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CaMKK2: A novel target for shaping the androgen-regulated tumor ecosystem

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Abstract

The androgen receptor (AR) is pivotal in the biology of sex hormone-regulated malignancies, with prostate cancer (PC) the most affected tumor. AR signals control the growth, survival, and migration of cancer cells, and they regulate the activation of macrophages, a cell type pivotal to the tumor ecosystem. Intriguingly, CaMKK2 has recently been identified as both an important AR-regulated gene in the context of PC and as a critical regulator of macrophage activation. By contrast, CaMKK2 is barely detectable in normal prostate or immune cells that mediate the response against tumorigenesis. These novel findings suggest that CaMKK2 resides at a critical molecular node that shapes the cancer ecosystem, and identifies this kinase as a novel therapeutic target for sex hormone-regulated cancers.

Keywords

CaMKK2; Androgen Receptor; Prostate cancer; Macrophage; Inflammation; Cancer Therapy

CaMKK2 meets AR-signaling

The androgen receptor (AR) is a ligand-activated transcription factor that plays a pivotal role in the biology of prostate cancer (PC) [1], as well as other sex hormone-regulated malignancies such as breast cancer [2]. Testosterone and dihydrotestosterone (DHT) in the cytoplasm can bind AR and drive its translocation into the nucleus, where it controls the expression of a large number of genes that regulate growth, survival, and the invasiveness of cancer cells [3, 4]. For these reasons, androgen-deprivation therapy (ADT) is the first choice for treating advanced PC, and in more than 90% of patients this treatment improves symptoms and/or decreases the levels of prostate-specific antigen (PSA) [5]. Unfortunately, over time PC loses responsiveness to the androgen blockade and in the majority of cases the tumors progress into castration-resistant prostate cancer (CRPC). Unexpectedly, *in vivo* knockdown studies and treatment with second generation AR antagonists or hormone synthesis inhibitors have demonstrated that AR-dependent signaling is also important in CRPC [6-9]. Therefore, a complete understanding of AR-signaling is mandatory for establishing novel and more effective therapies for advanced prostate cancer.

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Calcium (Ca^{2+}) is a universal second messenger in all eukaryotic cells, where it regulates many functions through forming a complex with the protein calmodulin (CaM) [10]. Upon Ca^{2+} binding, CaM can bind and activate a wide range of proteins, including several related kinase families: the CaM-dependent kinases (CaMK) [11, 12], the CaMKI subfamily (α, β, γ, δ), the CaMKII subfamily (α, β, γ, δ), CaMKIV, and the CaMKK subfamily (1 and 2, or α and β, respectively). The structures and biochemical functions of the CaMKs have been reviewed elsewhere, and will be not discussed in depth here [12-14]. Briefly, CaMKI and CaMKIV are the known primary targets of CaMKK2, and the full activation of these enzymes requires phosphorylation on Thr by CaMKK2 [13]. CaMKK2 is also a physiologically relevant upstream activator of the AMP-dependent protein kinase (AMPK) [15-17], and this enzyme is a crucial cellular energy sensor that promotes ATP production by increasing the catabolic pathways, while conserving ATP by switching off biosynthetic pathways. In addition, AMPK is involved in the regulation of many other physiological relevant processes, such as cell cycle, membrane excitability, cytoskeleton reorganization, authopahgy [18]. Specifically, the CaMKK2-AMPK signaling axis has been reported to play a role in the regulation of energy balance by acting in the hypothalamus [19], and in the control of macroauthophagy in cancer cells [20].

During a study aimed at investigating the temporal program of transcription that reflected the cellular response to androgen of LNCap adenocarcinoma cell lines, Nelson et al. identify 143 androgen-regulated genes, and described a putative androgen-responsive element (ARE) in the promoter of 25 of them. Of note, CaMKK2 was included in the list of AR-regulated genes, as well as in the small subsets of genes showing an ARE in their promoter [21]. Recently, several groups have demonstrated that CaMKK2 is an important node in signaling networks that control the proliferation and metabolism of prostatic cancer cells [22-25]. Frigo et al. provide the first evidence for the expression of this protein in PC, showing that a short isoform of CaMKK2 is expressed in the LNCaP prostate cancer cell line and is upregulated by androgens [22]. These authors also confirmed the presence of an ARE in the *Camkk2* promoter region, and found that genetic ablation or pharmacological inhibition of CaMKK2 is sufficient to blunt the effects of androgens on migration and invasion of of prostatic cancer cells to androgens. Finally, it was shown that inhibition of a single CaMKK2 target protein, the AMP-dependent protein kinase (AMPK), prevents the stimulatory effects induced by androgens on migration and invasiveness of LNCaP cells [22].

