Targeting TMEM176B enhances antitumor immunity and augments the efficacy of immune checkpoint blockers by unleashing inflammasome activation

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SUMMARY

Although immune checkpoint blockers have yielded significant clinical benefits in patients with different malignancies, the efficacy of these therapies is still limited prompting the identification of novel immunotherapeutic targets. Here, we show that disruption of Transmembrane Protein 176b (Tmem176b)/Tolerance-Related and Induced cation transporter (Torid) contributes to $CD8^+$ T cell-mediated tumor growth inhibition by unleashing inflammasome activation. Lack of *Tmem176b* enhances the antitumor activity of anti-CTLA-4 antibodies through mechanisms involving caspase-1/IL-1β activation. Accordingly, patients responding to checkpoint blockade therapies display an activated inflammasome signature. Finally, we identify BayK8644 as a potent Tmem176b inhibitor that promotes $CD8^+$ T cell-mediated tumor control and reinforces the antitumor activity of both anti-CTLA-4 and anti-PD-1 antibodies. Thus, pharmacologic de-repression of the inflammasome by targeting TMEM176B may enhance the therapeutic efficacy of immune checkpoint blockers.

KEY WORDS

Tmem176b, immune checkpoint blockers, cancer, inflammasome.

SIGNIFICANCE

Therapies targeting immune checkpoint pathways have revolutionized treatment of several cancers. However, innate and adaptive mechanisms may limit the clinical efficacy of this immunotherapeutic modality. Here, we identify the Transmembrane Protein 176B (Tmem176b) as an innate immune checkpoint that curtails $CD8^+$ T cell mediated immunity by repressing inflammasome activation. Genetic disruption or pharmacologic inhibition of Tmem176b potentiates antitumor immunity and enhances the efficacy of anti-CTLA-4 and anti-PD-1 antibodies in mice by unleashing inflammasome activation. Accordingly, an activated inflammasome signature delineates favorable clinical responses in patients receiving immune checkpoint blockers. Thus, targeting TMEM176B may influence antitumor effector mechanisms by de-repressing inflammasome activation.

HIGHLIGHTS

- TMEM176B inhibits the NLRP3 inflammasome by controlling cytosolic Ca^{++}.
- Lack of *Tmem176b* enhances antitumor immunity via activation of the caspase 1-IL-1β pathway.
- Patients responding to immune checkpoint blockers display an inflammasome-activated signature.
- BayK8644, a Tmem176b inhibitor, improves the antitumor activity of checkpoint blockers.

INTRODUCTION

Stimulation of adaptive antitumor responses has revolutionized the therapeutic management of a variety of cancers (Baumeister et al., 2016; Sharma and Allison, 2015; Topalian et al., 2015, 2016). Immune checkpoint blockers unleash antitumor T-cell responses by interrupting co-inhibitory signals that normally hold effector T cells in check. Blockade of immune checkpoints, including the cytotoxic T lymphocyte-associated protein (CTLA)-4 and the programmed cell death-1 (PD-1)/PD ligand-1 (PD-L1) pathway, have increased overall survival and progression-free survival of cancer patients. However, only a restricted number of patients show clinical benefits (Syn et al., 2017), suggesting that other immune inhibitory mechanisms may limit the efficacy of these treatments. Thus, identification of novel targets and biomarkers of response to treatment are necessary to develop novel immunotherapeutic drug combinations (Binnewies et al., 2018; Krieg et al., 2018; Pitt et al., 2016; Sharma et al., 2017). In this regard, an ionic disbalance within the tumor microenvironment has been shown to hinder the efficacy of immunotherapeutic strategies. High intratumoral K^+ led to T-cell dysfunction by inhibiting voltage and Ca^{++}-dependent K^+ channels expressed in anti-tumoral T lymphocytes (Eil et al., 2016), suggesting a role for ionic channels as emerging regulatory checkpoints and therapeutic targets to reinforce anti-tumor immunity.

A number of innate immune players can shape antitumor immunity and influence cancer immunotherapeutic strategies by positively or negatively controlling T-cell activation, differentiation, expansion and survival (Berraondo et al., 2016). Recognition of immunogenic tumors by innate immune sensors including the Tmem173 (STING) type I interferon (IFN) pathway leads to stimulation of $CD8^+$ T cell-mediated immunity and potentiation of CTLA-4- and PD-1-targeted therapies (Corrales et al., 2015; Demaria et al., 2015; Woo et al., 2014). Moreover, a type-I IFN signature is associated with ipilimumab sensitivity in melanoma patients (Chiappinelli et al., 2015). Furthermore, STING controls the NLRP3 inflammasome within the human myeloid cell compartment (Gaidt et al., 2017).

The inflammasome is a cytosolic multiprotein complex which, once activated, cleaves caspase 1 which then processes pro-IL-1β and pro-IL-18 to give the active and secreted forms of these pro-inflammatory cytokines (Rathinam and Fitzgerald, 2016). Altered levels of cytosolic cations have been shown to control secretion of active IL-1β through modulation of inflammasome activation (Gong et al., 2018; Muñoz-Planillo et al., 2013; Murakami et al., 2012). Interestingly, activation of the NLRP3 inflammasome in dendritic cells (DCs) following chemotherapy triggers vigorous $CD8^+$ T cell-mediated antitumor responses (Ghiringhelli et al., 2009) and immunogenic chemotherapy sensitizes tumors to immune checkpoint blockers (Pfirschke et al., 2016). However, the role of the NLRP3 inflammasome in modulating checkpoint blockade therapies has not yet been explored.

Transmembrane Protein 176B (TMEM176B) / Tolerance-related and induced (TORID) has been recently identified as an immunoregulatory cation channel (Anandasabapathy et al., 2014; Condamine et al., 2010; Drujont et al., 2016; Louvet et al., 2005; Segovia et al., 2014). This ubiquitously expressed intracellular protein is composed by four transmembrane domains containing an ITIM motif in its C-terminal (Condamine et al., 2010; Eon Kuek et al., 2016; Louvet et al., 2005). Tmem176a and its homologous Tmem176b are members of the CD20-like MS4A family of proteins (Eon Kuek et al., 2016; Louvet et al., 2005) and are highly expressed in monocytes, macrophages and $CD11b^+$ DCs (Condamine et al., 2010; Louvet et al., 2005).

Here we show that Tmem176b inhibits activation of the NLRP3 inflammasome and hinders development of antitumor T-cell responses by controlling cytosolic Ca^{++} homeostasis. Lack of *Tmem176b* promotes heightened T-cell mediated tumor growth inhibition and reinforces the antitumor activity of immune checkpoint blockers by activating inflammasome-dependent caspase 1/IL-1β pathway on myeloid cells. Accordingly, patients responding to immune checkpoint blockers exhibit an activated inflammasome signature. Finally, we identify BayK8644, a potent Tmem176b inhibitor, that reinforces the

anti-tumor responses unleashed by CTLA-4 or PD-1 blockade. Thus, inflammasome disinhibition by targeting TMEM176B may enhance the clinical efficacy of checkpoint blockade therapies in cancer.

RESULTS

Tmem176b/TMEM176B inhibits activation of the NLRP3 inflammasome

To investigate whether TMEM176B regulates inflammasome activation and antitumor immunity through the control of ion homeostasis, we first conducted *in vivo* experiments by injecting ATP in WT or *Tmem176b*$^{-/-}$ mice (Figure 1A). In this model, neutrophil recruitment to the peritoneal cavity relies on caspase-1/11 activation (Schroeder et al., 2017; Xiang et al., 2013). We observed that *Tmem176b*$^{-/-}$ mice recruited significantly more neutrophils than WT animals. To determine whether increased neutrophil recruitment upon ATP injection was dependent on inflammasome activation, we generated *Tmem176b*$^{-/-}$*Caspase1*$^{-/-}$ double knockout mice (Figure S1A). Peritoneal neutrophil recruitment was almost completely inhibited in *Tmem176b*$^{-/-}$*Caspase1*$^{-/-}$ as compared to *Tmem176b*$^{-/-}$ animals (Figure 1A). ATP-induced neutrophil recruitment in *Tmem176b*$^{-/-}$ mice was also interrupted by injection of a caspase-1 inhibitor (Figure S1B). We then stimulated WT and *Tmem176b*$^{-/-}$ bone marrow-derived DCs (BMDCs) with the well-established NLRP3 activators ATP and nigericin and determined IL-1β in culture supernatants as a readout of inflammasome activation. We observed that for both stimuli, *Tmem176b*$^{-/-}$ BMDCs secreted significantly higher levels of IL-1β than WT DCs in a dose and time-dependent manner (Figures 1B-C). Similar findings were observed when we stimulated BMDCs with aluminum particles (Figure S1C). Western blot studies confirmed that the mature (cleaved) form of IL-1β was more abundant in culture supernatants from *Tmem176b*$^{-/-}$ BMDCs as compared to those obtained from WT cells (Figure 1D). Moreover, we observed increased mature caspase-1 in supernatants from *Tmem176b*$^{-/-}$ BMDCs compared to WT cells when stimulated with ATP (Figure 1D). Although lower doses (2.5 μM) of nigericin induced expression of mature caspase-1 in culture supernatants from *Tmem176b*$^{-/-}$ but not WT BMDCs (Figure 1D), higher doses of this NLRP3 activator (5 μM) induced cleavage of caspase-1 in WT DCs whereas LPS alone did not (Figure S1D). In agreement with this observation, flow cytometry studies using the

