triggering the YAP signaling pathway. Further experiments using the
BRAF<sup>V600E</sup>PTEN<sup>-/-</sup> melanoma cell line indeed demonstrated that rWnt5a induced YAP stabilization while also stimulating CXCL5 expression in a YAP-dependent manner (Figure 2E). To confirm the role of Wnt5a in the upregulation of CXCL5 in the BRAF<sup>V600E</sup>PTEN<sup>-/-</sup> melanoma cell line, we further demonstrated diminished CXCL5 expression in a Wnt5a-silenced BRAF<sup>V600E</sup>PTEN<sup>-/-</sup> melanoma cell line (BRAF<sup>V600E</sup>PTEN<sup>-/-</sup>-Wnt5a<sup>KD</sup>) by qrt-PCR, an effect that could be rescued with the addition of rWnt5a (Figure 2F, Supplementary Figure 3B). Additional studies also showed BRAF<sup>V600E</sup>PTEN<sup>-/-</sup>-Wnt5a<sup>KD</sup> tumors to exhibit reduced CXCL5 expression and an associated decrease in intra-tumoral PMN-MDSCs using both flow cytometry and IHC analysis, respectively (Figure 2G,H). Based on these data, we proposed that the previously observed recruitment of PMN-MDSCs to the tumor bed in response to anti-PD-1 ab therapy would be eliminated in tumors silenced for Wnt5a expression. Indeed, PMN-MDSC recruitment to BRAF<sup>V600E</sup>PTEN<sup>-/-</sup>-Wnt5a<sup>KD</sup> tumors was significantly diminished relative to control BRAF<sup>V600E</sup>PTEN<sup>-/-</sup> tumors in response to anti-PD-1 ab therapy (Figure 2I). Consistent with an important role for PMN-MDSCs in driving immunotherapy resistance, we also found BRAF<sup>V600E</sup>PTEN<sup>-/-</sup>-Wnt5a<sup>KD</sup> tumors to be associated with increased CD8<sup>+</sup> T cell infiltration and to respond more favorably to anti-PD-1 ab therapy relative to control BRAF<sup>V600E</sup>PTEN<sup>-/-</sup> tumors (Figure 2J, Supplementary Figure 3C). Altogether, these findings support a role for tumor-derived Wnt5a as an important mediator of CXCL5-dependent PMN-MDSC recruitment to the tumor bed in response to anti-PD-1 ab checkpoint inhibitor immunotherapy.

**An Autocrine HSP70-TLR4 Signaling Pathway Stimulates Tumor Wnt5a Release in Response to Anti-PD-1 Immunotherapy.**

Given our previous data, we then investigated the underlying mechanism for Wnt5a upregulation in response to anti-PD-1 ab therapy in the BRAF<sup>V600E</sup>PTEN<sup>-/-</sup> melanoma model. Prior studies have
shown TLR signaling to modulate Wnt5a expression in macrophages (20). Heat shock proteins (HSPs) are known to bind and induce the activation of toll-like receptor (TLR) signaling pathways (29). A review of the previous whole transcriptomic data analysis performed in the autochthonous BRAF^{V600E}PTEN/- melanoma model showed anti-PD-1 ab therapy to result in increased expression of several genes associated with cellular stress, including a subset of HSPs (Figure 3A) (30). To investigate this further, we analyzed the secretome of resected BRAF^{V600E}PTEN/- melanoma tissues following escape from anti-PD-1 ab immunotherapy by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using the stable isotope labeling with amino acids in cell culture (SILAC) technique coupled with azidohomoalanine (AHA) labeling (31). This work showed further evidence of increased HSP release, including HSP70, in those tumors that have escaped anti-PD-1 ab immunotherapy relative to IgG isotype ab control-treated tumors (Figure 3B). Consistent with this data, we also found that melanoma-bearing mice undergoing anti-PD-1 ab treatment demonstrate increased circulating plasma HSP70 levels relative to those mice treated with an IgG isotype control ab (Figure 3C). A qrt-PCR-based screen identified elevated levels of Tlr2 and Tlr4 expression by BRAF^{V600E}PTEN/- melanoma cells relative to other TLRs and an analysis of the TCGA database also revealed a significant relationship between WNT5A and TLR2/TLR4 expression in human melanoma (Figure 3D, Supplementary Figure 4A). Consistent with this, we found HSP70 stimulation of the BRAF^{V600E}PTEN/- melanoma cell line to induce the upregulation of WNT5A expression in a dose-dependent manner while pharmacologic inhibition of HSP70 suppressed autocrine stimulation of WNT5A expression (Figures 3E,F). Subsequent pharmacologic inhibitor and genetic silencing studies using a shRNA-expressing lentiviral vector demonstrated HSP70 stimulation of melanoma WNT5A expression to be TLR4-
dependent (Figure 3G, Supplementary Figures 4B,C). Interestingly, these BRAF<sup>V600E</sup>PTEN<sup>−/−</sup> melanoma cells genetically silenced for *Tlr4* did not generate tumors in vivo following their implantation. Even with modest *Tlr4* silencing using small interfering RNA oligonucleotides (siRNAs), these melanomas exhibited diminished tumor growth, reduced WNT5A and CXCL5 expression based on whole tissue Western blot analysis, and a lower level of tumor-infiltrating PMN-MDSCs along with enhanced numbers of CD8<sup>+</sup> T cells compared to control BRAF<sup>V600E</sup>PTEN<sup>−/−</sup> melanomas (Figures 3H-J, Supplementary Figure 4D,E). To verify that HSP70 induction of the WNT5A-CXCL5 signaling axis is not a specific phenomenon related to melanoma, we also repeated these experiments in a murine lung epithelial cell line with similar results (Supplementary Figure 4F). In summary, these data suggest that tumor HSP70 release in response to anti-PD-1 ab treatment induces WNT5A-mediated upregulation of CXCR2-dependent chemokine expression in tumor tissues.

**CD8<sup>+</sup> T Cells Drive the HSP70-TLR4-Wnt5a-CXCL5 Signaling Axis in Tumors.**

Since our data indicate that PMN-MDSC recruitment is induced by anti-PD-1 ab therapy, we hypothesized that CD8<sup>+</sup> T cells play an important role in triggering the HSP70-TLR4-WNT5A-CXCL5 signaling cascade. We, therefore, co-cultured increasing numbers of OT-1 K<sup>b</sup>-SIINFEKL-specific CD8<sup>+</sup> T cells with a BRAF<sup>V600E</sup>PTEN<sup>−/−</sup> melanoma cell line engineered to express the ovalbumin xenoantigen (BRAF<sup>V600E</sup>-OVA) and measured the soluble production of HSP70 (Supplementary Figure 5A). This approach showed a direct correlation between antigen-specific CD8<sup>+</sup> T cells, tumor HSP70 secretion, and the induction of WNT5A expression in vitro (Figure 4A). Further flow cytometry analysis of resected BRAF<sup>V600E</sup>PTEN<sup>−/−</sup> melanoma tissues also revealed a linear
relationship between the number of infiltrating CD8⁺ T cells and the number of PMN-MDSCs per gram of tissue following anti-PD-1 ab therapy but not in response to IgG isotype control ab therapy (Figure 4B). In line with our previous results showing that the elimination of CD8⁺ T cells abolished the increase in tumor CXCL5 expression with anti-PD-1 ab therapy (Figure 1F), further in vivo experiments showed antibody-mediated ablation of CD8⁺ T cells to also diminish PMN-MDSC recruitment in response to anti-PD-1 ab therapy (Figures 4C, Supplementary Figure 5C). Together, these data suggest that CD8⁺ T cell activity contributes to the induction of PMN-MDSC recruitment and that this process involves tumor-dependent secretion of HSP70.

Since HSP release by tumors could be interpreted to be secondary simply to tumor cell death, we inquired whether cytotoxic chemotherapy could induce similar effects. Dacarbazine chemotherapy has historically been utilized for the management of metastatic melanoma. While dacarbazine was capable of inducing BRAF⁶⁰⁰E/PTEN⁻/⁻ melanoma cell line death in vitro, this process was not associated with increased HSP70 release (Figures 4D, Supplementary Figure 5B). Consistent with this effect, dacarbazine suppressed BRAF⁶⁰⁰E/PTEN⁻/⁻ melanoma progression in vivo but did not influence the recruitment of PMN-MDSCs or the numbers of tumor-infiltrating CD8⁺ T cells (Figure 4E,F).