To identify a core set of AR binding sites that regulate gene expression in prostate cancer cells, Massie et al. combined genome-wide AR binding profiles with an analysis of the integrated androgen-stimulated recruitment of the transcriptional machinery [23]. Similar to what was reported previously [21, 22] this study identified *Camkk2* as one of the several AR-regulated gene in PC that codify for hub proteins, and found increased CaMKK2 protein levels in prostate cancer versus adjacent normal tissue using two separate clinical cohorts. Interestingly, they suggested that AMPK was the relevant downstream target involved in the control of the anabolic transcriptional pathway that is required to sustain tumor growth [23]. Massie et al. also demonstrated the importance of CAMKK2 in tumor formation using the C4-2B xenograft model of CRPC. In this experimental model, they found that the CAMKK2 inhibitor STO-609 [26] effectively reduced the growth of prostate cancer, and this treatment was additive with AR inhibition in castrated mice. CAMKK2 inhibition had no demonstrable effect on normal mouse prostate size, whereas castration decreased prostate size and resulted in the atrophy of luminal epithelial cells [23].

Karacosta et al. recently investigated the expression of CaMKK2 during tumor progression in the transgenic adenocarcinoma model of mouse prostate (TRAMP), in CWR22 human xenografts and in clinical PC specimens [24]. They found CaMKK2 expression increases in

advanced cancer, and this protein is expressed at higher level in castration-resistant tumor xenografts compared with androgen-responsive grafts. Androgens appear to regulate CaMKK2 expression in LNCaP prostate cancer cells, given that treatment with DHT induced the accumulation of CaMKK2 mRNA and this effect was reversed by androgen withdrawal. Moreover, the silencing of CaMKK2 in LNCaP cells by siRNA decreased expression of the AR target gene prostate specific antigen (PSA), as well as cyclin D1 and phospho-Rb, two AR-regulated proteins that regulate the cell cycle. Of note, these authors also showed nuclear accumulation of CaMKK2 in response to androgens induced. Based on these results, they proposed a novel regulatory loop in which AR feeds forward to induce CaMKK2 expression and CaMKK2, in turn, feeds back to positively regulate the transcriptional activity of AR [24].

The relationship between CAMKK2 and PC has most recently been investigated by Shima et al. [25]. By performing genome-wide expression analysis on clinical samples, albeit a small set, they found a 6-fold increase in CaMKK2 expression in PC compared with normal prostate, confirming previous reports [22-24]. Interestingly, these authors also explored CaMKK2 protein expression by tissue microarray and found CaMKK2 to be barely detectable in normal prostate tissue. However, CaMKK2 accumulated in prostatic intraepithelial neoplasia and in more advanced PC, and this accumulation inversely correlated with prognosis. In contrast with previous reports [22-24], this study provided evidence for an inhibitory effect of CaMKK2 on AR-regulated transcriptional activity. For these reasons, the authors hypothesized that CaMKK2 exerts differential effects on PC by supporting the growth of tumors at an early stage or by limiting excessive proliferation in CRPC [25]. Although additional studies are required to validate this intriguing hypothesis, it seems possible that this phenomenon could be explained by the ability of CaMKK2 to bind and activate diverse downstream targets. For example, such targets could include factors that are tumor stage-specific or in the microenvironment, and the presence of inflammatory cells might switch CaMKK2 activity toward a specific downstream target, resulting in relevant biological consequences.

CaMKK2 regulates macrophage activation

Macrophages are one of the most active secretory cell types in the body and release a multitude of mediators that regulate all aspects of host defense, inflammation, and homeostasis [27]. The inflammatory response by macrophages is initiated by the binding of pathogen derivatives, irritants, or endogenous moieties to specialized pattern recognition receptors (PRRs), which include the Toll-like, NOD-like, and RIG-I-like receptors (TLRs, NLRs, and RLRs, respectively) [28]. During the past couple of decades, TLRs have been extensively investigated, and it has been demonstrated that stimulating this pathway can trigger a variety of biological responses in macrophages and dendritic cells that may result in cytokine release, terminal differentiation, cell adhesion, migration, survival, or the death of these cells types [29-36]. Notably, this biologically and clinically relevant property of TLR signaling largely depends on the extensive crosstalk between components of the canonical TLR pathway and signal cascades coupled to other immune receptors [36].