FLICA1 reagent revealed higher caspase-1 activation in *Tmem176b*$^{-/-}$ BMDCs (Figure 1E). These results suggest that increased caspase-1 activation contributes to higher secretion of mature IL-1β in *Tmem176b*$^{-/-}$ BMDCs. To confirm these findings, we then induced inflammasome activation in WT and *Tmem176b*$^{-/-}$ BMDCs in the presence or absence of a caspase-1 inhibitor. Remarkably, IL-1β secretion was completely inhibited in WT and *Tmem176b*$^{-/-}$ BMDC when caspase-1 activation was interrupted (Figure 1F). Moreover, IL-1β secretion was completely abrogated in *Tmem176b*$^{-/-}$*Caspase1*$^{-/-}$ BMDCs (Figure 1G). Thus, increased IL-1β secretion observed as a result of *Tmem176b*$^{-/-}$ deficiency requires intact caspase-1 activity. Moreover, *Tmem176b*$^{-/-}$ BMDCs also secreted higher amounts of IL-18 as compared to WT cells in a caspase-1-dependent manner (Figure 1H). We then speculated that TMEM176B overexpression may impair IL-1β secretion in cells in which the inflammasome was activated. To address this issue, THP-1 differentiated macrophages were transfected with *TMEM176B/GFP* or *GFP* alone (Figure S1E) and then treated with LPS and nigericin. TMEM176B over-expression impaired IL-1β secretion as compared to GFP-transfected cells (Figure 1I). This effect was not associated with increased cell death as compared to GFP-transfected cells (Figure S1F). Thus, cation channel TMEM176B inhibits activation of the NLRP3 inflammasome.

TMEM176B inhibits the inflammasome through the control of cytosolic Ca^{++}

TMEM176B is an endophagosomal non-selective monovalent cation channel (Segovia et al., 2014). Because the NLRP3 inflammasome is tightly regulated by cytosolic K^+ (Muñoz-Planillo et al., 2013) and Ca^{++} (Murakami et al., 2012) levels, we speculated that TMEM176B may inhibit inflammasome activation through the regulation of ion homeostasis. To address this question, we first determined cytosolic Ca^{++} levels using the ratiometric Ca^{++}-sensitive probe Fura-2 in WT and *Tmem176b*$^{-/-}$ BMDCs stimulated with ATP. BMDCs lacking *Tmem176b* showed greater cytosolic Ca^{++} as compared to WT BMDCs (Figure 1J). Interestingly, intracellular Ca^{++} chelation using BAPTA-AM completely

blocked IL-1β secretion in WT and *Tmem176b*$^{-/-}$ BMDCs (Figure 1K). This effect was also dependent on K^+ efflux in WT and *Tmem176b*$^{-/-}$ BMDCs (Figure 1L).

We recently proposed that Ca^{++}-activated K^+ channels are involved in ATP-triggered inflammasome activation (Schroeder et al., 2017). We therefore inhibited Ca^{++}-activated K^+ channels in ATP-treated WT and *Tmem176b*$^{-/-}$ BMDCs and determined the amounts of IL-1β in culture supernatants. Inhibition of channel function using iberiotoxin or hydroxychloroquine (Schroeder et al., 2017) led to dose-dependent reduction in IL-1β secretion by WT BMDCs. Interestingly, IL-1β secretion by *Tmem176b*$^{-/-}$ BMDCs was completely abrogated by both inhibitors at doses that partially inhibited IL-1β secretion in WT BMDCs (Figure 1M-N). Thus, heightened inflammasome activation in *Tmem176b*$^{-/-}$ BMDCs is highly dependent on Ca^{++}-activated K^+ channels. These results suggest that TMEM176B impairs ATP-induced cytosolic Ca^{++} accumulation, preventing Ca^{++}-dependent K^+ channel-driven inflammasome activation.

Lack of *Tmem176b* restrains tumor growth in an IL-1β- and caspase-1-dependent manner

To investigate whether TMEM176B-mediated regulation of inflammasome activation may influence anti-tumor immunity, we first examined the association of TMEM176B expression in human cancer. High stromal TMEM176B expression was associated with significantly lower overall survival in colon cancer patients (n=90; p=0.0194; Log-rank Mantel-Cox test) (Figure S2A-B). Moreover, we detected a striking negative correlation between *TMEM176B* and *NLRP3/IL1B* expression from single cell RNA-seq analysis in macrophages infiltrating human melanoma [data analyzed from (Jerby-Arnon et al., 2018)], suggesting a role for this axis in the tumor microenvironment (Figure S2C). Accordingly, *Tmem176b*$^{-/-}$ mice inoculated with MC38 (colon), LL/2 (LLC1; lung) or EG7 (thymic lymphoma) cell lines showed higher survival (Figure 2A) and

reduced tumor growth (Figure S2D) compared to WT mice. Although Tmem176b is expressed by the three tumor cell lines studied (Figure S2E), immune cells from tumor-bearing *Tmem176b*$^{-/-}$ animals did not show enhanced *in vivo* cytotoxicity against WT cells as compared to tumor-bearing *Tmem176b*$^{+/+}$ mice (Figure S2F), suggesting that tumor-associated Tmem176b is not immunogenic in *Tmem176b*$^{-/-}$ hosts.

To investigate the mechanisms underlying TMEM176B contribution to tumor growth, we then studied inflammasome activation in tumors developed in WT and *Tmem176b*$^{-/-}$ mice. We found no differences in caspase-1 activation when comparing those tumors lysates (Figure S2G). However, we did find increased caspase-1 activation in tumor-draining lymph nodes (TDLN) from *Tmem176b*$^{-/-}$ mice as compared to WT animals as shown by Western blot (Figure 2B-C) and immunofluorescence staining (Figure 2D-E). Moreover, flow cytometry analysis revealed augmented caspase-1 activation in resident CD11c^{hi} MHCII^{+} CD11b^{+} classical DCs (cDCs) in *Tmem176b*$^{-/-}$ versus WT TDLN from tumor-bearing mice (Figure 2F-G and Figure S2H). Migratory and resident DCs were discriminated based on CD11c and MHCII expression (Figure S2H) as described (Kissenpfennig et al., 2005; Laoui et al., 2016). Interestingly, CD11c^{hi} MHCII^{+} CD11b^{+} cDCs expressed considerable amounts of Tmem176b (Crozat et al., 2011) and TDLN contained high numbers of CD11b^{+} Tmem176b^{+} cells compared to lymph nodes from naïve animals (Figure S2I).

Since CD11b^{+} cDCs induce differentiation of Th17 cells (Durai and Murphy, 2016), we then speculated that this CD4^{+} T-cell subset may augment in TDLN from *Tmem176b*$^{-/-}$ mice. We observed increased frequency of TCRβ^{+} CD4^{+} RORγt^{+} cells in TDLN from *Tmem176b*$^{-/-}$ animals as compared to WT and anti-IL-1β-treated *Tmem176b*$^{-/-}$ mice (Figure S2J). Moreover, *in vitro* re-stimulation of TDLN cells with OVA showed increased proportion of IL-17^{+} CD4^{+} T cells in *Tmem176b*$^{-/-}$ as compared to WT mice (Figure S2K) and *in vivo* IL-17A blockade showed a clear trend toward suppression of the antitumor effect in tumor-bearing *Tmem176b*$^{-/-}$ mice (Figure S2L). Thus, *Tmem176b* deficiency is

associated with an enhanced frequency of functional TCRβ^+ CD4$^+$ RORγt$^+$ IL-17$^+$ T cells in an inflammasome-dependent manner.

To study whether increased inflammasome activation could be responsible of tumor control in mice lacking *Tmem176b*, we blocked IL-1β and studied EG7 tumor development. Whereas *Tmem176b*$^{-/-}$ mice showed reduced tumor growth and increased mice survival when treated with control IgG, this effect was eliminated when mice were treated with anti-IL-1β neutralizing antibodies (Figure 2H). Thus, *Tmem176b* deletion inhibits development of syngeneic tumors in an IL-1β-dependent fashion. Tumor growth control was also rescued in *Tmem176b*$^{-/-}$*Caspase1*$^{-/-}$ double knockout mice (Figure 2I), suggesting that the diminished tumor growth observed in *Tmem176b*$^{-/-}$ mice was dependent on inflammasome activation

To further examine the cellular effectors involved in tumor growth inhibition in *Tmem176b*$^{-/-}$ mice, we then analyzed a panel of immunological mediators by quantitative RT-PCR. Notably, no differences were found between tumors grown in WT or *Tmem176b*$^{-/-}$ mice (Figure S3A left panel). Moreover, we did not find significant changes in the percentage or absolute numbers of infiltrating myeloid, B, NK, NKT or CD4$^+\alpha\beta$ T cells between WT and *Tmem176b*$^{-/-}$ tumors (Figure S3B). However, the percentage of total CD8$^+$ TCRβ^+ cells within the tumor infiltrate as well as the absolute number of total and tumor-specific CD8$^+$ T cells were considerably increased in tumors grown in *Tmem176b*$^{-/-}$ mice compared to those developed in WT mice (Figure S4A-B). Notably, although increased absolute numbers of CD4$^+$ CD25$^+$ Foxp3+ regulatory T (Treg) cells were found within tumors developed in *Tmem176b*$^{-/-}$ as compared to WT animals (Figure S4C), an increased Teff (CD8) / Treg (Foxp3) ratio was apparent (Figure S4C). Moreover, tumor-infiltrating CD8$^+$ T cells from *Tmem176b*$^{-/-}$ mice showed greater proliferation compared to those obtained from WT animals when re-stimulated *in vitro* with OVA MHC-I peptide (Figure S4D). Interestingly, we found downregulation of the Treg-related molecules Foxp3, CTLA-4, CCL5, CCL19 and CCL22 in TDLN from *Tmem176b*$^{-/-}$ versus WT mice