In light of these findings, we investigated the mechanism regulating tumor HSP70 secretion in response to CD8⁺ T cell activity. Prior studies have implicated adenosine triphosphate (ATP) as a stimulator of HSP70 release (32). Since ATP is also a classic activator of the NLRP3 inflammasome, we hypothesized that NLRP3 played a role in promoting the release of HSP70 using a similar mechanism responsible for its induction of IL-1β and IL-18 secretion (33). To address this question, we conducted in vitro experiments demonstrating that a NLRP3
inhibitor can block both ATP stimulation and CD8\(^+\) T cell-mediated induction of HSP70 release from the BRAF\(^{V600E}\)PTEN\(^-\) melanoma cell line (Figures 4G,H). These results support a potential role for the tumor NLRP3 inflammasome in CD8\(^+\) T cell-mediated HSP70 release and the observed stimulation of PMN-MDSC recruitment.

**PD-L1 Triggers NLRP3 Activation and Downstream Activation of the HSP70-Wnt5a Signaling Axis in Tumors.**

In view of the relationship between infiltrating T cells and the induction of PD-L1 in the tumor microenvironment via interferon (IFN) signaling, we further conjectured that the release of HSPs from tumors and the stimulation of the NLRP3-HSP70 signaling axis was dependent upon PD-L1 upregulation (15). To address this hypothesis, we co-incubated BRAF\(^{V600E}\)PTEN\(^-\) melanoma cells with IFN\(\gamma\) in the absence and presence of an agonistic anti-PD-L1 ab and monitored for HSP70 release as well as for evidence of caspase-1 cleavage as a surrogate for NLRP3 activation. This study showed anti-PD-L1 ab/IFN\(\gamma\) treatment of BRAF\(^{V600E}\)PTEN\(^-\) melanoma cells indeed induces the activation of the NLRP3 inflammasome, the concurrent release of HSP70, and the upregulation of WNT5A (Figure 5A). This effect was also observed in the Lewis lung carcinoma cell line indicating that this phenomenon is not restricted to the BRAF\(^{V600E}\)PTEN\(^-\) melanoma model (Supplementary Figure 6A). To further confirm the sequence of this signaling pathway, we genetically silenced HSP70 using a CRISPR/Cas9 approach in the BRAF\(^{V600E}\)PTEN\(^-\) melanoma cell line and stimulated the upstream NLRP3 inflammasome in these BRAF\(^{V600E}\)PTEN\(^-\)-HSP70\(^-\) cells along with their non-target control cell line. These experiments showed that HSP70 ablation eliminates the ability of the NLRP3 inflammasome to stimulate the upregulation of Wnt5a in both
an autocrine and paracrine manner (Supplementary Figure 6B). Further experiments demonstrated that tumor cell PD-L1 cross-linking induces NLRP3 binding to the apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) adaptor protein while also promoting ASC polymerization, both of which are necessary to generate the inflammasome macromolecular complex (Figures 5B,C). We then verified that PD-1 blockade promotes CD8\(^+\) T cell induction of NLRP3 activation and tumor HSP70 release in an IFN\(\gamma\)-dependent manner in further OT-1 CD8\(^+\) T cell:BRAF\(^{V600E}\)-OVA melanoma co-culture experiments (Figure 5D). Notably, this induction in NLRP3 activation by CD8\(^+\) T cells was found to be antigen-specific as CD8\(^+\) T cells that recognize an irrelevant control peptide do not induce caspase-1 activation (Supplementary Figure 6C). Furthermore, this phenomenon requires T cell:tumor cell contact or close proximity as transwell assays failed to induce caspase-1 cleavage and HSP70 release, consistent with an important role for physical PD-1:PD-L1 interactions (Supplementary Figure 6D). Importantly, both the pharmacologic inhibition and genetic silencing of Nlrp3 effectively suppresses HSP70 release and subsequent WNT5A upregulation in response to anti-PD-1 ab activation of tumor antigen-specific CD8\(^+\) T cells (Figures 5E,F). A similar effect was observed with the genetic silencing of Pdl1 in the BRAF\(^{V600E}\)PTEN\(^{-/-}\) melanoma cell line, resulting in the elimination of ASC polymerization, caspase-1 activation, HSP70 secretion, and WNT5A upregulation (Figures 5C,F, Supplementary Figure 6E).

Based on these data, we examined the mechanism mediating PD-L1-dependent NLRP3 activation. Double-stranded RNA dependent protein kinase R (PKR) is a known activator of all inflammasome proteins including NLRP3 and has been demonstrated to regulate certain inflammatory pathways (34). Indeed, PKR inhibition suppresses caspase-1 cleavage in the
BRAF<sup>V600E</sup>PTEN<sup>-/-</sup> melanoma cell line in response to anti-PD-L1 ab/IFNγ stimulation (Supplementary Figure 6F). Consistent with these studies, further treatment of BRAF<sup>V600E</sup>PTEN<sup>-/-</sup> melanoma cells with anti-PD-L1 ab/IFNγ induced PKR-NLRP3 binding while the genetic silencing of Pdl1 also suppressed the phosphorylation of PKR (Figures 5G,H). A recent report has shown that tumor intrinsic PD-L1 signaling inhibits STAT3 activation (23). Previous work has also shown that cytosolic STAT3 inhibits PKR kinase activity and suppresses activation of the NLRP3 inflammasome (35, 36). Based on these data, we hypothesized that tumor PD-L1 promotes PKR-NLRP3 activation by suppressing STAT3 levels. Indeed, we also found Pdl1 silencing to upregulate total STAT3 levels concurrently with diminished PKR phosphorylation (Figure 5I). Consistent with a suppressive role for STAT3 in this pathway, constitutive activation of STAT3 (STAT3<sup>CA</sup>) suppresses NLRP3 activation based on diminished caspase-1 cleavage and WNT5A expression levels (Figure 5J). These data indicate that PD-L1 triggers PKR-dependent activation of the NLRP3 inflammasome in tumors by repressing STAT3 (Figure 5K). In summary, we have elucidated a previously unreported mechanistic link between PD-L1 and the tumor-intrinsic NLRP3 inflammasome and we have shown that this pathway drives adaptive immune evasion by promoting the recruitment of PMN-MDSCs.

**Genetic and Pharmacologic Inhibition of NLRP3 Blocks PMN-MDSC Recruitment and Enhances the Efficacy of Anti-PD-1 Antibody Immunotherapy.**

Given the central role of the NLRP3 inflammasome in mediating PMN-MDSC recruitment to the tumor bed in response to anti-PD-1 ab therapy, we hypothesized that inhibiting NLRP3 activation would promote anti-tumor immunity and suppress tumor progression. Utilizing a shRNA-
expressing lentiviral vector, we silenced \textit{Nlrp3} in BRAF^{V600E}PTEN\(^{-/-}\) melanoma cells and transplanted this cell line into syngeneic hosts (\textit{Supplementary Figure 7A}). After 20 days of tumor growth, \textit{Nlrp3}-silenced BRAF^{V600E}PTEN\(^{-/-}\) melanomas were associated with reduced plasma HSP70 levels, decreased levels of CXCR2 ligand expression, and diminished PMN-MDSC infiltration relative to control BRAF^{V600E}PTEN\(^{-/-}\) melanomas (\textit{Figures 6A-C}). Although \textit{Nlrp3} silencing did not influence tumor cell proliferation in vitro, it did increase the levels of tumor-infiltrating CD8\(^{+}\) T cells and suppress the growth of BRAF^{V600E}PTEN\(^{-/-}\) melanomas in vivo. (\textit{Figures 6C,D, Supplementary Figures 7B,C}). NLRP3-dependent regulation of the anti-tumor immune response was further confirmed by additional in vivo tumor experiments where CD8\(^{+}\) T cell ablation reversed tumor growth suppression in \textit{Nlrp3}-silenced BRAF^{V600E}PTEN\(^{-/-}\) melanomas (\textit{Supplementary Figure 7D}).

