Clinical & Experimental Immunology
The Journal of Translational Immunology
British Society for immunology
doi: 10.1111/cei.13433 IMMUNE CHECKPOINT INHIBITION: FROM MOLECULES TO CLINICAL APPLICATION
Clinical and Experimental Immunology REVIEW ARTICLE Series Editors: Leonie S. Taams and Tanja D. de Gruijl

# Role of inflammasome activation in tumor immunity triggered by immune checkpoint blockers

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Accepted for publication 19 March 2020
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## Summary

**Immune checkpoint blockers improve the overall survival of a limited number of patients among different cancers. Identifying pathways that influence the immunological and clinical response to treatment is critical to improve the therapeutic efficacy and predict clinical responses. Recently, a key role has been assigned to innate immune mechanisms in checkpoint blockade-driven anti-tumor responses. However, inflammatory pathways can both improve and impair anti-tumor immunity. In this review, we discuss how different inflammatory pathways, particularly inflammasome activation, can influence the clinical outcome of immune checkpoint blockers. Inflammasome activation may reinforce anti-tumor immunity by boosting $CD8^+$ T cell priming as well as by enhancing T helper type 17 (Th17) responses. In particular, we focus on the modulation of the cation channel transmembrane protein 176B (TMEM176B) and the ectonucleotidase CD39 as potential targets to unleash inflammasome activation leading to reinforced anti-tumor immunity and improved efficacy of immune checkpoint blockers. Future studies should be aimed at investigating the mechanisms and cell subsets involved in inflammasome-driven anti-tumor responses.**

**Keywords:** Cancer, checkpoint blockade, immunotherapy, inflammasome

## Inflammation and immune checkpoint blockers

Immunotherapy based on monoclonal antibodies has revolutionized oncology therapy by unleashing the breaks on T cells through the blockade of cytotoxic T lymphocyte antigen-4 (CTLA-4) and programmed cell death-1/PD-ligand-1 (PD-1/PD-L1) pathways [1,2]. However, a minority of patients experience clinical benefit when receiving these treatments. Thus, understanding primary and secondary resistance mechanisms is urgently needed to improve the clinical efficacy of immune checkpoint blockers (ICB) [3]. However, despite considerable research efforts, the precise molecular and cellular pathways that mediate tumor immunity elicited by ICBs remain incompletely understood [4]. Moreover, an in-depth investigation of the fundamental mechanisms triggered by ICB will be critical for developing predictive biomarkers of response to therapy. In this context, inflammation is certainly a key player in shaping ICB-triggered anti-tumoral immunity [5]. Nevertheless, the role of inflammation in cancer is well known to be ambiguous [6,7]. Inflammatory mediators impact upon cancer hallmarks such as increased survival, proliferation, angiogenesis, invasion and even immune escape. Conversely, the innate immune system also plays a critical role in developing anti-tumor adaptive immune responses.

Most human solid tumors show one of three distinct immunological phenotypes: immune inflamed, immune excluded or immune desert [8]. There is now growing evidence that inflamed tumors are associated with enhanced clinical responses to ICBs [8–10]. Thus, there is an increasing interest in modulating inflammatory pathways to enhance tumor immunity, particularly in the context of ICB. For instance, blocking transforming growth factor (TGF)-β in colon and urothelial cancer overcomes immune exclusion [11,12]. Furthermore, Toll-like receptor (TLR) ligands can