(Figure S3A right panel). Moreover, decreased percentages but not absolute numbers of TCRβ$^+$ CD4$^+$ Foxp3$^+$ Treg cells were observed in TDLN from *Tmem176b*$^{-/-}$ versus WT mice (Figure S5A-B). Moreover, the CD8/Treg ratio in TDLN was significantly increased in mice lacking *Tmem176b* (Figure S5B). *In vivo*, MHC I-dependent CD8$^+$ T-cell mediated cytotoxicity against the tumor-associated OVA antigen was increased in tumor-bearing *Tmem176b*$^{-/-}$ compared to WT mice (Figure 2J and Figure S4E). This effect was prevented in *Tmem176b*$^{-/-}$ animals treated with anti-IL-1β antibodies (Figure 2K) as well as in *Tmem176b*$^{-/-}$*Caspase-1*$^{-/-}$ animals (Figure 2L). Within the tumor microenvironment, CTLs from *Tmem176b*$^{-/-}$*Caspase-1*$^{-/-}$ showed lower expression of the degranulation marker CD107a as compared to *Tmem176b*$^{-/-}$ mice (Figure S4F). Interestingly, depletion of CTLs in *Tmem176b*$^{-/-}$ mice using an anti-CD8 monoclonal antibody rescued tumor growth to similar levels as those observed in WT mice (Figure 2M). Thus, *Tmem176b* deletion enhances CTL-mediated-tumor control through mechanisms involving the caspase-1-IL-1β pathway. This mechanism is associated with inflammasome-dependent induction of TCRβ$^+$ CD4$^+$ RORγt$^+$ cells. Altogether, these results support a role for TMEM176B as an emerging immune checkpoint that interrupts inflammasome activation and links innate and adaptive antitumor responses.

Inflammasome activation reinforces immune checkpoint blockade therapies

Given the influence of *Tmem176b* deletion in antitumor immunity, we investigated whether targeting this ion channel might control the efficacy of checkpoint blockade therapies. Remarkably, we found greatly diminished tumor growth when anti-CTLA-4 antibodies were inoculated in *Tmem176b*$^{-/-}$ as compared to WT mice. Improved survival observed in anti-CTLA-4-treated *Tmem176b*$^{-/-}$ mice was dependent on inflammasome activation as this effect was abrogated in *Tmem176b*$^{-/-}$*Caspase1*$^{-/-}$ animals (Figure 3A). To investigate this further, we injected anti-CTLA-4 or anti-PD-1 mAb in EG7 tumor-bearing C*aspase 1/11*$^{-/-}$ or *Nlrp3*$^{-/-}$ mice. Genetic deficiency of *Caspase 1/11* eliminated the anti-

tumor effects triggered by CTLA-4 or PD-1 blockade (Figure 3B). Although the experiments performed in *Nlrp3*$^{-/-}$ mice did not reach statistical significance, there was a clear tendency towards lower survival in those mice when treated with anti-CTLA-4 or anti-PD-1 mAb (Figure 3C). Moreover, we found no differences in tumor growth in mice lacking inflammasome components under control conditions (Figure 3B-C) in agreement with previous reports (Ghiringhelli et al., 2009). These results highlight the importance of triggering inflammasome activation to improve the efficacy of checkpoint blockade therapies.

Sensitivity to immune checkpoint blockers is associated with an 'inflammasome activated' signature in cancer patients

To further understand the clinical relevance of our findings, we then investigated whether inflammasome-related genes might be associated with clinical responses in patients treated with immune checkpoint blockers. We first analyzed data obtained from whole-exome sequencing and transcriptomics of a published cohort of melanoma patients treated with checkpoint inhibitors (Riaz et al., 2017). These studies focused on pretreatment and on treatment tumor biopsies from patients that progressed to anti-CTLA-4 therapy and were further treated with anti-PD-1 antibodies (IPI-progressing) and patients treated with anti-PD-1 antibodies who had not received anti-CTLA-4 antibodies (IPI-naïve). Strikingly, in the IPI-naïve population of non-responder patients, only two inflammasome-related genes were significantly up-regulated during treatment as compared to the pre-treatment stage. These genes were *TMEM176A and TMEM176B* (Figure 4A and Table S1). These observations emphasize the role of TMEM176 ionic channels as potential mediators of resistance to checkpoint blockade therapies.

Interestingly, when comparing patients responding or not to anti-PD-1 at the pre-treatment stage, we found no significant differences in inflammasome-related genes either in the bulk population (Table S2), IPI-naïve (Table S3) or IPI-progressed (Table S4)

groups. However, 8 inflammasome-related genes were significantly up-regulated in responders versus non-responder patients in the bulk population during anti-PD-1 treatment (Figure 4B). *TMEM176A* and *TMEM176B* were two of the inflammasome-related genes that were significantly upregulated in patients responding to anti-PD-1, suggesting that they could function as a counter-regulatory mechanism in response to treatment. Similar findings were observed in the IPI-naïve population (Table S5). We then performed a paired analysis comparing pre-treatment and on anti-PD-1 treatment tumor biopsies from responder patients. In this case, we found 11 inflammasome-related genes that were significantly up-regulated during anti-PD-1 therapy as compared to the pre-treatment biopsies (Figure 4C). Similar results were found when analyzing the IPI-naïve population (Table S6).

We then analyzed transcriptomic datasets from patient biopsies to estimate the diversity of leukocyte populations infiltrating tumors using the CIBERSORT method (Newman et al., 2015). We observed increased relative frequencies of $CD8^+$ T cells and activated memory $CD4^+$ T cells during anti-PD-1 treatment versus the pre-treatment stage in responders but not in progressor patients (Figure 4D). Absolute total number of leukocytes, $CD8^+$ T cells and activated memory $CD4^+$ T cells were also increased (Figure 4E). In patients responding to anti PD-1 therapy, the total number of leukocytes as well as the frequency of $CD8^+$ T cells and activated memory $CD4^+$ T cells were positively associated with the expression of NLRP3 during ongoing treatment (Figure 4F). These observations reinforce the concept that inflammasome activation controls T-cell immunity in patients treated with immune checkpoint blockers.

To validate further these observations, we analyzed the inflammasome gene expression profile in longitudinal tumor biopsies from melanoma patients treated sequentially with anti-CTLA-4 and anti-PD-1 antibodies (Chen et al., 2016). These authors studied gene expression profiling (GEP) via a custom 795-gene NanoString panel composed of immune and cancer-related genes. Of note, *TMEM176A* and *TMEM176B* were not included in the NanoString panel used by Chen et al. The authors found no

significant differences in GEP when comparing responders versus progressors at baseline (before CTLA-4 or PD-1 blockade) or following anti-CTLA-4 or anti-PD-1 therapy. Consistently, we found no significant expression of inflammasome-related genes at these stages (Figures S6A-B and S7A-B). These results are in agreement with our findings from the analysis from the Riaz et al cohort at the pre-treatment stage (Tables S1-4). However, the authors found 411 genes that were significantly regulated (mostly up-regulated) in responding versus progressing patients following early PD-1 blockade. In those patients, 15/16 inflammasome-related genes were significantly upregulated in responders compared to progressors (Figure 5A). We then performed a paired analysis of the 16 inflammasome-related genes in biopsies of 5 responding patients and 7 progressors comparing gene expression prior and during anti-PD-1 therapy. All these patients had progressed to anti-CTLA-4 therapy. Critically, 5/5 patients responding to anti-PD-1 showed a significant up-regulation of inflammasome-related genes during anti-PD-1 treatment (Figure 5B). Moreover, 4/7 patients who did not respond to anti-PD-1 therapy significantly downregulated the inflammasome signature during PD-1 blockade (Figure 5B). Thus, gene expression profiles from biopsies of two independent cohorts of melanoma patients treated with immune checkpoint blockers revealed strong association between inflammasome activation and clinical responses. These findings support the notion that inflammasome activation contributes to antitumor responses triggered by immune checkpoint blockers and highlights the potential role of an 'inflammasome activation' signature as a biomarker of response to immune checkpoint blockade.