We subsequently pursued additional studies to determine whether systemic pharmacological inhibition of NLRP3 could also suppress tumor growth and augment anti-PD-1 ab immunotherapy in the BRAF^{V600E}PTEN\(^{-/-}\) melanoma model. Using the MCC950 NLRP3 inhibitor, we also found systemic NLRP3 inhibition to diminish PMN-MDSC recruitment in response to anti-PD-1 ab therapy, enhance levels of tumor-infiltrating CD8\(^{+}\) T cells, and suppress tumor progression in vivo beyond what is observed with anti-PD-1 ab monotherapy (\textit{Figures 6E,F}) (37). Whole tissue Western blot, plasma ELISA, and tumor qrt-PCR studies further showed these effects correlate with suppressed caspase-1 cleavage, WNT5A expression, Cxcl5 levels, HSP70 release, and enhanced expression of the cytolytic T cell marker, Gzmb (\textit{Figures 6G,H, Supplementary Figure 7E}). Notably, neither pharmacologic inhibition of NLRP3 nor genetic silencing of \textit{Nlrp3} exhibited any impact on tumor cell proliferation in vitro (\textit{Supplementary Figure
Altogether, these data are consistent with our previous studies supporting the critical role of the NLRP3 inflammasome in driving PMN-MDSC recruitment as an adaptive response to CD8+ T cell activation and suggest that NLRP3 is a viable pharmacologic target for enhancing the efficacy of anti-PD-1 ab therapy.

**PMN-MDSC Recruitment as a Mechanism of Adaptive Resistance to Anti-PD-1 Antibody Therapy in Human Melanoma.**

In order to investigate whether PD-L1 can trigger NLRP3 activation and HSP70 release in human melanoma, we performed in vitro experiments utilizing the WM266 human melanoma cell line. Similar to our previous observations, tumor PD-L1 cross-linking following IFNγ stimulation induces caspase-1 cleavage and HSP70 release (Figure 7A). Further studies in the WM266 human melanoma cell line also showed HSP70 to induce WNT5A upregulation in a TLR4-dependent manner and pharmacologic NLRP3 inhibition to suppress ATP-mediated HSP70 release (Figures 7B,C). These data were consistent with a significant correlation between the expression of the myeloid markers ITGAM and CD33 as well as NLRP3 and the cytolytic T cell markers CD8A, GZMB, and PRF1 in human metastatic melanoma specimens based on RNA expression data in the SKCM-TCGA database (Figure 7D). These data are consistent with our previous observations in the autochthonous murine melanoma model and suggest that CD8+ T cell activation results in tumor release of HSP70, WNT5A upregulation, and the recruitment of infiltrating myeloid cells (Figure 1A). To address this mechanism further, human melanoma tissue specimens were harvested at baseline and disease progression following nivolumab anti-PD-1 ab immunotherapy and were analyzed by RNAseq differential whole transcriptomic sequencing analysis. Consistent with our
pre-clinical studies, this work demonstrated an elevation in the expression of several myeloid markers including *CXCL1*, *CD33*, *ITGAM*, *CXCR2*, *S100A8*, and *S100A9* following disease progression through checkpoint inhibitor immunotherapy (*Figure 7E*).

Our previous work noted that tumors release HSP70 in response to CD8+ T cell responses and that this increase in HSP70 levels can be measured in the plasma of mice undergoing anti-PD-1 ab immunotherapy (*Figures 3C, Supplementary Figure 7E*). Based on these data, we conducted a plasma-based ELISA study to quantitate HSP70 levels at baseline and at week 12 of anti-PD-1 ab immunotherapy in advanced melanoma patients undergoing anti-PD-1 ab immunotherapy. This study revealed HSP70 levels to increase in patients that progress on anti-PD-1 ab therapy while these changes were seldom observed in responders (*Figures 7F*). Indeed, mean changes in plasma HSP70 levels with anti-PD-1 ab therapy were significantly greater in non-responding melanoma patients relative to responders (*Figure 7G*). This relationship was also observed after normalizing quantitative HSP70 levels to tumor burden based on CT imaging, indicating that this observation was not strictly due to disease progression. These findings suggest that the process leading to HSP70 release correlates with resistance to checkpoint inhibitor therapy, providing further support for the key role of the NLRP3 inflammasome in the evolution of adaptive resistance to anti-PD-1 ab immunotherapy.
DISCUSSION

Using several pre-clinical models in addition to clinical specimens, we have characterized a PD-L1-dependent tumor-intrinsic signaling pathway that directly links CD8+ T cell activity with the recruitment of PMN-MDSCs to the tumor bed in response to anti-PD-1 ab immunotherapy. We propose that this signaling cascade constitutes an adaptive resistance pathway that when blocked can enhance the efficacy of anti-PD-1 ab checkpoint inhibitor immunotherapy. After recognizing a consistent increase in PMN-MDSCs in tumor tissues following anti-PD-1 ab escape in several tumor models, we traced the underlying mechanism of this phenomenon to a process that involves PD-L1-dependent activation of the NLRP3 inflammasome in tumor tissues. Together, this data highlights several potential pharmacologic targets that may be capable of enhancing immunotherapy efficacy as well as promising candidate biomarkers that may contribute to improved patient selection and management of cancer patients undergoing immunotherapy.

Prior studies demonstrating that non-canonical Wnt ligands can induce the activation of YAP-dependent signaling pathways coupled with data showing that YAP signaling can regulate the expression of several CXCR2-dependent chemokines, prompted us to explore a role for WNT5A in mediating the observed influx of PMN-MDSCs in response to checkpoint inhibitor therapy (27, 28). These studies demonstrated that tumor WNT5A expression is induced in response to anti-PD-1 ab therapy and that the genetic silencing of tumor Wnt5a expression eliminates adaptive recruitment of PMN-MDSCs in response to checkpoint inhibitor immunotherapy. A recent RNAseq-based study found WNT5A to be significantly upregulated in
PD-1-refractory melanoma tissues while other studies have found WNT5A to be a marker of dedifferentiation, disease aggressiveness, and therapeutic resistance (17, 18, 38, 39). However, a mechanistic description of how WNT5A promotes immune tolerance and immunotherapy resistance has remained incomplete. This study indicates that autocrine WNT5A signaling can promote PMN-MDSC recruitment to the tumor bed which is consistent with our prior studies demonstrating a role for paracrine WNT5A signaling in the induction of dendritic cell (DC) indoleamine 2,3-dioxgenase expression and enzymatic activity and the subsequent promotion of DC-mediated Treg differentiation (19, 40). Overall, these data provide further support that Wnt ligand-mediated signaling in the tumor microenvironment promotes immune evasion and that targeting Wnt ligand signaling is a promising option for modulating tumor immunity and responses to checkpoint inhibitor immunotherapy.

When evaluating the underlying mechanism driving WNT5A upregulation in response to anti-PD-1 ab therapy in these models, we noted evidence of a significant level of HSP release by tumor cells based on a LC-MS/MS secretome analysis. Tumor release of HSP70 was further confirmed by Western blot and ELISA in response to cytolytic CD8+ T cell activity in vitro and in vivo, respectively. This finding was of interest since 1) previous studies have demonstrated that HSP70 can promote cancer progression and 2) that TLRs both mediate HSP70 signaling and have been implicated in the regulation of WNT5A expression in macrophages (20, 29, 41, 42). We subsequently demonstrated that HSP70 is capable of stimulating WNT5A expression in various cell lines and that genetic silencing and pharmacologic inhibition of tumor TLR4 blocked tumor WNT5A and CXCL5 expression while also inhibiting PMN-MDSC recruitment to developing tumors. Notably, the release of HSP70 was not found to be due simply to a cell death-dependent
mechanism as dacarbazine chemotherapy readily induced tumor apoptosis in the BRAF<sup>V600E</sup> melanoma model but did not induce significant levels of HSP70 release. While HSP70 has been identified within tumor-derived exosomes, prior studies have also suggested that soluble HSP70 may be released by tumor cells by an alternative, previously uncharacterized, mechanism (43). Extracellular ATP has been implicated as a regulator of HSP70 release by tumor cells (32). Since ATP is a known modulator of the NLRP3 inflammasome which, in turn, regulates the release of other inflammatory cytokines such as IL-1β and IL-18 also lacking leader peptide sequences, we initiated studies that confirmed that the NLRP3 inflammasome serves as an upstream regulator of HSP70 secretion by tumors (44). Indeed, these data demonstrate that both the pharmacologic inhibition and genetic silencing of Nlrc3 effectively inhibits tumor release of HSP70 and ultimately eliminates the adaptive recruitment of PMN-MDSCs in response to anti-PD-1 ab therapy. It is important to note that we also conducted a series of experiments which found no evidence that anti-PD-L1 ab in vitro or anti-PD-1 ab in vivo resulted in significant IL-1β expression or release in tumor cells and that IL-1β does not induce WNT5A expression by tumor cells as is observed by HSP70, thus suggesting that tumor-derived IL-1β does not contribute to MDSC recruitment in response to checkpoint inhibitor immunotherapy (Supplementary Figures 7F-H) (45). This finding is also consistent with further studies demonstrating that genetic knock-out of HSP70 eliminates the stimulation of WNT5A in response to NLRP3 activation (Supplementary Figure 6B). Whether NLRP3-dependent IL-18 secretion may contribute to the overall mechanism by promoting IFNγ expression is unclear and is currently under investigation.