Several reports have shown that CaMKs control important functions in myeloid progenitors as well as in dendritic cells and macrophages [14, 37, 38]. CaMKIV is a critical component of the molecular mechanisms that regulate the survival of activated dendritic cells and the amplitude of the antibody response induced by vaccines [34, 39]. In immune cells, CaMKK2 is selectively expressed in macrophages, and its ablation impairs the ability of this cell type to spread, phagocytose bacterial particles, and release cytokines and chemokines in response to lipopolysaccharides (LPS) [38]. In agreement with this finding, *in vivo* studies show that genetic ablation of CaMKK2 prevents the accumulation of macrophages and

inflammatory cytokine mRNAs in adipose tissue of mice fed a high fat diet [38]. Moreover, CaMKK2-null mice are profoundly resistant to irritants that lead to systemic inflammation and fulminant hepatitis [38]. Mechanistically, the loss of CaMKK2 seems to uncouple TLR4 signaling from the phosphorylation of protein tyrosine kinase 2 (PYK2; also known as PTK2B), and from signal transduction to the PYK2 downstream effectors involved in the activation program of macrophages [38]. PYK2 was originally identified as a signaling protein highly expressed in brain [40], and was later implicated in macrophage activation [41]. More recently PYK2 has been found to regulate prostate cancer development [42, 43]. Based on the above findings in macrophages, it is tempting to hypothesize a similar role for CaMKK2 in PYK2-related signaling in both the brain and PC.

The involvement of CaMKK2 in circuits controlling macrophage activation in response to PAMPs or DAMPs (pathogen-associated or damage-associated molecular patterns, respectively) is corroborated by several reports implicating the CaMKK2 downstream targets CaMKI and AMPK in TLR signal cascades. Stimulation of TLR4 in macrophages by LPS induces, at later time point, accumulation of the phosphorylated form of CaMKI, which is the only CaMK downstream target of CaMKK2 in macrophages; this effect is inhibited by STO-609, a pharmacological inhibitor of CaMKK2 [44]. In agreement with this observation, *in vivo* silencing of CaMKI attenuates serum accumulation of cytokines and HMGB1; together this attenuates the systemic inflammatory response and tissue damage in an experimental model of sepsis [44]. Thus, the CaMKK2-CaMKI signaling axis seems to modulate the inflammatory response initiated by the stimulation of TLR4 in macrophages. AMPKα, the most recently identified target of CaMKK2, is expressed in murine macrophages, and its genetic ablation boosts the release of proinflammatory cytokines in response to LPS stimulation, supporting the development of macrophages exhibiting an anti-inflammatory phenotype and contributing to protection against obesity, inflammation, and insulin resistance [45-48]. Accordingly, the CaMKK2-AMPK axis has been recently identified as a component of a signaling pathway that controls the expression of the inhibitory protein SHP that is induced by the macrophage-stimulating protein (MSP) [47]. It can be hypothesized that the kinetics and amplitude of calcium flux are responsible for switching CaMKK2 toward specific downstream effectors, such as CaMKI or AMPK. For example, although generation of calcium transients in the case of TLR4 signaling is still under question [49], it has been established that tonic or low, constitutive calcium-dependent signaling sustained by integrin plays an important role in fine-tuning the amplitude and nature of cellular responses to heterologous receptors such TLR4 [50-52]. Under this condition, CaMKK2 seems to be coupled to a CaMKI-mediated signaling pathway that sustains secretion of pro-inflammatory cytokines and chemokines [38]. On the other hand, the generation of CaMKK2/AMPK complexes seems to depend on CaM/Ca^{2+} availability [53], and because certain PAMP/DAMP are capable of triggering Ca^{2+} and K+ transients, this type of ligands might switch CaMKK2 towards AMPK-dependent regulatory circuits [54-56]. In my opinion, CaMKK2 is emerging as an important hub that can be coupled to different signal networks to regulate the initiation, progression, and termination of the inflammatory response.

Is CaMKK2 a major regulator of the prostate cancer ecosystem?