Pharmacologic inhibition of Tmem176b triggers inflammasome-dependent tumor control and improves the efficacy of immune checkpoint blockers

The up-regulation of inflammasome-related genes in patients responding to immune checkpoint blockers (Figures 4 and 5), the contribution of caspase 1/11 to the success of anti-CTLA-4 and anti-PD-1 therapies (Figure 3) and the central role of

TMEM176B as a potent inflammasome inhibitor (Figure 1), suggested the possibility of targeting this pathway to improve the efficacy of immune checkpoint blockers. To identify drugs capable of inhibiting Tmem176b-dependent ion flux and triggering inflammasome activation, we set up an *in vitro* assay. Briefly, CHO-7 cells were transfected with *Tmem176b* and *Tmem176a*-mCherry. Cells were then loaded with the Na^+-sensitive fluorescent dye Asante NaTRIUM Green 2 (ANG-2). We observed increased ANG-2 MFI in $mCherry^+$ compared to $mCherry^-$ cells. We then screened a compound library known to modulate ion channels activity (Supplementary Dataset 1). We found that both enantiomers (+) and (-) of BayK8644 potently inhibited Tmem176b-a-dependent Na^+ influx, while minimally affecting Tmem176b-a negative cells (Figure 6A-B and Figure S8A). Therefore, we decided to focus on these compounds also because they are members of the dihydropyridin family, which are drugs currently used in clinical settings. Whereas (+) BayK8644 is known to inhibit L-type voltage-dependent Ca^{++} channels, the (-) stereoisomer activates those channels (Grove et al., 1991; Hamilton et al., 1987; Yatani et al., 1988). Since both isomers inhibit Tmem176b/a activity, it is unlikely that our observations could be explained by indirect effects on Na^+ influx through the modulation of Ca^{++} channels. Although we cannot rule out the possibility that BayK8644 could inhibit Tmem176a in the ANG-2 experiments, in electrophysiology studies using Tmem176b-overexpressing Xenopus oocytes, (+) BayK8644 completely inhibited Tmem176b-dependent current (Figure 6B). Therefore, we focused on the (+) isomer for functional experiments.

Because BayK8644 inhibits Tmem176b-dependent ion fluxes, we asked whether this drug could trigger inflammasome activation in BMDCs. We observed that BayK8644 induced IL-1β secretion and caspase-1 activation in LPS-primed WT BMDCs but not in *Tmem176b*$^{-/-}$ cells (Figure 6C and Figure S8B-D). Furthermore, BayK8644-induced IL-1β was inhibited by the KCa inhibitors TEA and HCQ (Figure 6D). Thus, BayK8644 treatment on WT BMDCs phenocopied *Tmem176b*$^{-/-}$ cells as IL-1β secretion was highly dependent on Ca^{++}-activated K^+ channels. In THP-1 macrophages, TMEM176B-dependent inhibition

of IL-1β secretion was prevented when these cells were treated with BayK8644 (Figure 6E). These results indicate that BayK8644 triggers inflammasome activation through inhibition of TMEM176B.

To evaluate the pre-clinical relevance of these findings, we then explored whether BayK8644 treatment may inhibit tumor growth. Administration of BayK8644 (i.p) significantly increased survival of tumor-bearing WT but not *Tmem176b*$^{-/-}$ mice (Figure 6F) compared to injection of vehicle control, highlighting the ability of the drug to restrain tumor growth through inhibition of Tmem176b. Of note, *in vitro* treatment of EG7 thymic lymphoma cells with BayK8644 did not induce apoptosis at similar doses as those detected in plasma after i.p injection (Figure S8E). To explore whether BayK8644 recapitulated the effects in tumor growth control observed in untreated *Tmem176b*$^{-/-}$ mice, we evaluated the effects of the drug in inflammasome activation by disrupting important components of this pathway. We found that BayK8644 significantly improved survival of WT but not *Caspase 1/11*$^{-/-}$ tumor-bearing mice (Figure 6G). Consistent with this observation, BayK8644 increased the frequency of CD11b^{+} cDCs expressing active caspase-1 in TDLN (Figure 6H).

Similar to *Tmem176b*$^{-/-}$ mice, which controlled tumor growth via IL-1β- and CD8^{+} T cell-dependent mechanisms, we observed that BayK8644 increased CD8^{+} T cell-mediated tumor cytotoxicity *in vivo* (Figure 6I). Moreover, BayK8644-induced tumor control was dependent on CD8^{+} T cells, since depletion of these cells completely abolished the antitumor effect of this inhibitor (Figure 6J and Figure S8F). Thus, BayK8644 restrains tumor growth in a Tmem176b-, caspase-1/11- and CD8^{+} T cell-dependent manner in EG7 tumors. Moreover, BayK8644 significantly impaired growth of CT26 colon cancer cells in BALB/c mice (Figure S8G-H). Thus, BayK8644 emerges as a new immunotherapeutic agent that limits tumor growth by licensing inflammasome activation.

Finally, we evaluated whether BayK8644 administration may enhance the antitumor activity of immune checkpoint blockers. Compared with mice treated with

monotherapy, administration of BayK8644 in combination with anti-CTLA-4 antibodies significantly improved survival of tumor (EG7)-bearing mice (Figure 6K). Moreover, therapeutic administration of BayK8644 in mice with EG7 established tumors significantly improved the anti-tumoral effect of anti-PD-1 treatment (Figure 6L), whereas BayK8644 monotherapy was not effective in this therapeutic protocol (data not shown). Interestingly, combination of anti-PD-1 with BayK8644 was associated with an increased absolute number and percentage of $TCR\beta^+CD4^+ROR\gamma t^+$ T cells in TDLN (Figure S8I) and increased frequency of tumor-specific $CD8^+$ T cells within the tumor microenvironment (Figure 6M) as compared to anti-PD-1 monotherapy. Depletion of $CD8^+$ T cells in anti-PD-1 + BayK8644-treated animals abrogated antitumor immunity (Figure 6N). This observation might be explained by concomitant CTL-mediated mechanisms required for both the antitumor activity of BayK8644 (Figure 6J and S8F) and anti-PD-1 therapy (Sharma and Allison, 2015). Thus, as expected, combination treatment strongly relies on the $CD8^+$ T-cell compartment. Furthermore, BayK8644 significantly enhanced the anti-tumoral effect of anti-PD-1 therapy in mice bearing 5555 melanoma (Figure 6O), whereas this effect was also apparent in LL/2 lung cancer (Figure S8J-K) and MC38 colon cancer (Figure S8L-M) models, although not reaching statistical significance. Thus, unleashing inflammasome activation by targeting Tmem176b improves the anti-tumor activity of checkpoint inhibitors in different tumor models. Moreover, whereas BayK8644 reinforced the anti-tumor effects of anti-PD-1 treatment in mouse melanoma, it did not enhance tumor growth inhibition induced by anti-CTLA-4 and anti-PD-1 combination therapy, at least in this model (Figure S8O). Given the pharmacologic impact of channel inhibitors in cardiomyocyte function, we finally examined whether dihydropyridin BayK8644 may lead to acute cardiac toxicity. Notably, Bayk8644 treatment was not associated with electrocardiographic nor echocardiographic alterations 30 min after i.v. injection compared to mice treated with vehicle control (Tables 1-2). Thus, pharmacological inhibition of TMEM176B by Bayk8644, represents a novel therapeutic approach to unleash

inflammasome activation, leading to potentiation of $CD8^+$ T-cell-dependent antitumor immunity and greater efficacy of immune checkpoint blockers.

DISCUSSION

In this study, we demonstrate a central role of the inflammasome as a novel target to reinforce $CD8^+$ T cell-dependent antitumor immunity and enhance the efficacy of immune checkpoint blockade therapies. In particular, we demonstrate the key role of TMEM176B, an ionic channel expressed on myeloid cells (including cDCs) as a negative regulator of inflammasome activation. Furthermore, we identify BayK8644 as a potent TMEM176B inhibitor that reinforces antitumor immunity and enhances the efficacy of immune checkpoint blockers via inflammasome activation. Finally, we highlight the predictive value of NLRP3 inflammasome activation in clinical responses to immune checkpoint blockade in two independent cohorts. Thus, patients who do not respond to immune checkpoint blockers are expected to benefit from therapeutic strategies capable of triggering inflammasome activation.

Most immunotherapeutic approaches have focused on drugs targeting adaptive components of the immune system. However, innate immune pathways have recently emerged as potential targets amenable of intervention to trigger and control downstream adaptive players. In this regard, stimulation of STING activity has been shown to enhance the anti-tumoral effect of checkpoint blockers (Corrales et al., 2015; Demaria et al., 2015; Woo et al., 2014). Importantly, a type-I IFN pathway signature correlates with sensitivity to anti-CTLA-4 antibodies in melanoma patients (Chiappinelli et al., 2015). Nevertheless, the role of the IL-1β/IL-18/inflammasome in modulating anti-tumor responses is still controversial (Karki et al., 2017; Apte and Voronov, 2008). It has been proposed that the cell type in which inflammasome is activated and the nature of acute versus chronic inflammasome activation may dictate the outcome of the antitumor responses (Karki et al., 2017).