trigger innate immune pathways which potentiate anti-PD-1 therapy. In fact, intratumoral and peritumoral injection of the TLR-9 ligand cytosine–phosphate–guanosine (CpG) increased the survival of anti-PD-1-treated tumor-bearing mice [13–15]. A Phase Ib multi-center study showed that intratumor injection of a synthetic CpG combined with anti-PD-1 blockade was well tolerated, and had a high response rate in a small number of patients who were naive to PD-1 blockade at baseline in advanced melanoma [16]. Combined treatment resulted in enhanced tumor infiltration by $CD8^+$ T cells. Alternative intratumoral injection of TLR-9 or TLR-7 agonists, combined with anti-PD-1 therapy, suppressed growth of experimental head and neck squamous cell carcinoma (HNSCC) at the primary tumor and metastatic sites [17]. Nanoparticles (NP) loaded with the TLR-7 and TLR-8 agonist R848 polarized tumor-associated macrophages (TAMs) to an M1 phenotype and sensitized mice to the anti-tumoral effects of PD-1 blockade [18]. Also, PD-1-targeted delivery of NPs loaded with R848 improved the survival of MC38 colon cancer-bearing mice treated with anti-PD-1 antibody [19]. Furthermore, the TLR-3-specific RNA agonist, ARNAX, triggers cross-priming of antigen-specific $CD8^+$ T cells by dendritic cells (DCs) in an *Ifnar*-dependent manner [20]. Systemic co-injection of $ARNAX^+$ tumor antigens followed by therapy with anti-PD-L1 antibodies effectively controlled tumor growth. This anti-tumoral effect was associated with enhanced tumor infiltration by total and tumor-specific $CD8^+$ T cells [20]. Thus, combination therapies using ICB and TLR agonists are expected to generate more efficient and durable anti-tumor responses.

With regard to other proinflammatory mediators, it has been appreciated that proinflammatory cytokines enhance the anti-tumor efficacy of ICBs [19]. Accordingly, lowering the cytotoxicity threshold of TNF-α by TNF receptor-associated factor 2 (TRAF2) inactivation in melanoma cells enhanced tumor necrosis factor (TNF)-α-dependent anti-tumor efficacy of anti-PD-1 therapy [21]. Furthermore, high TNF-α in tumor biopsies was associated with clinical responses to PD-1 blockade in melanoma patients [21]. However, the role of TNF-α in anti-tumor immunity seems to be complex, as contradictory observations have been reported. Thus, concomitant blockade of TNF-α and PD-1 has been proposed to reinforce the anti-tumor efficacy of anti-PD-1 antibodies [22,23].

Type I IFNs constitute another relevant inflammatory pathway. Type I IFNs play a critical role in tumor immunity triggered by ICBs. In human melanoma, a type I IFN signature was associated with clinical responses to anti-CTLA-4 [24]. The combination of anti-CTLA-4 + anti-PD-L1 therapy in mouse melanoma significantly lost efficacy in animals deficient in *TMEM173* (stimulator of interferon genes; STING) [25]. Moreover, activation of STING by intratumoral injection of cyclic dinucleotide-adenosine monophosphate (cGAMP) before anti-CTLA-4/anti-PD-1 therapy significantly enhanced anti-tumor immunity triggered by ICBs [26]. Accordingly, a STING-activating nanovaccine showed great synergy with PD-1 blockade in controlling murine lung cancer growth [27]. Interestingly, the cGAS–STING axis controlled the nucleotide-binding oligomerization domain and leucine-rich repeat receptors (NLR)P3 inflammasome activation in human myeloid cells [28]. Thus, inflammasome activation might enhance tumor immunity in the context of ICBs.

## Is there a role for inflammasome activation in cancer immunotherapy?

Inflammasomes are cytosolic multi-protein complexes that sense cellular stress [29]. Sensor proteins can be divided into nucleotide-binding oligomerization domain and leucine-rich repeat receptors (NLRs), absent in melanoma 2 (AIM2)-like receptors as well as pyrin. Once activated, they cleave caspase-1, which then processes pro-IL-interleukin (IL)-1β and pro-IL-18 to give the active and secreted forms of these proinflammatory cytokines [29]. Furthermore, active caspase-1 cleaves gasdermin D (GSDMD), which forms pores in the cell membrane leading to cell death [30]. Inflammasomes are involved in different hallmarks of cancer development, performing tumor-promoting as well as tumor-suppressive functions [31–33]. The inflammasome-related cytokines IL-1β and IL-18 promote proliferation and survival of malignant cells. IL-18 has been shown to control caspase 8-mediated apoptosis in gastric cancer cells [34]. In breast cancer, IL-1β induces malignant cell proliferation by inducing nuclear translocation of β-catenin [35]. Additionally, IL-1β has been shown to regulate angiogenesis in several settings [36,37] as well as immune suppression mechanisms, by inducing myeloid-derived suppressor cells [38]. The Canakinumab Anti-Inflammatory Thrombosis Outcome Study (CANTOS) has shown a significant decrease in lung cancer incidence and lung cancer mortality when IL-1β was blocked compared to placebo in patients with atherosclerosis [39]. In contrast, NLRP3-dependent secretion of IL-18 triggers tumoricidal activity of NK cells against metastatic colon cancer cells in the liver in murine models [40]. In the context of tumor therapies, inflammasomes play an anti-tumoral role in immunogenic chemotherapy [14] and probably in response to BRAF inhibitors [15]. Furthermore, pharmacological activation of inflammasome components has been proposed as a potential strategy to enhance the anti-tumor efficacy of ICBs [41].