Using in vitro co-culture assays and in vivo ablation experiments, these studies establish CD8<sup>+</sup> T cell activity as an important driver for this tumor-intrinsic signaling pathway. This finding
further prompted experiments that found that IFNγ and its downstream modulation of PD-L1 is necessary for activation of the NLRP3 inflammasome. While NFκB-dependent priming signals are required for the induction of NLRP3 activation in certain cell types such as macrophages, it appears that this signal is not necessary in the tumor models investigated here (33). Whether IFNγ stimulation also serves to facilitate NLRP3 priming by an alternative mechanism in tumor cells is currently being investigated. Interestingly, HSP70-TLR4 signaling as described above may also provide a positive feedforward priming pathway capable of perpetuating NLRP3 activation in tumors (46). These studies reveal that NLRP3 inhibition phenocopies downstream TLR4 and CXCR2 inhibition, suppressing the recruitment of PMN-MDSCs as an adaptive resistance mechanism initiated by local CD8+ T cell activity. This finding is in-line with a previous study showing that NLRP3 can mitigate against DC vaccine therapies by promoting the migration of MDSCs into tumors (47).

Due to the role of PD-L1 in the induction of this pathway as well as reports describing downstream signaling effects of PD-L1, we also conducted studies to better understand the underlying mechanism of NLRP3 activation in tumor cells (48). The previously described role of PKR in the regulation of inflammasome activation and its modulation by STAT3 led to a series of experiments culminating in our finding that PD-L1 induces PKR-mediated NLRP3 activation by inhibiting STAT3 (34, 35). Interestingly, these findings were consistent with those of other investigators who recently reported that the cytoplasmic domain of PD-L1 negatively regulates STAT3 in tumor cells (23). However, to our knowledge, this study is the first to report a mechanistic link between tumor PD-L1 and the activation of the NLRP3 inflammasome in response to checkpoint inhibitor immunotherapy.
The overall findings of this study support further interrogation of this pathway as a source of both pharmacologic targets to augment the efficacy of immunotherapy and biomarkers to predict clinical responses and outcomes following exposure to immunotherapy. It should be noted that the use of CXCR2 inhibitors as a strategy to enhance anti-PD-1 ab immunotherapy is a concept that has been tested in pre-clinical tumor models and is currently being evaluated in early phase clinical trials (NCT02583477, NCT03161431, NCT03473925) (8, 10). However, much like how PD-1/PD-L1 antibody antagonists offer a more tissue selective treatment approach over alternative immunotherapies, we conjecture that targeting the upstream NLRP3 inflammasome in this pathway will allow for more tumor-selective inhibition of CXCR2 chemokine-dependent recruitment of PMN-MDSCs, thus reducing the risk of systemic toxicity such as neutropenia. The NLRP3 target is also of particular interest in light of its described role in several inflammatory and autoimmune conditions, suggesting that the PD-L1-NLRP3 signaling axis may play an important role in driving some immune-related adverse events (irAEs) associated with checkpoint inhibitor immunotherapy (49, 50). These findings have prompted further study by our group to determine if NLRP3 inhibition may mitigate against anti-PD-1 ab-induced irAEs and whether any genetic alterations of NLRP3 or its regulators may predict for the development of specific irAEs. Finally, it is tempting to speculate a potential relationship between this IFNγ-dependent resistance pathway and recent reports describing chronic interferon signaling to promote immunotherapy resistance (51).

In summary, we present evidence of an adaptive resistance signaling pathway that is inexorably linked to tumor PD-L1 and drives the recruitment of PMN-MDSCs to the tumor bed in response to anti-PD-1 checkpoint inhibitor therapy. This process extinguishes local cytotoxic anti-
tumor T cell activity and serves as a rheostat for modulating effector T cell responses, thus making this signaling axis a promising target for immunotherapeutic intervention.
METHODS

Clinical Samples

All plasma samples were collected from 17 advanced melanoma patients undergoing anti-PD-1 ab immunotherapy at week 0 and week 12 on an ongoing tissue acquisition protocol investigating checkpoint inhibitor resistance at Duke Cancer Institute (NCT02694965). Three paired tumor specimens obtained at week 0 and at the time of disease progression while undergoing anti-PD-1 ab immunotherapy were collected at Vanderbilt University Medical Center (Institutional Protocol #: 100178). Treatment responses were evaluated based on RECIST (Response Evaluation Criteria in Solid Tumors v1.1).

In vivo Animal Studies

C57BL/6J (C57, H-2b) (Stock number 000664), B6.CgBraf^{tm1Mmcpten^{tm1Hwu}}Tg(Tyr-cre/ERT2)13Bos/BosJ (Braf^{V600E} Pten^{-/-}, H-2b) (Stock number 012328) and C57BL/6Tg(TcraTcrob)1100Mjb/J (OT-1, H-2b) (Stock number 003831) mice were obtained from Jackson Labs. All experimental groups included randomly chosen littermates of both sexes, were ages 6-8 weeks, and were of the same strain.

Cell Lines and Culture Conditions.

Braf^{V600E}Pten^{-/-} (male, BPD6 (40)), Braf^{V600E}Pten^{-/-}-Wnt5a^{KD} (40), Braf^{V600E}Pten^{-/-}-CXCL5^{KD}, Braf^{V600E}Pten^{-/-}-PDL1^{KD}, Braf^{V600E}Pten^{-/-}-NLRP3^{KD}, OVA-expressing Braf^{V600E}Pten^{-/-}, BRAF^{V600E}PTEN^{-/-}-STAT3^{CA}, and Braf^{V600E}Pten^{-/-}-NTC (40) cell lines were generated using shRNA-expressing lentiviral vectors and cultured as previously described (40): shNLRP3 (Sigma, SHCLN-
NM_145827), shPDL1 (Sigma, SHCLND-NM_021893), shWnt5a (Sigma, SHCLND-NM_009524), shTLR4 (Sigma, SHCLNG-NM_025817, and pLKO.1-puro empty vector control (NTC) (Sigma, SHC001). Stable cell lines were selected by puromycin resistance (Sigma-Aldrich, P8833). Murine Lewis Lung Carcinoma (LLC) cell line is from ATCC (1704526). All cell lines were tested Mycoplasma-free by Duke University Cell Culture Facility shared services. All Braf^{V600E}Pten^{-/-} cell lines and the LLC cell line were maintained at 37°C in DMEM (Invitrogen) with 2 mM L-glutamine, supplemented with 10% fetal bovine serum, 100 units/ml penicillin. Depending on the experiment, cell lines were treated with Wnt5a (100-200ng/ml, R&D Systems/Bio-techne, 645-WN-010), IFNγ (100ng/ml, BioAbChem, 42-IFNg), anti-PD-L1 ab (1-2 µg/ml), Hsp70 (1 µM-10 µM, Enzo, ADI-ESP-502-D), Hsp70 inhibitor (ThermoFisher, VER155008), CLI-095 TLR4 inhibitor (3 µM-10 µM, Invivogen, ttrl-cli95), TLR2-IN-C29 TLR2 inhibitor (1 µM- 10 µM , Glixx, GLXC-06203), MPLA TLR4 agonist (10 µM, Enzo, ALX-581-205-C100), LPS (Lipopolysaccharide) (10ng, Sigma-Aldrich, L4391-1MG), recombinant IL-1β (100-200ng, Biolegend , 575102), ATP (1 mM-5 mM, Invivogen, ttrl-atpl), MCC950 NLRP3 inhibitor (2.5 µM-10 µM, Invivogen, inh-mcc), XAV939 β-catenin inhibitor (0.5-1.0 µM, Sigma-Aldrich, X3004-5MG), 2-Aminopurine PKR inhibitor (1 mM-5 mM, Invivogen, ttrl-april), Verteporfin YAP inhibitor (0.1 µM- 1 µM, R&D Systems/Bio-techne, 530510) or vehicle control either for 24 or 48 hrs prior to in vitro and in vivo experiments. Control siRNA (Santa Cruz, sc-37007). TLR4 siRNA (SantaCruz, sc-40261).