Immunogenic cell death triggered by chemotherapeutics, which sensitizes tumors to checkpoint blockade therapies, relies in NLRP3 inflammasome activation by DCs (Ghiringhelli et al., 2009; Pfirschke et al., 2016). Although recently proposed to play a role

in the outcome of immunotherapeutic approaches (Mangan et al., 2018), the direct contribution of the inflammasome to adaptive checkpoint blockade remains elusive. Here we identified a new strategy to reinforce antitumor responses by targeting TMEM176B and promoting inflammasome disinhibition. Our results suggest that inflammasome activation plays a central role in antitumor immunity triggered by anti-CTLA-4 and anti-PD-1 antibodies. It is worth noting that experiments using *Nlrp3*$^{-/-}$ animals did not reach statistical significance, whereas experiments in animals lacking downstream effectors *Caspase-1/11* did. These observations suggest that different inflammasomes may be involved in the anti-tumoral effect triggered by anti-CTLA-4 and anti-PD-1 therapies. Accordingly, analysis of anti-PD-1-treated melanoma patients (Figures 4 and 5) suggest that NLRP6, NLRP7, AIM2 and NLRC4 inflammasomes might contribute to antitumor responses unleashed by checkpoint blockers. Although the requirement of caspase-1 autoproteolysis can differ within different inflammasomes (Broz et al., 2010) and caspase-1 may cleave other proteins than IL-1β and IL-18 (Sokolovska et al., 2013), to our knowledge, caspase-1 and caspase-11 activation mostly depend on inflammasomes. Thus, it is unlikely that results obtained with both *Caspase-1/11*$^{-/-}$ and *Tmem176b*$^{-/-}$ *Caspase-1*$^{-/-}$ mice might rely on inflammasome-independent mechanisms.

Our results suggest that TMEM176B might be a predictive marker of response to anti-PD-1 therapy. In addition, *TMEM176B* expression in the tumor stroma was associated with poor survival in colorectal cancer patients suggesting a potential role of this ion channel as a prognostic factor. Interestingly, TMEM176B was associated with diminished *NLRP3* and *IL1B* expression in macrophages infiltrating human melanoma, suggesting that this ion channel may function as an innate checkpoint signal that hinders immune responses in the tumor microenvironment. However, our results in experimental models support a key role for Tmem176b in the modulation of inflammasome activation mostly in TDLN during the induction phase of anti-tumor responses. Thus, Tmem176b-dependent immune inhibitory mechanisms may operate within the tumor microenvironment and TDLN. Although further mechanistic studies are warranted to

elucidate the molecular pathways that control Tmem176b expression, we found a reciprocal regulation of this ion channel by inflammasome activation (Figure S9A). Notably, LPS+ATP treatment induced a striking up-regulation of IL-6 and TNF-α (Figure S9A) and a very modest increase in cell death (Figure S9B) as compared to untreated BMDCs, excluding the possibility that down-regulation of Tmem176b by inflammasome activation could be related to massive induction of cell death.

Recently, it has been proposed that pharmacologic manipulation of ion channels may influence NLRP3 inflammasome activation (Gong et al., 2018). Here we show that BayK8644 attenuates tumor growth by inhibiting TMEM176B activity, leading to inflammasome disinhibition and $CD8^+$ T cell responses with no evident signs of cardiotoxicity. Remarkably, BayK8644 treatment recapitulated *Tmem176b* deficiency as shown by increased caspase-1 activation in $CD11b^+$ cDCs in TDLN. These observations are in line with the high expression of TMEM176B in these cells (Anandasabapathy et al., 2014; Crozat et al., 2011).

Remarkably, in this study we showed that *Tmem176b* deficiency is associated with increased inflammasome activation both *in vitro* and *in vivo.* Moreover, TMEM176B overexpression inhibited LPS and nigericin-induced IL-1β secretion by macrophages. Increased inflammasome activation in *Tmem176b*$^{-/-}$ DCs was dependent on K^+ efflux and increased cytosolic Ca^{++}. In this regard, the hierarchy between Ca^{++} and K^{++} in inflammasome activation is a matter of debate (Gong et al., 2018). We have previously shown that Ca^{++}-activated K^+ channels such as KCa1.1 can mediate NLRP3 inflammasome activation and are inhibited by hydroxychloroquine (Schroeder et al, 2017). Here we found complete inhibition of IL-1β secretion in *Tmem176b*$^{-/-}$ DCs by the specific KCa1.1 inhibitor iberiotoxin as well as by hydroxychloroquine. Secretion of IL-1β by *Tmem176b*$^{-/-}$ DCs seems therefore to be highly dependent on KCa1.1 channels. In this sense, sustained K^+ efflux in T cells through the voltage-gated (K_v1.3) or Ca^{++}-activated (KCa3.1) K^+ channels has been shown to reinvigorate tumor-infiltrating T cells (Eil et al.,

2016). Our results suggest that K^+ efflux in DCs may also promote anti-tumor immunity by triggering inflammasome activation, a process that is repressed by TMEM176B. Taken together, these observations highlight a regulatory role for ion homeostasis in controlling anti-tumor immunity through modulation of both innate and adaptive immune programs.

In conclusion, our study links inflammasome activation to antitumor responses triggered by immune checkpoint blockers, highlighting a central role for TMEM176B, an ion channel expressed on myeloid cells, in repression of T-cell dependent immunity. Further efforts should be aimed at evaluating the clinical efficacy and safety of inflammasome disinhibition in the treatment of cancer patients, particularly those resistant to current immunotherapeutic modalities.

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AUTHOR CONTRIBUTIONS

Conceptualization, M.S., S.R. MCC, G.A.R. and M.H.; Methodology, M.S and S.R.; Validation, R.A.F., M.C.C., B.V., M.R.G., G.A.R and M.H.; Investigation, M.S., S.R., M.J., V.P., M.D., Y. D. M., S.V., F.V., M.R.G., P.C. and M.R.; Writing – Original Draft, M.S., S.R., M.R.G, G.A.R. and M.H.; Funding Acquisition, M.H.; Resources, C.L., R.A.F, I.A. and M.C.C.; Supervision, M.H.

DECLARATION OF INTERESTS

BV declares conflict of interest with OSE Immunotherapeutics. MH is founder and CSO of ARDAN Immuno Pharma.

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FIGURE LEGENDS

Figure 1. The ionic channel Tmem176b/TMEM176B inhibits the NLRP3 inflammasome

A) Six-to-eight weeks-old male WT, *Tmem176b*$^{-/-}$ and *Tmem176b*$^{-/-}$*Caspase1*$^{-/-}$ mice were injected i.p with vehicle control (PBS) or 20 mg/kg ATP. Four h later, peritoneal lavage was performed and absolute numbers of neutrophils (CD11b^{+} Ly6C^{int} Ly6G^{+}) were determined by flow cytometry. The left panel shows representative dot plots and the right panel shows quantifications for the different groups. In the plots, CD11b^{+} cells were analyzed for Ly6C and Ly6G expression. At least six animals were studied in each group in two independent experiments. ns: not significant; * $p<0.05$; One-way ANOVA test.

B-C) WT and *Tmem176b*$^{-/-}$ bone marrow-derived DCs (BMDCs) were treated with LPS (0.25 µg/ml) for 4 h, then washed and treated with the indicated doses of ATP or nigericine (Nig) for the indicated times. Dose-response experiments are shown in B) whereas time-response experiments are depicted in C). Culture supernatants were harvested and IL-1β was determined by ELISA. One experiment representative of five is shown. * $p<0.05$; ** $p<0.01$; Two-way ANOVA test.

D) Western blot analysis of pro-IL-1β and pro-caspase-1 (lysates) or IL-1β and caspase-1 (supernatants) in WT and *Tmem176b*$^{-/-}$ BMDCs stimulated with LPS as in B-C) and then treated for 90 min with 2.5 µM nigericin or 0.5 mM ATP. Culture supernatants and cell lysates were analyzed. One experiment representative of three is shown.

E) WT and *Tmem176b*$^{-/-}$ BMDCs were treated with LPS and then with 0.5 mM ATP or 2.5 µM nigericin (Nig) for 45 min. Cells were harvested and stained with FLICA1 660 to determine active caspase-1. One experiment representative of three is shown. * $p<0.05$; Two-way ANOVA test.

F) IL-1β secretion by WT and *Tmem176b*$^{-/-}$ BMDCs treated as in E) and compared to an experimental condition where caspase-1 was inhibited by adding 10 µM Z-WEHD-FMK

15 min before ATP. One experiment representative of three is shown. ** p<0.01; **** p<0.0001; Two-way ANOVA test.

G-H) WT, *Tmem176b*$^{-/-}$ and *Tmem176b*$^{-/-}$*Caspase1*$^{-/-}$ BMDCs were treated as in E). IL-1β (G) or IL-18 (H) were determined in culture supernatants by ELISA. One experiment representative of two is shown. ND: Not detected. * p<0.05; ** p<0.01; **** p<0.0001; Two-way ANOVA test.

I) THP-1 cells were differentiated to macrophages by treatment with 0.1 µM PMA for a 48 h-period. Cells were then electroporated with *GFP* or *GFP-TMEM176B* encoding pcDNA1.3 plasmids. Sixteen hours later, cells were left untreated or treated for 3 h with 0.25 µg/ml LPS and then for 2 h with 2.5 µM nigericin (Nig). IL-1β was determined in culture supernatants by ELISA. One experiment representative of four is shown. ** p<0.01; *** p<0.001; Two-way ANOVA test.

J) WT and *Tmem176b*$^{-/-}$ BMDCs were treated for 3 h with 0.25 µg/ml LPS. Cells were then loaded with the Ca^{++}-sensitive probe Fura-2. Emission at 340/380 nm was recorded in time-laps experiments. 0.5 mM ATP was added when indicated by the arrow.

K) NLRP3 inflammasome was activated in BMDCs as described in E) but in the presence of the intracellular Ca^{++} chelator BAPTA (100 µM) or DMSO vehicle control. One experiment representative of three is shown. *p<0.05. Two-way ANOVA test.