The NLRP3 inflammasome activation is tightly regulated by cytosolic levels of ions such as $K^+$, $Ca^{++}$ and $Cl^-$ [42–47]. We have recently shown that unleashing inflammasome activation by targeting transmembrane protein 176B (TMEM176B), a cation channel expressed on antigen-presenting cells, reinforces tumor immunity triggered by

CTLA-4 and PD-1 blockade [48]. TMEM176B inhibits adenosine triphosphate (ATP) and nigericin-induced NLRP3 inflammasome activation through ionic mechanisms in human and mouse DCs and macrophages. Furthermore, anti-PD-1 and anti-CTLA-4 therapies lost anti-tumor efficacy in *Nlrp3*$^{-/-}$ and *Casp1/11*$^{-/-}$. Accordingly, an inflammasome signature in tumor biopsies was associated with a response to PD-1 blockade in two cohorts of advanced melanoma patients [48].

The NLRP3 inflammasome is activated, among other stimuli, by extracellular ATP (eATP) [42]. eATP levels increase by 100–1000-fold as a consequence of tissue stress such as inflammation, hypoxia or ischemia in the tumor microenvironment [49-51]. ATP release can occur not only upon membrane damage, but also independently of cell death through ATP-binding cassette (ABC) transporters, vesicular release or pannexins and connexins [51]. eATP is recognized through its interaction with the ionotropic P2X and metabotropic purinergic P2Y receptors [52]. Dying tumor cells release ATP, which binds to P2X7R on DCs and reinforces CD8$^+$ T cell responses in an inflammasome-dependent manner [14]. Interestingly, breast cancer patients with a loss-of-function substitution (E496A) in P2X7R showed shorter metastatic disease-free survival [14]. The levels of eATP are also determined by CD39, the rate-limiting enzyme in the hydrolysis of this nucleoside. CD39 plays a critical immunoregulatory role by modulating effector and regulatory T cells, macrophages, natural killer (NK) and myeloid-derived suppressor cells (MDSCs), among other mechanisms [49,50]. Thus, it would be expected that CD39 blockade with specific antibodies may lead to increased eATP levels within the tumor microenvironment, enhanced inflammasome activation and probably reinforced tumor immunity triggered by ICBs. In agreement with this hypothesis, anti-CD39 therapy showed synergistic effects with PD-1 and CTLA-4 blockade in controlling lung metastasis of B16F10 murine melanoma [53]. More recently, Li *et al*. have shown in MC38 tumors that anti-CD39 is less effective in *Casp1/11*$^{-/-}$, *Nlrp3*$^{-/-}$, *Pycard*$^{-/-}$ and *P2x7r*$^{-/-}$ mice [54]. Using syngeneic and humanized tumor models, the authors also showed that CD39 and PD-1 blockade have synergistic anti-tumoral effects [54].

In contrast to those studies, IL-1β blockade was shown to improve the anti-tumoral efficacy of anti-PD-1 therapy in a mouse model of breast cancer [55]. In this model, IL-1β was neutralized before the commencement of anti-PD-1 therapy. Thus, although the inflammasome/IL-1β pathway may play different roles in different tumor types [48,55], unknown mechanisms may explain these apparent contradictory observations.