Beyond the insight into CaMKK2 signaling in the context of inflammation outlined above, it appears likely that this kinase is major regulator of the microenvironment surrounding prostate tumors based on the effects modulating AR. As I discussed previously, CaMKK2 regulates activation programs of macrophages, a stoma cell type that play a critical role in tumor microenvironment [38]. Intriguingly, this kinase also controls important metabolic pathways in cancer cells and is a relevant component of the AR-signaling [22-25]. For these

reasons, CaMKK2 might represent a prototype of molecular hubs that operate as master regulator of the sex-hormone dependent cancer cell niche.

A large number of diseases are associated with a chronic inflammatory status, which is mainly sustained by macrophage activation and occurs in the absence of any detectable microorganisms [57]. This process, also named sterile inflammation, plays a pivotal role in the cancer microenvironment, which is a unique ecosystem characterized by a low level of oxygen, abundant cell death, and the massive accumulation of “danger” molecules [58]. These extreme conditions fuel a chronic inflammatory state and, in turn, promote the recruitment of myeloid progenitors, which differentiate into tumor-associated macrophages (TAM) that foster tumor growth [58, 59]. Diverse TAM subsets have been identified on the basis of their ability to blunt the immune response against cancer, support blood vessel formation, and help tumor healing after chemo and radiotherapy [27, 60-62]. The inflammatory response plays an important inhibitory role in PC [63, 64]. Macrophages, lymphocytes, and, less frequently, plasma cells and eosinophils, are histologically identified in PC. Of note, TAM infiltration has been found to correlate negatively with prognosis after hormonal therapy [65]. Inflammatory cells and cytokines can also regulate the responsiveness of prostate cancer cells to androgen and ADT. For example, IL-6 has been shown to stimulate the growth of tumor prostate cells, and the serum levels of IL-6 and soluble IL6R parallel PC progression [66].

Zhu et al. have highlighted an unexpected connection between inflammation and sex steroid hormones in the promotion of prostate cancer [67]. These authors compared tissue arrays containing patient-matched sections with both normal and cancer-containing regions of prostate, and found that almost all prostate tumor samples exhibited macrophage infiltration as well as stromal interactions with macrophages. By employing an *in vitro* experimental model, they found that the integrin-mediated adhesion of macrophages to cancer cells promotes the release of interleukin-1 (IL-1), which in turn converts selective androgen-receptor modulators (SARMs) from their intended function as inhibitors of androgen-receptor-induced gene expression to activators of expression [67]. They identified TAB2, a component of a co-repressor complex N-COR/HDAC, as a downstream target of an IL-1-mediated cascade. Mechanistically, IL-1 signaling impinges on this pathway by inducing TAB2 phosphorylation, which causes N-COR/HDAC to detach with the resultant activation of AR-mediated gene transcription. These findings add an additional layer of complexity to the mechanisms regulating prostate cancer development and establish a novel function for TAM, which might serve as an important component of new generation of AR antagonists resistance in prostate cancer [67, 68].

Recent studies from immune cell-specific androgen receptor (AR) knockout mice have established a role for AR-signaling in innate and adaptive immune responses [69]. Conditional MARKO mice, which lack AR expression in macrophages, fail to recruit this cell type to the wounds and show accelerated healing [70]. This seems to be a consequence of the impaired ability of MARKO monocytes/macrophages to express the chemokine receptor CCR2 and synthesize TNF-α. Prostate cancer cells exposed to androgens display a significantly enhanced ability to promote macrophage migration, and this identifies an additional function of AR in mechanisms shaping the PC ecosystem [71]. Overall, these findings reveal AR to be a major regulator of pathways that control tumor cells growth and shape the tumor ecosystem, and leads to the hypothesis that CaMKK2 is an important component of AR-signaling.

Concluding remarks and perspectives

CaMKK2 is expressed in only a few cell types outside the brain [13]. In normal prostate, this protein is expressed at very low levels, but it accumulates in prostate cancer cells where it facilitates AR signaling [22-25]. In the immune system, CaMKK2 expression is restricted to macrophages, including TAM (L.R. data not published) [38]. In dendritic cells CaMKK2 is barely detectable (L.R., data not published), and it is not expressed in NK or T-cells, which are cell types capable of mediating an effective immune response against cancer. In macrophages, CaMKK2 regulates metabolic responses and cytokine release in response to PRR/integrin stimulation, and this might have important consequences on the pharmacological effects induced by the new generation of AR antagonists (i.e. MDV3100, ARN509), which are used in PC (Figure 1) [68, 72]. In prostate cancer cells, the CaMKK2-AMPK axis controls glucose metabolism, growth, and cell migration. In my opinion, these novel findings suggest that CaMKK2 resides at the apex of a pivotal molecular node that shapes the cancer ecosystem, and identifies this protein as a global regulator of the PC ecosystem.