L) IL-1β determination in BMDSCs following inflammasome activation in the presence of control buffer (5 mM) or high K^+ buffer (120 mM). One experiment representative of three is shown. * p<0.05; Two-way ANOVA test.

M-N) IL-1β determination in BMDSCs following inflammasome activation in the presence or absence of the Ca^{++}-activated K^+ channels iberiotoxin (IbTx, in M) or hydroxychloroquine (HCQ, in N). One experiment representative of three is shown in each case. * p<0.05; ** p<0.01; Two-way ANOVA test.

See also Figure S1.

Figure 2. Mice lacking *Tmem176b* control tumor growth through an IL-1β and caspase-1-dependent manner

A) MC38 colon cancer cells (1 x 10^6), LL2 lung cancer cells (1 x 10^5) or EG7 thymic lymphoma cells (1 x 10^6) were s.c. injected in WT and *Tmem176b*$^{-/-}$ mice. Tumor growth and mice survival were monitored every three days. Tumors were measured by its longer and shorter diameters. Mice were sacrificed when one of the diameters reached 2 cm. The ratio shows the number of surviving animals/ total injected mice from three experiments. * p<0.05; ** p<0.01; Log-rank (Mantel-Cox) test.

B) Western blot analysis of caspase-1 expression and cleavage in tumor-draining lymph nodes (TDLN) from WT and *Tmem176b*$^{-/-}$ mice.

C) Densitometric analysis of Western blots shown in B. At least 4 animals/group are shown. * p<0.05; Student's *t* test.

D) Confocal microscopy of activated caspase-1 expression using the FLICA1 fluorescent probe in TDLN.

E) Semiquantification of staining depicted in D is shown. WT and *Tmem176b*$^{-/-}$ animals (n=3 each group) were studied. * p<0.05; *** p<0.001; One-way ANOVA test.

F) Flow cytometry analysis of FLICA1$^+$ cells within TDLN. SSC: Side Scatter. One experiment representative of two is shown.

G) Evaluation of FLICA1$^+$ CD11b$^+$ and CD11b$^-$ classical DCs (cDCs) in the TDLN is shown. ns = not significant, *** p<0.001; Student's *t* test.

H) Survival of *Tmem176b*$^{-/-}$ EG7 tumor-bearing mice treated with anti-IL-1β or IgG control antibodies. The ratio shows the number of surviving animals/ total injected mice from one experiment. * $p<0.05$; Log-rank (Mantel-Cox) test.

I) Survival of untreated *Tmem176b*$^{-/-}$ and *Tmem176b*$^{-/-}$*Caspase1*$^{-/-}$ EG7 tumor-bearing mice. The ratio shows the number of surviving animals/ total injected mice pooled from three independent experiments. * $p<0.05$; Log-rank (Mantel-Cox) test.

J) *In vivo* cytotoxicity assay against OVA peptide-pulsed EG7 cells as a model tumor antigen. Naïve splenocytes were loaded with high dose DDAO and SIINFEKL peptide. Cells were mixed (1:1 ratio) with naïve splenocytes exposed to low dose DDAO without peptide. The mix was injected i.v. into naïve and tumor-bearing mice and 4 h later the ratio between the high and low DDAO populations in the spleen was determined by flow cytometry. Representative histograms are shown in Figure S4E. Data from four different animals of one experiment in each group are shown. ** $p<0.01$; Student's *t* test.

K) *In vivo* cytotoxicity assay as described in J) in *Tmem176b*$^{-/-}$ mice previously treated with anti-IL-1β or control IgG antibodies. * $p<0.05$; Student's *t* test.

L) *In vivo* cytotoxicity assay as described in J) comparing CTLs from tumor-bearing *Tmem176b*$^{-/-}$ and *Tmem176b*$^{-/-}$*Caspase1*$^{-/-}$ mice. Data from two experiments are shown. * $p<0.05$; Student's *t* test.

M) Survival of tumor-bearing WT and *Tmem176b*$^{-/-}$ mice left untreated or treated with anti-CD8 depleting antibodies. The ratio depicts the number of surviving animals/ total injected mice. Data from one experiment are shown. * $p<0.05$; Log-rank (Mantel-Cox) test.

The genetic background of the animals used was C57BL/6.

See also Figures S2-S5.

Figure 3. Inflammasome activation reinforces immune checkpoint blockade

Survival analysis of the indicated groups of animals in response to different treatments. The ratio depicts the number of surviving animals/total injected mice.

A) Tumor (EG7)-bearing WT, *Tmem176b*$^{-/-}$ and *Tmem176b*$^{-/-}$*Caspase1*$^{-/-}$ mice were injected with anti-CTLA-4 or control IgG antibodies and mice survival was studied. *p<0.05; Log-rank (Mantel-Cox) test.

B) Tumor (EG7)-bearing WT and *Caspase1/11*$^{-/-}$ mice were injected with anti-CTLA-4, anti-PD-1 or control IgG antibodies and mice survival was studied. *p<0.05; Log-rank (Mantel-Cox) test.

C) Tumor (EG7)-bearing WT and *Nlrp3*$^{-/-}$ mice were injected with anti-CTLA-4, anti-PD-1 or control IgG antibodies and mice survival was studied. Log-rank (Mantel-Cox) test.

Data from 3 (A-B) or 2 (C) experiments are shown.

The genetic background of the animals used was C57BL/6.

Figure 4. Analysis of the inflammasome signature in tumor biopsies from melanoma patients treated with immune checkpoint blockers (cohort by Riaz *et al.* 2017)

A) Paired analysis of patients who did not respond to anti-PD-1 therapy and were not treated previously with anti-CTLA-4 antibody (IPI-naïve) comparing pre-treatment vs on-treatment melanoma biopsies. *TMEM176A* and *TMEM176B* were the only inflammasome-related genes that were significantly up-regulated in the on-treatment as compared to pre-treatment population. * p<0.05; Paired Student's *t* test.

See also Table S1.

B) Heat maps of transcriptomic analysis from tumor biopsies of melanoma patients responding (responders) or not (non responders) to anti-PD-1 therapy. The indicated inflammasome-related genes were significantly up-regulated in responder patients *p<0.05; unpaired Student's *t* test.

C) Paired analysis of patients responding to anti-PD-1 therapy comparing pre-treatment vs on-treatment melanoma biopsies. The indicated inflammasome-related genes were significantly up-regulated during therapy. p<0.05; Paired Student's *t* test.

D-E) Paired study of pre-treatment vs on-treatment tumor biopsies from bulk patients responding to anti-PD-1 therapy analyzed through the CIBERSORT method. The relative frequency of $CD8^+$ T cells, activated and non-activated memory $CD4^+$ T cells (D) as well as the absolute number of total leukocytes, $CD8^+$ T cells and activated memory $CD4^+$ T cells (E) were analyzed. * p<0.05; ** p<0.01; Paired Student's *t* test.

F) Association of NLRP3 expression with the frequency of total leukocytes, $CD8^+$ T cells and activated memory $CD4^+$ T cells in patients responding to anti-PD-1 therapy. Results show transcriptomics data obtained from tumor biopsies at the on-treatment stage.

See also Tables S1-6.

Figure 5. Analysis of the inflammasome signature in tumor biopsies from melanoma patients treated with immune checkpoint blockers (cohort by Chen *et al.* 2016)

A) The log2-transformed normalized NanoString counts from melanoma tumor biopsies for the indicated inflammasome-related genes is shown for patients being treated with anti-PD-1. The results for responding and non-responding patients as defined by Chen et al. 2016 * p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001; Unpaired Student's *t* test.

Paired analysis of the 16 inflammasome-related genes studied in (A) comparing pre-treatment and on-treatment tumor biopsies from melanoma patients responding (n= 5) or not responding (n= 7) to anti-PD-1 therapy. See color code to identify each gene in Figure S7C. * p<0.05; *** p<0.001; **** p<0.0001; Paired Student's *t* test.

See also Figures S6 and S7.

Figure 6. Targeting Tmem176b with Bayk8644 triggers inflammasome-dependent antitumor immunity

A) Identification of Tmem176b inhibitors. CHO-7 cells were transfected with *Tmem176b* and *Tmem176a*-mCherry-coding pSecTag2B plasmids. Cells were then loaded with the Na^+-sensitive fluorescent dye Asante NaTRIUM Green 2 (ANG-2). Left panels show representative flow cytometry histograms assessing ANG-2 fluorescence in the indicated conditions. The right panel shows quantification of ANG-2 mean fluorescence intensity (MFI) subtracting in each condition the MFI obtained in Na^+-free buffer. Untreated and (+) BayK8644-treated cells were studied. One experiment representative of five is shown. ** p<0.01; *** p<0.001; Two-way ANOVA test.

B) *Tmem176b*-coding mRNA was injected in *Xenopus* oocytes. Forty-eight h later, oocytes were left untreated or activated (treated for 30 min with 0.1 µM PMA and extracellular pH 5.0 as previously described (Segovia et al., 2014)). Tmem176b-dependent conductance was assessed in conditions were 10 µM (+) BayK8644 was added to extracellular buffer during PMA stimulation. Representative currents are shown in the left panel. Determination of Tmem176b current at 800s post-extracellular acidification is depicted in the right graph. *** p<0.001; One-way ANOVA test.