## How does inflammasome activation reinforce tumor immunity triggered by ICBs?

In spite of considerable progress showing that CD8$^+$ T cells mediate anti-tumor immune responses when inflammasome is unleashed [48,54], the mechanisms by which inflammasomes promote anti-tumor immunity in the context of ICBs remain unclear. Inflammasomes may regulate priming of cytotoxic T lymphocytes. The response of some PD-L1 negative tumors, which also lack tumor-infiltrating T cells to anti PD-1 therapy, suggest that PD-1 blockade can trigger *de-novo* anti-tumor responses. Mice devoid of *Sec22b*, which lack the ability to cross-present antigens to CD8$^+$ T cells, showed a compromised response to anti-PD-1 therapy [56]. This observation suggests that anti-PD-1 may facilitate the priming of CD8$^+$ T cells, although antigen cross-presentation may be necessary before PD-1 blockade. In non-small-cell lung cancer (NSCLC), neoadjuvant anti-PD-1 induced the expansion of mutation-associated, neoantigen-specific T cell clones in peripheral blood [57]. Accordingly, PD-1 blockade has been shown to enhance early stages of T cell activation in lymph nodes [58]. However, anti-PD-1 therapy in transplantable mouse tumor models such as MC38 colon cancer does not depend upon lymph node priming [59,60]. Nevertheless, recent single-cell RNA and T cell receptor sequencing data from basal or squamous cell carcinoma tumors showed that the expansion of T cell clones did not derive from pre-existing tumor-infiltrating T lymphocytes [61]. Novel clones may, therefore, derive from tumor-extrinsic sources including lymph nodes. Similarly, inflammasome activation may enhance anti-PD-1 therapy by reinforcing CD8$^+$ T cell *de-novo* priming. In fact, the expression of IL-1R1 enhances CD8$^+$ T cell responses in viral infections [62,63], *Mycobacterium tuberculosis* [64] and tumors [14]. Accordingly, IL-1 reinforces expansion, effector function, tissue localization and memory response of CD8$^+$ T cells in response to antigen stimulation [65]. Interestingly, activated CD8$^+$ T cells can promote inflammasome activation in antigen-presenting cells (APCs) through perforin-dependent mechanisms, suggesting a positive feedback leading to tumor rejection [66].

Conversely, anti-tumoral CD8$^+$ T cell responses can be reinforced by T helper type 17 (Th17) cells [67]. Different lines of evidence suggest that Th17 cells may play an important role as downstream effectors of unleashed inflammasome activation. Accordingly, genetic deletion or pharmacological blockade of TMEM176B are associated with increased numbers of CD4$^+$ retinoid-related orphan receptor γt (RORγt$^+$) T cells in tumor-draining lymph nodes [48]. Moreover, blockade of IL-17A undermined the capacity of *TMEM176B*$^{-/-}$ mice to control tumor growth [48]. However, the role played by Th17 cells in cancer is controversial