**Autochthonous Tumor Studies.**

B6.Cg-Braf^{tm1Mmcm}Pten^{tm1Hwu}Tg(Tyr-cre/ERT2 H-2b)13Bos/BosJ (BRAF^{V600E}-PTEN^{-/-}) transgenic mice were sub-dermally injected with 4-Hydroxytomoxifen (4-HT) (Sigma, H6278-50MG CCF,
38.75 µg/mouse) to induce primary melanoma development at the base of the tail. Mice were randomly assigned to treatment cohorts once tumor volumes reached 64 mm$^3$ (19, 30, 52). Depending on the experiment, mice were treated with the following agents: CXCR2 inhibitor (AZD5069, AstraZeneca) at 100 mg/kg per os (po) twice daily, NLRP3 inhibitor (MCC950) 10 mg/kg i.p every other day, anti-PD1 ab (BioXCell) or rat IgG2a isotype control (BioXCell) at 200 µg by i.p. injection every 3 days, Dacarbazine (50 mg/kg or 75 mg/kg, Sigma-Aldrich, D2390) by i.p. injection once every other day. Melanoma growth was monitored by orthogonal caliper measurements every 3 days.

**Syngeneic Transplant Tumor Studies.**

$\text{Braf}^{\text{V600E} \text{Pten}^-/-}$, $\text{Braf}^{\text{V600E} \text{Pten}^-/-}\text{-NTC}$, $\text{Braf}^{\text{V600E} \text{Pten}^-/-}\text{-Wnt5a}^{\text{KD}}$, $\text{Braf}^{\text{V600E} \text{Pten}^-/-}\text{-CXCL5}^{\text{KD}}$, $\text{Braf}^{\text{V600E} \text{Pten}^-/-}\text{-PDL1}^{\text{KD}}$, and $\text{Braf}^{\text{V600E} \text{Pten}^-/-}\text{-NLRP3}^{\text{KD}}$ cell lines ($0.5\times10^5 – 1\times10^5$ cells) were implanted by subcutaneous injection into the base of the tail of syngeneic C57BL/6 mice. Tumor growth was monitored by caliper measurement every 3 days and treatment was initiated when tumor volumes reached 64 mm$^3$. Tumor volume was calculated according to the formula: cm$^3 = [(\text{length (cm)} \times \text{width (cm)})^2]/2$.

**Murine Cell Isolation.**

Tumors were resected and mechanically disaggregated by a gentleMACS dissociator (Miltenyi), filtered through 70-µm filters and digested with RPMI containing collagenase IV (1 mg/mL, Sigma-Aldrich), hyaluronidase (0.1 mg/mL, Sigma-Aldrich), and deoxyribonuclease (20 U/mL, Sigma-Aldrich) on a shaker at 250 rpm at 37°C for 1 hour (23). Resected splenic and lymph node tissues
were mechanically disaggregated using 1 cc syringe plunger and 40 μm filters followed by treatment with RBC lysis buffer (Sigma-Aldrich).

**In vivo CD8 Depletion**

Hybridoma clone 53-6.7 was expanded at the Duke Cell Culture Facility in hollow fiber cartridges; 10 ml of serum free supernatant was harvested every 2 days. Anti-mouse CD8 antibody was purified using a Pierce Gentle Ag/Ab Binding and Elution Buffer Kit (ThermoFisher, 21030) according to the manufacturer’s protocol (ThermoFisher). Antibody concentration was determined by BCA protein assay. Anti-CD8 ab or IgG isotype control ab was delivered daily for the first three days then every 7 days thereafter by intraperitoneal injection (500 μg/dose). For CD8 depletion of progressing Braf\(^{V600E}Pten^{-/-}\)-NTC and Braf\(^{V600E}Pten^{-/-}\)-NLRP3\(^{KD}\) tumors, anti-CD8a antibody delivery was initiated when NLRP3\(^{KD}\) tumor growth became stagnant and followed the schedule described above. CD8 depletion was verified by splenocyte flow cytometry analysis.

**In vitro Tumor Killing Assays**

OT-1 CD8 cell activation was performed by incubating isolated splenocytes of OT-1 transgenic mice with IL-2 (100 U/ml) and SIINFEKL peptide (1 μg/ml, New England Peptide, BP10-915) for 3 days followed by magnetic bead CD8 purification according to the manufacturer’s instructions (Miltenyi biotec, 130-104-075). Activated OT-1 CD8 cells were incubated with Braf\(^{V600E}Pten^{-/-}\)-OVA cells and treated with anti-PD1 ab (1 μg/ml) for 72 hrs at a tumor cell:CD8\(^+\) T cell ratio of 1:5. In separate experiments, Braf\(^{V600E}Pten^{-/-}\) cells were treated with increasing concentrations of Dacarbazine (10 μmol - 400 μmol).
**Immunohistochemistry and Immunofluorescence Analysis**

Paraffin-embedded tissues were processed and stained following standard protocols and imaged with a Zeiss CLSM 700 confocal microscope. The following antibodies were used in immunohistochemistry and immunofluorescence experiments: anti-Wnt5a (1 μg/ml), anti-Ly6G (0.5μg/ml), anti-CD8a (0.5μg/ml) and anti-CXCL5 (1 μg/ml). Tissue was permeabilized by incubation in 0.4% Triton-X in TBS for 20 min. Goat anti-rabbit conjugated to Alexa Fluor 564 and goat anti-mouse Alexa 488 were used as secondary antibodies for the appropriate primary antibody. For immunohistochemistry, anti-rat polymers were used as secondary antibodies and Warp Red chromogen detection system (BioCare, WR806S) was used for antigen visualization.

**Flow Cytometry Analysis**

One million cells were stained with 1 μg of each fluorochrome conjugated antibody or commercially available dyes according to the standard protocols and analyzed using a FACSCanto II or LSRII (Becton Dickinson). Cells were stained with Fc receptor blocking antibodies followed by a live/dead discriminator (CellTrace Violet, ThermoFisher, C34571), then by conjugated antibodies for 30 min at 4°C. Cell number was calculated by hemocytometer. Flow cytometry data was analyzed using Flowjo software v10.3.

**Immunoblot, Immunoprecipitation, and ASC Polymerizations Studies**

Tumor tissue or cells were homogenized in NP40 lysis buffer (Sigma-Aldrich) supplemented with complete protease inhibitor and phosphatase inhibitor (Roche). Cells were lysed in Laemmli
sample buffer after treatment and subjected to SDS-polyacrylamide gel electrophoresis. After transferring onto PVDF membranes (Bio-Rad), monoclonal and polyclonal primary antibodies and appropriate HRP-conjugated secondary antibodies were utilized for blotting. For the immunoprecipitation assays, cells were lysed with TBS buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl) containing 0.5% Triton X-100, EDTA-free protease inhibitor cocktail and phosphatase inhibitor cocktail, pre cleared with protein A/G beads, and then incubated with 1 μg of appropriate antibodies or isotype control IgG on a rotator overnight at 4°C then incubated with protein A/G-agarose at 4°C for 1 hour. After 5 washes with TBS buffer, immunoprecipitated complexes were eluted in sample buffer by boiling and subjected to immunoblot analysis. Immunoblots were visualized by chemiluminescence substrate (ThermoFisher) and imaged by a ChemiDoc XRSplus system (BioRad). For the ASC polymerization assay, cells were lysed with TBS buffer containing 0.5% Triton X-100, EDTA-free protease inhibitor cocktail and phosphatase inhibitor cocktail. The lysates were centrifuged at 4,000 g at 4°C for 15 min. For the detection of ASC oligomerization, the Triton-insoluble pellets were washed twice with TBS buffer and then resuspended in 500 μl TBS buffer. The resuspended pellets were crosslinked for 30 min at room temperature with 2 mM Disuccinimidyl suberate (DSS) (Thermo Fisher Scientific, 21655) and then centrifuged for 15 min at 6,000g. The pellets were dissolved in SDS sample buffer.

**Antibodies**

Anti-β-actin, mouse monoclonal (Santa Cruz Biotechnology, sc-47778). Anti-NLRP3, rabbit monoclonal (Cell Signaling, 15101S). Anti-ASC, mouse monoclonal (Santa Cruz Biotechnology, sc-514414). Anti-Caspase-1 p20, mouse monoclonal (Adipogen, AG-20B-0042-C100). Anti-Caspase-

**RNA Isolation and RT-qPCR Analysis**

Total RNA was isolated by RNeasy Plus Mini Kit (Qiagen, 74134). RNA (1000ng) was used in cDNA Synthesis (iScript Reverse Transcription Supermix, BioRad, 1708841). Real-time PCR was performed using an ABI7500 Real-Time PCR system (Life Technologies) and the primers listed in [Supplementary Table 1](#). Data analysis utilized the PrimePCR Analysis Software (BioRad). Conventional qPCR was performed using validated primers and SsoAdvanced Universal SYBR Green Super mix (BioRad, 1725271) or SsoAdvance Universal Probes Supermix (BioRad, 1725281). All data were normalized to Actb expression, and relative gene expression was quantitated based on the 2^DDCt method.