C) WT and *Tmem176b*$^{-/-}$ BMDCs were primed for 3 h with LPS and then left untreated or treated with 2.5 µM BayK8644. IL-1β was determined in culture supernatants by ELISA. One experiment representative of three is shown. * p<0.05; Two-way ANOVA test.

D) LPS-primed WT BMDCs were treated with 10 µM BayK8644 alone or in combination with TEA (2 mM) or HCQ (10 µM) to inhibit Ca^{++}-activated K^{+} channels. IL-1β was determined in culture supernatants by ELISA. One experiment representative of three is shown. * p<0.05; ** p<0.01; One-way ANOVA test.

E) The human macrophage cell line (THP-1) was transfected with *GFP* or *TMEM176B/GFP*-coding plasmids and treated with LPS + nigericine (LPS/Nig) or left untreated in the presence of ethanol (vehicle) or 5 µM BayK8644. IL-1β was determined in culture supernatants by ELISA. To calculate the extent of TMEM176B-dependent inhibition, IL-1β levels (pg/ml) were incorporated to the formula: [GFP/LPS/Nig – GFP untreated] – TMEM176B/LPS/Nig x 100. One experiment representative of three is shown. * p<0.05; Student's *t* test.

In F, G, H, K, L, M and O the ratio shows the number of surviving mice/ total injected mice. For these experiments, we used C57BL/6 mice.

F) Tmem176b WT and *Tmem176b*$^{-/-}$ mice were inoculated with EG7 tumor cells and treated with 1 mg/kg Bayk8644 i.p on days 2-15 after tumor cell injection. Mice survival was monitored. * p<0.05; Log-rank (Mantel-Cox) test.

G) WT (C57BL/6JN) or *Caspase 1/11*$^{-/-}$ mice were inoculated with EG7 tumor cells and left untreated or treated with BayK8644 as in F. Mice survival was monitored. ** p<0.01; Log-rank (Mantel-Cox) test.

H) WT mice were injected with EG7 tumor cells and left untreated or treated with BayK8644 as in G. TDLN were resected 14 days after tumor injection and caspase-1 activation was studied by flow cytometry using the FLICA1 reagent. * p<0.05; Student's *t* test.

I) WT mice were inoculated with EG7 tumor cells and left untreated or treated with BayK8644 from days 2-15. At day 15, *in vivo* cytotoxicity against OVA antigen was studied. * p<0.05; Student's *t* test.

J) WT mice were inoculated with EG7 tumor cells and left untreated or treated with BayK8644 or BayK8644 in the absence or presence of anti-CD8 depleting antibody. Mice survival was studied. ns: non significant. WT + Vehicle vs WT + BayK8644: * $p<0.05$; WT + BayK8644 vs WT + BayK8644 + anti-CD8: * $p<0.05$; WT + Vehicle vs WT + BayK8644 + anti-CD8: ns; Log-rank (Mantel-Cox) test.

K) WT mice were inoculated with EG7 tumor cells and left untreated or treated with BayK8644, anti-CTLA-4 antibodies or BayK8644 plus anti-CTLA-4 antibodies. Mice survival was monitored. ns: non significant. Untreated vs Bayk8644 + anti-CTLA-4: ** $p<0.01$; BayK8644 vs BayK8644 + anti-CTLA-4: ns; anti-CTLA-4 vs BayK8644 + anti-CTLA-4: ns; Untreated vs anti-CTLA-4: ns; Untreated vs BayK8644: ns; Log-rank (Mantel-Cox) test.

L) WT mice were inoculated with EG7 tumor cells and then treated with 250 µg anti-PD-1 antibody at days 6, 9 and 12 after tumor inoculation. BayK8644 was injected every day since day 9 (all mice had established tumors) until day 21. Mice survival was monitored. * $p<0.05$; Log-rank (Mantel-Cox) test.

M) OVA-specific $CD8^+$ T cells were assessed using fluorescent MHC pentamers and analyzed by flow cytometry in EG7 tumor suspensions from WT mice treated with anti-PD-1 alone or anti-PD-1 + BayK8644 in a therapeutic protocol as in M**.** * $p<0.05$; Unpaired Student's *t* test.

N) WT mice were inoculated with EG7 tumor cells and left untreated or treated with BayK8644 + anti-PD-1 or BayK8644 + anti-PD-1 + anti-CD8 depleting antibody. Mice survival was monitored. * $p<0.05$; Log-rank (Mantel-Cox) test.

O) WT mice were inoculated with 5555 melanoma cells and left untreated or treated either with anti-PD-1 antibody (days 6, 9 and 12), BayK8644 (days 9-21) or both. All animals had established tumors when BayK8644 treatment was started. Mice survival was monitored. ns: non significant. Untreated vs Bayk8644 + anti-PD-1: * $p<0.05$; BayK8644

vs BayK8644 + anti-PD-1: ns; PD-1 vs BayK8644 + anti-PD-1: ns; Untreated vs anti-PD-1: ns; Untreated vs BayK8644: ns; Log-rank (Mantel-Cox) test.

See also Figure S8.

STAR METHODS

Contact for reagent and resource sharing

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Marcelo Hill (mhill@pasteur.edu.uy).

Experimental models

Animals

Six-to-ten weeks old male or female C57BL/6 or BALB/c mice were used (Jackson Lab; Bar Harbor, ME) and bred for up to 20 generations at the Institut Pasteur Montevideo or at the Institute of Biology and Experimental Medicine, Buenos Aires. All experiments were performed according to local regulation and approved by the local Ethics Committee for animal experimentation.

Tmem176b$^{-/-}$ mice were generated in the 129/SvJ strain and heterozygous mice were backcrossed for 10 generations onto the C57BL/6 background (Janvier, Saint Berthevin, France) as reported (Segovia et al., 2014). *Nlrp3*$^{-/-}$ (B6.129S6-*Nlrp3*^{tm1Bhk}/J; 021302) and *Caspase1/11*$^{-/-}$ (B6N.129S2-*Casp1*^{tm1Flv}/J; 016621) were from The Jackson Laboratory. *Nlrp3*$^{-/-}$ animals were compared to 000664 C57BL/6J, and *Caspase1/11*$^{-/-}$ mice to 005304 C57BL/6NJ. *Tmem176b*$^{-/-}$*Caspase1*$^{-/-}$ mice were generated by microinjecting Crispr/Cas9 targeting *Caspase-1* in *Tmem176b*$^{-/-}$ embryos. F1 animals were genotyped and heterozygous mice were crossed to generate F2 homozygous *Tmem176b*$^{-/-}$*Caspase 1*$^{-/-}$ animals. Caspase-1 deficiency was confirmed by Western blot (Figure S1). All animal strains including *Tmem176b*$^{-/-}$, *Tmem176b*$^{+/+}$ *WT* (issued from littermate controls), C57BL/6J, *Nlrp3*$^{-/-}$, C57BL/JN, *Caspase-1/11*$^{-/-}$ and *Tmem176b*$^{-/-}$*Caspase1*$^{-/-}$ were bred at a specific pathogen-free animal facility (Institut Pasteur, Montevideo).

Cell lines

EG7 (expressing the OVA antigen), LL2, CT26, THP-1 and CHO-K1 cell lines were purchased from ATCC (Manassas, VA). MC38 cells were from Kerafast (Boston, MA). The 5555 melanoma cell lines were kindly provided by R. Marais (Cancer Research UK, Manchester) and cultured as described (Hirata et al., 2015).

Tumor models and treatments

C57BL/6 mice were injected s.c. with 1 x 10^6 MC38 colon cancer cells, 1 x 10^5 LL2 lung cancer cells, 2.5 x 10^5 5555 melanoma cells or 1 x 10^6 EG7 thymic lymphoma cells. BALB/C animals were injected with 1 x 10^5 CT26 colon cancer cells. Injection was performed alternating one WT and one *Tmem176b*$^{-/-}$ mouse until completing both groups. In treated animals, alternation was done between drug- and vehicle-treated animals. Tumor growth was measured manually every 2-3 days with a caliper. The two major diameters were taken. Mice were sacrificed when one of the diameters reached 2 cm. In experiments where anti-IL-1β, anti-IL-17A or control goat IgG were used, 4 µg antibody was injected i.p 7 days after inoculation of tumor cells. Injections were repeated every five days until day 27 post-injection or euthanasia. For depletion of $CD8^+$ T cells, 100 µg YTS 169.4 antibody was injected every three days starting from the day before tumor inoculation. Depletion was confirmed in the spleen by flow cytometry. For administration of anti-CTLA-4 or control IgG, 100 µg antibody was given i.p starting from day 6 after tumor inoculation. Injections were repeated every three days until day 12. Anti-PD-1 or control IgG was injected (250 µg i.p) starting from day 6 and every three days until day 12. Bayk8644 or vehicle control (ethanol) was given i.p at 1 mg/kg since day 3 after tumor inoculation and until day 15. In animals treated with BayK8644 and anti-CTLA-4 antibody, BayK8644 was injected at days 3-15 every day and anti-CTLA-4 at days 6, 9 and 12 after tumor inoculation. In mice treated with BayK8644 and anti-PD-1 antibody, treatment with the former started at day 9 and repeated every day until day 21 after tumor inoculation.

Anti-PD-1 treatment started at day six after tumor inoculation and repeated every three days until day 12.