[68]. Intratumoral Th17 cells have been associated not only with a good, but also with a bad prognosis [68,69]. In fact, Th17 cells are known to be a heterogeneous population behaving as either regulatory or effector cells, depending on the dominant cytokine microenvironment. Importantly, IL-1β is a key factor in determining effector properties of Th17 cells [70,71]. Interestingly, CD39 and CD73 are markers of regulatory Th17 cells which promote tumor growth, suggesting that the ATP/inflammasome pathway promotes effector Th17 responses [70,72,73]. Adoptive transfer of intratumoral $CD39^+CD4^+ROR\gamma t^+$ intratumoral T cells promoted tumor growth in murine models, whereas this effect was lost in cells from *CD39*$^{-/-}$ animals [73]. Furthermore, eATP inhibits the suppressive potential of regulatory T cells ($T_{regs}$) through P2X7R expressed on these cells [74]. eATP also induces the differentiation of $T_{regs}$ into Th17 cells [74]. Conversely, CD39 expression by $T_{regs}$ prevented conversion into Th17 cells [75,76]. Interestingly, $CD39^+$ $T_{regs}$ were proposed to specifically suppress pathogenic Th17 cells [77,78]. Thus, TMEM176B and CD39 may serve as two different physiological strategies to impair ATP-induced inflammation mediated by inflammasomes and Th17 cells. Pharmacological blockade of TMEM176B and CD39 led to inflammasome-dependent tumor immunity and enhanced anti-tumor efficacy of ICBs [48,54]. Thus, concomitant blockade of both TMEM176B and CD39 may trigger Th17-dependent responses and enhance the efficacy of ICBs. However, it remains to be determined whether inflammasome activation *in vivo* skews Th17 differentiation into effector cells as well as whether bona fide effector Th17 responses promote tumor immunity in the context of ICBs. In melanoma as well as in prostate cancer patients, clinical responses to PD-1 blockade have been associated with increased peripheral $CD4^+IL\text{-}17^+$ T cells [79,80]. Although these observations suggest a role for Th17 cells in anti-PD-1 therapy, the effector or regulatory nature of these cells has not been assessed. In this regard, it has been recently reported that IL-17A blockade abrogates immune-related adverse events in a 50-year-old man with metastatic colon cancer treated with anti-PD-1 antibodies. However, IL-17A blockade was also associated with an increase in the tumor marker carcinoembryonic antigen (CEA) in plasma, suggesting that IL-17A was, in fact, mediating an anti-tumoral immune response [81]. Recently, Sharma's team showed that $CD4^+$ T cells polarize to Th17 rather than Th1 in a TGF-β-dependent manner in bone metastases of prostate cancer patients, leading to the failure of ICB [82]. It remains to be determined whether unleashing inflammasome activation by blocking TMEM176B and/or CD39 can commit Th17 cells into an effector phenotype that may reinforce tumor immunity in the context of ICBs (Fig. 1).

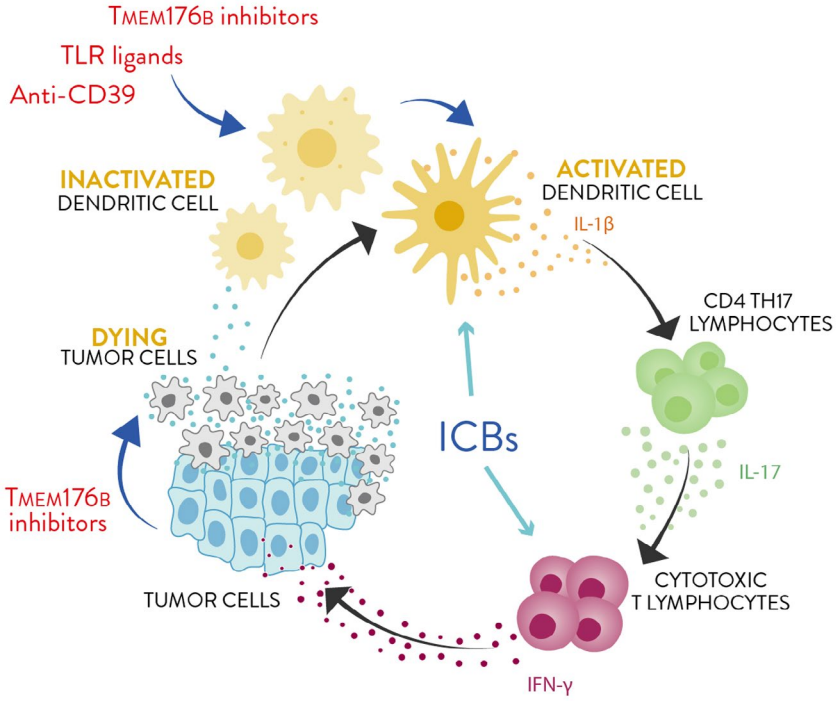


**Fig. 1.** Manipulating innate players to improve the efficacy of immune checkpoint blockers (ICBs). Anti-CD39 antibodies, Toll-like receptor (TLR) ligands and transmembrane protein 176B (TMEM176B) inhibitors trigger dendritic cell (DC) activation and tumor immunity through the cancer immunity cycle. Interleukin (IL)-1β secreted by activated DCs may reinforce T helper type 17 (Th17) cells leading to improved cytotoxic T lymphocyte (CTL) responses. Alternatively, IL-1β might directly impact upon $CD8^+$ T cells priming to promote the differentiation of CTLs. Moreover, TMEM176B inhibitors might induce malignant cell death fueling the whole process.