**RNA-seq Assays**

RNA-seq on tumor tissue-derived from the BRAF^{V600E}-PTEN^-/- transgenic murine model was performed by the Duke Sequencing and Genomic Technologies Shared Resource and previously published (30). A complementary DNA library was prepared via oligo-dT–directed reverse transcription (Ambion) and subjected to deep sequencing on IlluminaHiSeq4000 (50-bp single-read sequencing; Anti–PD-1 resistance Study RNA-seq, accession number: SAMN09878780). This
data was processed by Duke Center for Genomic and Computational Biology using the TrimGalore toolkit that uses Cutadapt to trim low-quality bases and Illumina sequencing adapters from the 3’ end of the reads. Only reads that were of 20 nucleotides (nt) or longer after trimming were kept for further analysis. Reads were mapped to the GRCm38v68 version of the mouse genome and transcriptome using the STAR RNA-seq alignment tool. Reads were kept for subsequent analysis, if they mapped to a single genomic location. Gene counts were compiled using the HTSeq tool. Only genes that had at least 10 reads in any given library were used in subsequent analysis. Normalization and differential expression were carried out using the DESeq2 Bioconductor package with the R statistical programming environment. The false discovery rate was calculated to control for multiple hypothesis testing. Gene set enrichment analysis was performed to identify differentially regulated pathways and gene ontology terms for each of the comparisons performed. Human melanoma tissues obtained from Vanderbilt University (IRB: 10078) were also subjected to RNAseq analysis and this data was previously published and deposited at https://prod.tbilab.org/balko_lab/LAG3_JCI_Insight (53).

ELISA
Levels of CXCL5 and HSP70 (R&D Systems, DYC166-2) in mouse plasma were evaluated using ELISA kit as per manufacturers’ instructions (R&D Systems, Inc.). IL-1β (BioLegend, 432601) level in cell lysate, tumor lysate, and supernatant were measured based on ELISA kit according to manufacturers’ instructions (Biolegend).

Human Plasma ELISA
Human plasma HSP70 concentrations were measured using the human DuoSet assay (R&D Systems, Inc., Catalog number DY1663, MN, USA) according to manufacturer’s protocol. Human melanoma plasma samples obtained from Duke Cancer Institute (IRB: Pro00059349, Clinical trial number: NCT02694965).

**Secretome Assays**

Single cell suspensions were prepared by enzymatic and mechanical digestion. $2 \times 10^6$ cells were plated in 2ml of 1% dialyzed FBS containing Light or heavy amino acids without L-methionine in 6 well plates, then incubated in CO$_2$ for 30 min. L-methionine or azidohomoalanine (AHA), an azide-bearing analogue of methionine, was added to the plates and incubated in 5% CO$_2$ at 37°C overnight. Culture supernatants were collected for secretome assay. Cell supernatants were enriched for AHA labeled proteins by incubating with DBCO-agarose overnight. Resins were washed, followed by reduction and alkylation of cys residues, and peptides were recovered after overnight digestion with trypsin. Samples were analyzed by quantitative one-dimensional liquid chromatography, tandem mass spectrometry. Using Proteome Discoverer 2.3, the data was searched against SwissPro Mouse database with semitrypsin specificity, fixed modification on Cys, and variable modifications on Met (oxidation), Gln/Asn (deamidation), Pro (hydroxylation) and Arg/Lys (15N-13C).

**TCGA Data Analysis**

The Cancer Genome Atlas (TCGA) skin cancer melanoma (TCGA-SKCM) annotated RNA expression files were extracted from the GDC portal (https://portal.gdc.cancer.gov/) for 376
cases of metastatic melanoma. Data preprocessing was performed using HTSeq-Counts from the TCGA, and raw counts were normalized using the relative log expression method implanted in R and its extension package DESeq2. Co-expression of genes of interest were tested using Chi-square statistic of sample-quantile based contingency (SQUAC) table, scatter plots were generated using normalized counts and Bonferroni corrections on the group p-values to account for multiple testing.

Statistics

Specific statistical tests are reported in the Figure Legends. GraphPad Prism 8 Windows version was used for all statistical analyses. Unpaired 2-tailed t-tests were used to compare mean differences between control and treatment groups. Univariate one-way ANOVA followed by Sidak post-hoc multiple comparison test was performed to analyze data containing three or more groups. Data correlation analyses were conducted using either Kendall Tau or Spearman calculations. A P value less than 0.05 was considered significant. All quantitative data is presented as a mean ± SEM.

Study Approval

Mouse tumor experiments were performed based on a protocol approved by the Institutional Animal Care and Use Committee at Duke University Medical Center. All patients provided written informed consent under approval from the Institutional Review Boards at Duke University (NCT02694965) and Vanderbilt University Medical Center (Institutional Protocol #: 100178), respectively.
AUTHOR CONTRIBUTIONS

B.T. and B.A.H. conceptualized the project, designed all experiments, and analyzed all data. K.S.E., M.S., Mark S., L.W., Y.K., and D.H. provided technical support. A.K.S.S., J.M.B., and D.B.J. provided clinical resources for the project. B.A.H. and A.N. supervised all experiments. B.A.H. wrote the manuscript. B.A.H., A.H., B.T., N.D., and M.P. reviewed and edited the manuscript.

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REFERENCES


Figure 1.

A. 

B. 

C. 

D. 

E. 

F. 

G. 

H. 

I.
Figure 1. PMN-MDSC Accumulation Contributes to Tumor Progression Following Anti-PD-1 Antibody Immunotherapy. (A) Overview schematic of described adaptive resistance pathway. (B) RNAseq differential gene expression analysis of tumor tissues following treatment of the autochthonous BRAFV600E/PTEN+/− melanoma model with anti-PD-1 ab therapy versus IgG isotype control (ctrl) (n=3). (C) Qrt-PCR analysis of target genes of interest in serial tumor FNA biopsy specimens harvested from the transgenic BRAFV600E/PTEN+/− melanoma model undergoing anti-PD-1 ab therapy versus IgG isotype ctrl (n=5). (D) Gr-1 immunohistochemical (IHC) analysis of transgenic BRAFV600E/PTEN+/− melanoma tissues following anti-PD-1 ab therapy versus IgG isotype ctrl. 40x. Gr-1, red. Representative of 3 tumors per group. (E) PMN-MDSC flow cytometry analysis of transgenic BRAFV600E/PTEN+/− melanoma tissues following anti-PD-1 ab therapy versus IgG isotype ctrl. PMN-MDSCs defined as live+CD45+CD11b+Ly6G+Ly6CintF4/80− cells. left, representative flow dot plot. right, quantification of PMN-MDSC flow cytometry data (n=5). (F) Qrt-PCR analysis of CXCR2 ligands in BRAFV600E/PTEN+/− melanoma tissues undergoing anti-PD-1 ab therapy following CD8+ T cell ablation in vivo (n=3). (G) In vivo tumor study of BRAFV600E/PTEN+/− melanoma genetically silenced for CXCL5. top, quantitation of tumor-infiltrating PMN-MDSCs by flow cytometry. bottom, In vivo tumor growth curve of CXCL5-silenced BRAFV600E/PTEN+/− melanoma vs BRAFV600E/PTEN+/− NTC melanoma control tumors undergoing anti-PD-1 ab therapy. Data normalized to tumors undergoing IgG isotype ctrl therapy (n=5). KD, knockdown. NTC, non-target control. (H) Anti-PD-1 ab - CXCR2i combination in vivo BRAFV600E/PTEN+/− melanoma study (n=5). Right, flow cytometry analysis of tumor-infiltrating PMN-MDSCs (top) and live+CD45+CD3+CD8+ T cells (bottom). Significance assessed in panels B,C,F using t-tests corrected usingHolm-Sidak post-hoc multiple comparisons test; Student’s t test in panels E,G; one-way ANOVA with Sidak post-hoc multiple comparison test in panel H. *P < 0.05. **P < 0.005. See also Supplemental Figures 1, 2, 5C.
Figure 2.