In vivo inflammasome activation

C57BL/6 animals were injected with 20 mg/kg ATP i.p. Four hours later, peritoneal lavage was performed using 5 ml PBS. Peritoneal cells were centrifuged and then stained with anti-CD11b, anti-Ly6C and anti-Ly6G antibodies. Cells were analyzed by flow cytometry. The percentage of $Ly6C^{int}$ $Ly6G^{hi}$ cells within the $CD11b^{+}$ cell compartment (neutrophils) was determined. The absolute number of neutrophils was calculated for each condition.

In vitro inflammasome activation

Bone marrow-derived DCs (BMDCs) were differentiated by culturing bone marrow cells for 8 days in the presence of 0.4 ng/ml GM-CSF. At day 8, adherent cells were >95% $CD11c^{+}CD11b^{+}$MHC class II^{int}. Cells were stimulated for 3 h with 0.25 µg/ml LPS, washed and treated with the indicated doses of ATP or nigericin. The presence of IL-1β was assessed in culture supernatants by ELISA (Biolegend, 432603) . To determine caspase-1 activation, BMDCs were stained with FLICA1 45 min after ATP or nigericin stimulation and analyzed by flow cytometry. For Western blot experiments, culture supernatants from BMDCs stimulated in the absence of FBS were precipitated with 20 % (v/v) TCA and washed with acetone. Cell lysates were generated with RIPA buffer in the presence of a protease inhibitor cocktail. Cell lysates and precipitates from culture supernatants were electrophoresed, blotted and probed with anti-caspase-1 (Adipogen, AG-20B-0042) or anti-IL-1β (Santa Cruz Biotechnol, sc-7884) antibodies.

THP-1 transfection and inflammasome activation

THP-1 monocytes were differentiated into macrophages by treatment with 0.1 µM PMA for 48 h. Macrophages (2.5×10^{6}) were then detached using trypsine and nucleofected with the *GFP* or *GFP-TMEM176B* coding pcDNA1.3 plasmids using the Amaxa Cell Line

Nucleofector Kit V-Lonza and nucleofector device from Amaxa. Sixteen hours later, cells were treated for 3 h with 0.25 µg/ml LPS. Cells were washed and treated for 2 h with 2.5 µM nigericin. IL-1β was assessed by ELISA in culture supernatants.

Method details

Cytosolic Ca^{++} determination

BMDCs cultured on glass coverslips were loaded with 1 µM Fura-2 for 45 min in the dark. Cells were then washed and analyzed by time-lapse microscopy at 37°C. Fluorescence emission intensity at 510 nm was determined in individual wells alternating excitation wavelengths of 340 and 380 nm every 3 sec. ATP was added when indicated at 0.5 mM.

In vivo cytotoxicity assay

Splenocytes from naïve C57BL/6 mice were stained alternatively with 0.8 (high) or 0.08 µM (low) DDAO-SE probe. The high DDAO population was loaded for 60 min at 37°C with 50 µM SIINFEKL OVA peptide. After three washes, the high and low population were mixed at 1:1 ratio. The mixed cells (2 x 10^6) were injected i.v in WT, *Tmem176b*$^{-/-}$ or *Tmem176b*$^{-/-}$*Caspase1*$^{-/-}$ naïve and tumor-bearing mice. Four hours later, mice were sacrificed and the spleens harvested. Splenocytes were analyzed by flow cytometry to assess DDAO high and low populations. Specific cytotoxicity was calculated with the following formula:

% specific lysis= $(1-[r_{naïve}/r_{tumor\ bearing}])x100$

r = $\%DDAO^{low}$ cells/$\%DDAO^{high}$ cells

Screening of Tmem176b inhibitors

CHO cells were transfected using Lipofectamine2000 with pSecTag2B-PS-Tmem176a-mCherry and pSecTag2B-PS-Tmem176b-V5His plasmids for 4 h, washed and cultured for 24 h. Cells were then loaded with 1 µM ANG-2 for 30 min at 37°C, washed and incubated

in 140 mM Na+-containing phosphate buffer or 140 mM NMDG to substitute Na^+ in the presence of different doses of tested drugs or vehicle controls. Cells were then analyzed by flow cytometry using a BD Accuri C6 cytometer equipped with a 488 nm laser. ANG-2 emission was detected using band-pass filter 670 LP. FlowJo vX.0.7 software was used for data analysis. MFI from NMDG-containing solutions was subtracted to MFI from Na+-containing solutions. A maximum of two drugs was studied in each experiment. Screened drugs were from SCREEN-WELL® Ion Channel ligand library (Enzo Life Sciences; Farmingdale, NY).

Immunohistochemistry staining of human colon microarrays

Expression of TMEM176B was analyzed by immunohistochemistry on 90 specimens of human colon tumors (US Biomax, Inc; Rockville, MD). Briefly, antigenic recovery was done by boiling slides in a pressure cooker for 10 min in the presence of alkaline buffer (10 mM Tris, 1 mM EDTA, 0.05% Tween 20, pH 9.0). 2.5 μg/ml anti-TMEM176B antibody (Abcam; ab103929) or control rabbit IgG were incubated ON at 4ºC. Staining was verified using EnVision+ System- HRP-labelled polymer anti-rabbit (Dako/Agilent, Santa Clara CA). Slides were counterstained with Meyer´s hematoxylin, analyzed by two independent researchers in a blind fashion and categorized as high or low/negative TMEM176B expression in the stroma and parenchyma. Expression levels were then correlated with survival information provided by US Biomax.

Electrophysiology experiments

Oocytes were surgically removed from MS222 (0.4%)-anesthetized *Xenopus laevis* female and dissociated under gentle agitation by a 2–3 h incubation in an OR2 solution (82 mM NaCl; 2 mM KCl; 1 mM $MgCl_2$; 5 mM HEPES pH 7.2) supplemented with collagenase 1A (1 mg/ml). Oocytes were then injected with 40 nl of *in vitro* synthesized *Tmem176b* mRNA at 1 µg/µL (mMESSAGE mMACHINE Ultra

kit). *Tmem176b* was fused to a signal peptide sequence (N-terminal) from pSecTag2B (Invitrogen, Carlsbad, CA) and to V5 + 6-His tags (C-terminal). The day after injection, oocytes were placed in a pH 8.0 solution (100 mM NaCl, 3 mM KCl, 2 mM $MgCl_2$, 15 mM HEPES pH 8.0) changed daily. Two to three days later, currents were recorded in two-electrode voltage-clamp using a genclamp500 amplifier (Axon Inst., Foster City, CA) interfaced to a personal computer using the Digidata 1200 interface and the pClamp software (v 7.0; Axon Inst.). Prior to recording, oocytes were incubated in PMA at 0.1 µM in pH 8.0 solution for 20–30 min. Currents were filtered at 100 Hz and digitalized at 0.5 kHz before storage and further analysis. During recording, oocytes were continuously superfused with the pH 8.0 solution. The currents were quantified 5–15 min after holding the extracellular pH at 5.0. In *Tmem176b*-expressing oocytes, induction of an inward current was obtained by switching to a pH 5.0 solution.

Gene expression analysis

Normalized NanoString nCounter data were analyzed from Chen *et al* (2016). Gene expression data from Riaz *et al* (2017) were obtained from their GitHub repository (https://github.com/riazn/bms038_analysis/tree/master/data). RNA-seq count data were normalized to FPKM (Fragment per kilobase per million) through the Bioconductor R package *DESeq2* 1.18.1. The on-treatment biopsy from patient 32 was excluded from further analyses since it presented extreme expression values.

CIBERSORT analysis

The leukocyte signature matrix LM22 (547 genes) that discriminates 22 types of tumor-infiltrating immune cells was used for analysis. Normalized gene expression data from Riaz *et al*. (2017) cohort were processed with the CIBERSORT web tool (http://cibersort.stanford.edu/) setting no quantile normalization and 1.000 permutations as

parameters. All samples were run with both relative and absolute modes. The first mode infers the relative cellular fraction for each cell of the LM22 matrix and the second calculates a score that reflects the absolute proportion of each cell type in the mixture.

Single cell RNA-Seq data analysis

Normalized single cell expression data from Jerby-Arnon *et al.* (2018) was obtained from Gene Expression Omnibus (Accession number GSE115978). To study gene correlations, the expression matrix was processed with the software MAGIC (van Dijk et al., 2018) to deal with the under-sampling of mRNA known as dropouts. R implementation of the MAGIC algorithm with default parameters (Rmagic v1.3.0) was applied. For correlation analysis, Spearman's Rank Correlation test was used.

Statistical analyses

Statistical analyses were performed either by R project or GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA). Survival analyses were done with the Log-rank (Mantel-Cox) test. Comparison of two experimental conditions was done with paired or unpaired Student's *t* test. Comparison of multiple conditions was done with one or two-way ANOVA tests. Differences in gene expression and CIBERSORT scores between responder and non-responder groups were assessed using the unpaired *t*-test when normality assumption was met. Otherwise, Mann-Whitney *U* test was used. Differences between matched samples pre- and on-treatment were evaluated with paired *t*-test when normality assumption was met or otherwise with Wilcoxon signed-rank test. For correlation analysis, the Pearson coefficient was used when samples passed the normality test. Spearman coefficient was used for all other cases. Shapiro-Wilk test was performed to evaluate the normality assumption for all samples.

Data availability

Mendeley dataset: https://data.mendeley.com/datasets/publish-confirmation/gvj6fc2b8v/1