Although CaMKK2 has been identified in human and rodents many years ago as a brain kinase, for many reasons studies on its role in physiopathology are still in their infancy [13]. AR signaling in PC has been investigated employing the wide genome analysis by several authors, and interrogation of these databases has led to identify CaMKK2 as a relevant component of this pathway [21-23]. The AR-network has been much less explored in macrophages, and at best of my knowledge there are no studies that have explored the role of CaMKK2/AR circuit in prostate cancer-associated macrophages. This might represent a promising field of investigation for identifying the functions of the primary targets of CaMKK2/AR, as well as metabolic pathways and genes sets regulated by CaMKK2 in macrophages associated with cancer cell niche. As mentioned above, early investigations have proposed a role for CaMKK2 in mechanism that control macroautophagy [20]. With this in mind, future studies should be aimed at extending these original observations, and defining the role of CaMKK2 in calcium signaling that regulates stress response in endoplasmic reticulum in TAM/PC. This will help to establish the role of CaMKK2/AR-signaling in cancer prostate ecosystem under clinically relevant conditions that stimulate ER-stress such as microbial infection, obesity and therapeutic cytotoxic treatments (i.e. chemo- and radiotherapy). I suspect that these studies will uncover CaMKK2 as an important hub of circuits governing the sex hormone regulated cancer ecosystems.

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Box 1

Glossary

Calmodulin (CaM): a 148 amino acid protein comprising four helix-loop-helix protein folding motifs called EF hands, each of which binds a Ca^{2+} ion. CaM is a key protein that transduces signals in response to transient increases in intracellular Ca^{2+}.

Ca2+/CaM dependent kinases (CaMK): a family of structurally related serine/threonine protein kinases that are activated by calmodulin (CaM) binding. Members of this family are grouped according to whether they are dedicated kinases having a single substrate (e.g., phosphorylase kinase, CaMKIII, and MLCK) or multiple substrates (CaMKI, CAMKII, and CAMKIV).

Ca2+/CaM-dependent kinases kinases (CaMKK): a structurally related subfamily of CaMKs. Two distinct CaMKK isoforms have been identified, namely, CaMKK1 and CaMKK2. Both CaMKKs can phosphorylate and activate CaMKI and CaMKIV. In addition, CaMKK2 can phosphorylate other substrates such as AMPK.

CaM kinase cascade: an enzymatic cascade consisting of a CaMKK that phosphorylates and activates one of two CaM kinases, CaMKI or CaMKIV.

AMPK: 5′ adenosine monophosphate-activated protein kinase is an enzyme that has important roles in cellular energy homeostasis. It consists of three subunits: α, β and γ.

PAMP: molecules associated with groups of pathogens. PAMPs are usually recognized by cells of the innate immune system, which express pattern recognition receptors, or PRRs.

DAMP: danger-associated molecular patterns released by stressed or dying cells. DAMPs are usually recognized by cells of the innate immune system, which express PRR.

PRR: pattern recognition receptors are class of receptors that recognizes PAMP and DAMP. PRR are usually expressed on immune cells.

AR: Androgen Receptor is a nuclear receptor that is activated by the binding of testosterone or dihydrotestosterone (DTH) in the cytoplasm, and then translocates into the nucleus.

ADT: androgen-deprivation therapy, the first choice for advanced prostate cancer. The treatment is based on lowering the amount of testosterone available to fuel cancer growth either by preventing testosterone production using drugs such as luteinizing hormone-releasing hormone analogs and antagonists, or by administering anti-androgens that are competitive inhibitors of testosterone binding to androgen receptors.

CRPC: castration-resistant prostate cancer refers to cancer that is still growing despite the fact that androgen-deprivation therapy is keeping the testosterone levels in the body at very low, or “castrated”, levels.

Box 2

Outstanding questions and future perspectives

- What are the molecular targets of CaMKK2 in macrophages and prostate cancer cells?
- How does CaMKK2 modulate AR-regulated transcriptional activity?
- Is CaMKK2 required for AR signaling in macrophages?
- Does CaMKK2 play a differential role in androgen-regulated and castration-resistant prostate cancer?
- Which is the role of CaMKK2/AR-network in ER-stress response in macrophages and prostate cancer cells?