A. CXCL5, CXCL2, CXCR2

B. IgG-isotype Ctrl, anti-PD1-Ab

Wnt5a
Yap-1
CXCL5
Vinculin
β-actin

C. Normalised CXCL5 Plasma Levels

D. Cxcl1, Cxcl2, Cxcl5

E. Wnt5a

F. Cxcl5

G. NTC, Wnt5a

H. Gr-1, Ly6G

I. IgG Isotype Ctrl, Anti-PD1 ab

N. Tumor Volume

J. Relative mRNA

\( \tau = 0.275 \)
\( \tau = 0.260 \)
\( \tau = 0.286 \)

\( \% \) PMN - MDSCs
Figure 2. Wnt5a Induces CXCR2-dependent Chemokine Expression in Response to Anti-PD-1 Ab Immunotherapy. (A) TCGA human melanoma database gene expression association analysis of CXCL5, CXCL2, and CXCR2 with WNT5A. (B) Whole tumor tissue Western blot analysis of WNT5A, YAP1, CXCL5, and vinculin/β-actin (loading controls). Representative of 3 independent experiments. (C) Plasma CXCL5 ELISA following anti-PD-1 ab therapy vs IgG isotype ctrl therapy in the transgenic BRAFV600E PTEN-/- melanoma model (n=6). Representative of 3 independent experiments. (D) Qrt-PCR analysis of Cxcl1, Cxcl2, and Cxcl5 in the BRAFV600E PTEN-/- melanoma cell line following treatment with recombinant WNT5A (rWNT5A) vs vehicle ctrl (n=3). (E) Western blot analysis of YAP1 expression in total cellular lysates (top) and nuclear lysates (middle) following treatment of BRAFV600E PTEN-/- melanoma cells with rWNT5A at various time points. (bottom) rWNT5A induction of CXCL5 ± verteporfin(YAP inhibitor) or XAV939(β-catenin inhibitor). Representative of 3 independent experiments. (F) Qrt-PCR analysis of Cxcl5 in BRAFV600E PTEN-/-NTC and WNT5A-silenced BRAFV600E PTEN-/- melanoma cells (BRAFV600E PTEN-/-Wnt5aKD). Upper right, dot blot analysis of secreted CXCL5 in BRAFV600E PTEN-/-NTC and BRAFV600E PTEN-/-Wnt5aKD cells (n=3). (G) CXCL5 IHC (red) in BRAFV600E PTEN-/-NTC and BRAFV600E PTEN-/-Wnt5aKD tumors. Representative of 3 tumors. white arrows, CXCL5+ tumor cells. 20x. (H) Top, Gr-1 IHC of BRAFV600E PTEN-/-NTC and BRAFV600E PTEN-/-Wnt5aKD tumors. Bottom, PMN-MDSC flow cytometry analysis of BRAFV600E PTEN-/-NTC and BRAFV600E PTEN-/-Wnt5aKD tumors (n=3). (I) PMN-MDSC flow cytometry analysis of BRAFV600E PTEN-/-NTC and BRAFV600E PTEN-/-Wnt5aKD tumors following anti-PD-1 ab therapy vs IgG isotype ctrl (n=5). (J) Tumor volume change based on an anti-PD-1 ab:IgG ctrl ratios for BRAFV600E PTEN-/-NTC and BRAFV600E PTEN-/-Wnt5aKD tumors (n=5). UT, untreated control. Kendall’s Tau correlation coefficient calculated for panel A; Significance assessed using Student’s t test in panels C,D,I; one-way ANOVA with Sidak post-hoc multiple comparison test in panel F. *P < 0.05. ***P < 0.0005. ns, non-significant. See also Supplemental Figure 3.
Figure 3.

A. GO Pathway

B. HSP70

C. HSP70 (pg/mL plasma)

D. Relative mRNA Levels

E. Total Cell Lysates

F. HSP70i

G. TLR4i

H. Tumor Volume (cm³)

I. Ctrl siRNA TLR4 siRNA

J. Ctrl siRNA TLR4-siRNA
Figure 3. HSP70-TLR4 Induces Wnt5a Expression in Response to Anti-PD-1 Ab Immunotherapy. (A) Top 12 pathways enriched in autochthonous BRAF^{V600E}PTEN^-/- melanomas following escape from anti-PD-1 ab therapy based on RNAseq gene set enrichment analysis. Arrows indicate pathways associated with cellular stress (n=3/group). (B) SILAC-AHA LC/MS-MS secretome analysis of resected autochthonous BRAF^{V600E}PTEN^-/- melanoma tissues following anti-PD-1 ab therapy vs IgG isotype ctrl. Secreted protein levels normalized to cell number (n=3/group). (C) Plasma HSP70 ELISA analysis following anti-PD-1 vs IgG isotype ctrl treatment of autochthonous BRAF^{V600E}PTEN^-/- melanoma mice (n=6). (D) Qrt-PCR analysis of TLR expression in BRAF^{V600E}PTEN^-/- melanoma cells. Data normalized to Tlr9 expression levels (n=3). (E) Treatment of BRAF^{V600E}PTEN^-/- melanoma cells with titrated concentrations of recombinant HSP70 (rHSP70) followed by WNT5A Western blot analysis of total cell lysates (top) and supernatant (SNT) (bottom). Representative of 2 independent experiments. (F) Treatment of BRAF^{V600E}PTEN^-/- melanoma cells with titrated concentrations of HSP70 inhibitor (HSP70i, VER155008). Representative of 2 independent experiments. (G) Treatment of BRAF^{V600E}PTEN^-/--NTC cells with rHSP70 ± TLR4 inhibitor (TLR4i, CLI-095) and treatment of Tlr4-silenced BRAF^{V600E}PTEN^-/- melanoma cells (TLR4^{KD}) with HSP70 followed by Wnt5a Western blot. Representative of 3 independent experiments. (H) BRAF^{V600E}PTEN^-/- melanoma growth curve following TLR4 siRNA vs Ctrl siRNA treatment (n=5). (I) Whole tissue Western blot analysis of WNT5A, CXCL5, and β-actin in TLR4 siRNA-treated and Ctrl siRNA-treated BRAF^{V600E}PTEN^-/- melanomas. Representative of 2 independent experiments. (J) Left, PMN-MDSC flow cytometry analysis of TLR4 siRNA-treated and Ctrl siRNA-treated BRAF^{V600E}PTEN^-/- melanomas (n=4). Right, CD8+ T cell flow cytometry analysis of TLR4 siRNA-treated and Ctrl siRNA-treated BRAF^{V600E}PTEN^-/- melanomas (n=4). Significance assessed using Student’s t test to compare treatment groups. *P< 0.05. See also Supplemental Figure 4.
Figure 4.
A. 

B. 

C. 

D. 

E. 

F. 

G. 