 

## TMEM176B as a potential target in cancer therapy

A growing body of studies are being conducted to develop and characterize inflammasome inhibitors to control immune-mediated inflammatory diseases. There are, however, far fewer examples of pharmacological strategies aiming at inducing inflammasome activation to trigger tumor immunity. We will focus on an emerging potential target, TMEM176B, the therapeutic blockade of which leads to enhancement of anti-tumor responses.

TMEM176B and TMEM176A are members of the CD20-like MS4A family [83,84]. These are ubiquitous proteins highly expressed by macrophages and dendritic cells [84,85], although they can also be expressed by NK and NK T cells [86] and by RORγt$^+$ [type 3 innate lymphoid cells (ILC3), Th17 and γδT] cells [87]. TMEM176B is associated with allograft tolerance [84], and its expression is strongly down-regulated during DC maturation [84,85]. TMEM176B controls the immunoregulatory properties of tolerogenic DCs [88] and is localized in the endophagosomal membrane [88,89] as well as in the trans-Golgi network [53]. In DCs, TMEM176B controls phagosomal pH and promotes antigen cross-presentation to activate CD8$^+$ $T_{reg}$ cells [88]. TMEM176A and TMEM176B interact physically to form cation channels [87,90]. Their expression is dysregulated in different cancers when compared to normal tissues [90]. In human gastric cancer, low mRNA expression of *TMEM176A* and *TMEM176B* are correlated with better overall survival [91]. Accordingly, low TMEM176B protein levels in the stroma were associated with improved overall survival in colorectal carcinoma [48]. In glioblastoma, TMEM176A was shown to inhibit Bcl2 expression and to induce apoptosis [92]. As well, TMEM176B was up-regulated in tumor vessels of human renal cell carcinoma specimens, suggesting a role as a potential target for anti-angiogenic intervention in these tumors [93]. In contrast, epigenetic silencing of *TMEM176A* has been associated with tumor progression in colorectal [94], esophageal [95] and hepatocellular [96] cancer.

Thus, targeting TMEM176B has the potential to reinforce anti-tumoral CD8$^+$ T cell responses in an inflammasome-dependent manner while directly killing malignant cells, at least in some tumor types. Although not directly demonstrated, pharmacological inhibition of TMEM176B might also impact upon tumor angiogenesis [93]; therefore, TMEM176B seems so far to be a safe target. Our studies have not demonstrated acute toxicity upon pharmacological inhibition of TMEM176B or spontaneous autoimmunity in our *TMEM176B*$^{-/-}$ mice [48].

## Concluding remarks

Recent evidence suggests that inflammasome activation leading to IL-1β and IL-18 secretion can modulate the immunological outcome of ICB in cancer immunotherapy settings. However, further work is needed to understand the mechanisms underlying these effects. Defining the timing of this process and determining which DC subsets and helper T cells are critical for inflammasome activation is of paramount importance to improve the clinical efficacy of immunotherapy. Moreover, characterization of molecular targets critical to trigger inflammasome activation is needed to design more selective and potent inhibitors. Combinatorial approaches aimed at blocking TMEM176B and CD39 may help unleash inflammasome activation, leading to enhancement of anti-tumor immunity. It remains to be determined, however, whether Th17 cells are downstream *in-vivo* effectors, and whether and how these cells may promote anti-tumoral responses upon blockade of immune checkpoints.

## Acknowledgements

Work in M. H.'s laboratory is supported by Uruguay INNOVA-2, FMV from ANII, CABBIO, PEDECIBA, ECOS-SUD AUF/FAPESP and FOCEM (MERCOSUR Structural Convergence Fund) COF 03/11 grants to M. H., CSIC UDELAR and FCE from ANII to M. S. and FCE from ANII to S. R. Work in G. A. R.'s laboratory is supported by grants from Agencia Nacional de Promoción Científica y Tecnológica (2017-0494 to G. A. R) as well as Bunge & Born, Sales and Richard Lounsbery Foundations.

## Disclosures

M. H. is founder and CSO of ARDAN Pharma.

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