H.
Figure 4. CD8+ T Cells Induce Tumor HSP70 Release in a NLRP3-dependent Manner in Response to Anti-PD-1 Ab Immunotherapy. (A) left, Co-culture of OT-I CD8+ T cells with OVA-expressing BRAFV600EPTEN+/− melanoma cells followed by HSP70 Western blot analysis of isolated SNT. right, Harvested SNT co-incubated at increasing concentrations with wild type BRAFV600EPTEN+/− melanoma cells followed by WNT5A Western blot analysis. Representative of 2 independent experiments. (B) PMN-MDSC and CD8+ T cell flow cytometry analysis of resected autochthonous BRAFV600EPTEN+/− melanoma tissues following anti-PD-1 ab and IgG isotype ctrl therapy. Expressed per gram of tumor tissue (n=6). (C) Tumor-infiltrating PMN-MDSC flow cytometry analysis of autochthonous BRAFV600EPTEN+/− melanomas following anti-PD-1 ab and IgG isotype ctrl therapy ± anti-CD8 antibody. Data normalized to IgG ctrl-treated tumors (n=3). (D) HSP70 and β-actin Western blot following treatment of BRAFV600EPTEN+/− melanoma cells with increasing concentrations of dacarbazine. Representative of 3 independent experiments. (E) Tumor growth curve of syngeneic BRAFV600EPTEN+/− melanomas following vehicle control or low (lo, 50 mg/kg ip qod) vs high (hi, 75 mg/kg ip qod) dose dacarbazine therapy (n=5). (F) Left, PMN-MDSC flow cytometry analysis of BRAFV600EPTEN+/− melanomas following vehicle control or dacarbazine therapy (n=5). Right, CD8+ T cell flow cytometry analysis of BRAFV600EPTEN+/− melanomas following vehicle control or dacarbazine therapy (n=5). ns, non-significant. (G) HSP70 Western blot analysis of SNT and tumor cell lysates following ATP stimulation of BRAFV600EPTEN+/− melanoma cells at different time points (top) ± NLRP3 inhibitor (NLRP3i, MCC950) treatment (bottom). Representative of 3 independent experiments. (H) HSP70 Western blot following co-incubation of OT-1 CD8+ T cells and OVA-expressing BRAFV600EPTEN+/− melanoma cells ± increasing concentrations of NLRP3i. Representative of 3 independent experiments. Spearman correlation calculation performed in panel B; Student’s t test in panel C; one-way ANOVA with Sidak post-hoc multiple comparison test in panels E,F. See also Supplemental Figure 5.
Figure 5. CD8$^+$ T cells Trigger a PD-L1:NLRP3 Signaling Pathway to Drive PMN-MDSC Recruitment to the Tumor. (A) SNT HSP70, Caspase-1 p20, and WNT5A Western blot analysis of BRAF$^{V600E}$PTEN$^{-/-}$ melanoma cells with IFN$\gamma$ ± anti-PD-L1 ab. (B) Immunoprecipitation (IP) of NLRP3 after treatment of BRAF$^{V600E}$PTEN$^{-/-}$ melanoma cells with IFN$\gamma$, anti-PD-L1, or both followed by ASC and NLRP3 Western blots. IgG-IP, IP control. ATP, positive control. (C) left, ASC polymerization assay following treatment of BRAF$^{V600E}$PTEN$^{-/-}$ melanoma cells with IFN$\gamma$, anti-PD-L1, or both. Right, ASC polymerization assay following treatment of Pdl1-silenced (PD-L1$^{KD}$) and NTC BRAF$^{V600E}$PTEN$^{-/-}$ melanoma cells with IFN$\gamma$. (D) Co-culture of OT-I CD8$^+$ T cells with OVA-expressing BRAF$^{V600E}$PTEN$^{-/-}$ melanoma cells ± anti-PD-1 ab alone or anti-PD-1 ab + anti-IFN$\gamma$ blocking ab followed by HSP70 and Caspase-1 p20 Western blots. (E) Co-culture of OT-I CD8$^+$ T cells with BRAF$^{V600E}$PTEN$^{-/-}$-OVA melanoma cells ± anti-PD-1 ab alone or anti-PD-1 ab + NLRP3i followed by Caspase-1 p20, HSP70, and WNT5A Western blots. (F) Caspase-1 p20, HSP70, and WNT5A Western blots of BRAF$^{V600E}$PTEN$^{-/-}$-OVA melanoma cells following co-culture with OT-I CD8$^+$ T cells after genetically silencing either Nlrp3 (NLRP3$^{KD}$) or Pdl1 (PD-L1$^{KD}$). (G) IP of NLRP3 after treatment of BRAF$^{V600E}$PTEN$^{-/-}$ melanoma cells with IFN$\gamma$, anti-PD-L1, or both followed by PKR and NLRP3 Western blots. (H) Phospho- and total PKR Western blot analysis of control and Pdl1-silenced BRAF$^{V600E}$PTEN$^{-/-}$ melanoma cells. GAPDH, cytoplasmic loading control. Laminin B, nuclear loading control. (I) STAT3, phospho-PKR, and total PKR Western blot analysis of control and Pdl1-silenced BRAF$^{V600E}$PTEN$^{-/-}$ melanoma cells. (J) Caspase-1 p20 and WNT5A Western blot analysis of wild type and constitutively active (CA) STAT3-expressing BRAF$^{V600E}$PTEN$^{-/-}$ melanoma cells following treatment with IFN$\gamma$, anti-PD-L1, or both. (K) Schematic diagram describing the PD-L1-STAT3-PKR-NLRP3 signaling axis in tumor cells. All Western blots representative of 2-3 independent experiments. See also Supplemental Figure 6.
Figure 6. Genetic and Pharmacologic Inhibition of NLRP3 Suppresses PMN-MDSC Recruitment and Enhances the Efficacy of Anti-PD-1 Ab Immunotherapy. (A) Plasma HSP70 ELISA analysis following growth of BRAF<sup>600EPTEN<sup>-/-</sup>NTC or Nlrp3-silenced BRAF<sup>600EPTEN<sup>-/-</sup> melanomas (n=5). (B) Qrt-PCR analysis of CXCR2-dependent chemokine expression in BRAF<sup>600EPTEN<sup>-/-</sup>NTC and BRAF<sup>600EPTEN<sup>-/-</sup>-NLRP3<sup>KD</sup> melanomas (n=3). (C) top, CD8<sup>+</sup> T cell flow cytometry analysis of resected BRAF<sup>600EPTEN<sup>-/-</sup>-NTC and BRAF<sup>600EPTEN<sup>-/-</sup>-NLRP3<sup>KD</sup> melanomas (n=5). bottom, PMN-MDSC flow cytometry analysis of resected BRAF<sup>600EPTEN<sup>-/-</sup>-NTC and BRAF<sup>600EPTEN<sup>-/-</sup>-NLRP3<sup>KD</sup> melanomas (n=5). (D) Tumor growth curve of BRAF<sup>600EPTEN<sup>-/-</sup>-NTC and BRAF<sup>600EPTEN<sup>-/-</sup>-NLRP3<sup>KD</sup> melanomas (n=5). (E) Treatment of syngeneic BRAF<sup>600EPTEN<sup>-/-</sup> melanomas with IgG isotype ctrl ab (200 µg ip every 3 days), NLRP3i (MCC950 10 µg ip every 3 days), anti-PD-1 ab (200 µg ip every 3 days), or NLRP3i and anti-PD-1 ab combination therapy (n=8). (F) PMN-MDSC and CD8<sup>+</sup> T cell flow cytometry analysis of resected BRAF<sup>600EPTEN<sup>-/-</sup> melanomas following treatment with IgG isotype ctrl ab, NLRP3i, anti-PD-1 ab, or NLRP3i and anti-PD-1 ab combination therapy. Representative dot blots shown. Right, flow cytometry analysis of tumor-infiltrating PMN-MDSCs (top) and CD44<sup>+</sup>CD8<sup>+</sup> T cells (bottom). (G) Whole tumor tissue Western blot analysis for Pro-caspase-1, Caspase-1 p20, and WNT5A following in vivo treatment with IgG isotype ctrl, anti-PD-1 ab, and anti-PD-1 ab/NLRP3i combination therapy. Representative of 2 independent experiments. (H) Qrt-PCR analysis of Cxcl5 and Gzmb (Granzyme B) expression in resected BRAF<sup>600EPTEN<sup>-/-</sup> melanoma tissues (n=5). Significance assessed using Student’s t test in panels A,B,C,D; one-way ANOVA with Sidak post-hoc multiple comparison test in panels E,F,H. *P < 0.05. **P < 0.005. See also Supplemental Figure 7.
Figure 7.

A. SNT

B. TLR4i

C. NLRP3i

D. ITGAM

E. TPM

F. Anti-PD-1 Ab Therapy

G. Δ Plasma HSP70 Levels with Anti-PD-1 Ab Therapy

Δ Plasma HSP70 Levels

Normalized Δ Plasma HSP70 Levels with Anti-PD-1 Ab Therapy
Figure 7. The PD-L1:NLRC3:HSP70 PMN-MDSC Adaptive Recruitment Pathway in Human Melanoma. (A) SNT HSP70 and Caspase-1 p20 Western blot analysis following treatment of human WM266 melanoma cells with IFNγ ± anti-PD-L1 ab. Representative of 3 independent experiments. (B) WNT5A Western blot analysis HSP70-treated human WM266 melanoma cells ± TLR4i. Representative of 3 independent experiments. (C) HSP70 and Caspase-1 p20 Western blot analysis following treatment of human WM266 melanoma cells with ATP in the absence and presence of MCC950. Representative of 2 independent experiments. (D) Cytolytic T cell markers correlate with ITGAM (CD11B), CD33, and NLRP3 gene expression in the melanoma TCGA SKCM database. (E) RNAseq analysis of human melanoma tissue specimens collected before anti-PD-1 ab therapy and at the time of disease progression on anti-PD-1 ab therapy. TPM, transcript per million. (F) Plasma HSP70 ELISA at week 0 and week 12 in advanced melanoma patients undergoing anti-PD-1 ab immunotherapy. left, responders (R). right, non-responders (NR). (G) Change in HSP70 plasma levels following anti-PD-1 ab immunotherapy in R and NR advanced melanoma patients. Response based on week 12 CT imaging. Right, HSP70 changes normalized to target tumor burden based on week 12 CT imaging. Whisker plot, central line represents the median, box represents 1st and 3rd quartiles, and error bars represent the data range. Significance assessed using Student’s t test in panels E,G. *P < 0.05. **P < 0.005.