Highlight

- Outside the brain, CaMKK2 is expressed in few cell types.
- CaMKK2 is barely detectable in normal prostate.
- CaMKK2 is overexpressed in prostate cancer, and is an important AR-regulated gene.
- CaMKK2 is expressed in macrophage and regulates the inflammatory response.
- CaMKK2 is barely detectable in Granulocytes, DC, NK, or B and T cells.

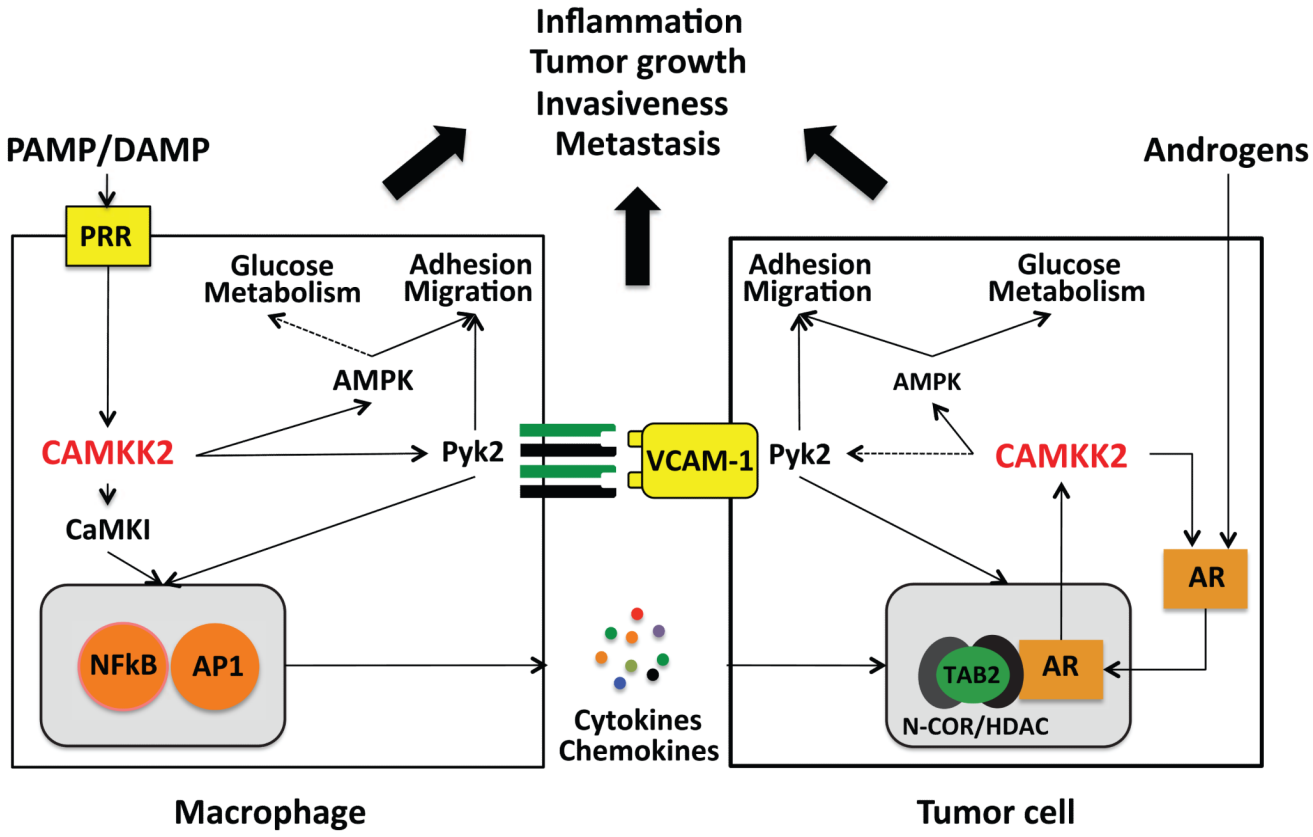

FIGURE 1. Model for CaMKK2 functions in prostate cancer biology

In macrophages, PAMPs and DAMPs bind PRRs and trigger downstream signals, which synergize with integrin-signaling generated at the macrophage-cancer interface. In macrophages: CaMKK2 regulates crosstalk between PPR- and integrin-signals required for maximal activation of PYK2. In turn, PYK2 controls cytoskeleton organization and cytokine gene expression. CaMKI and AMPK are downstream targets of CaMKK2, which regulates metabolic response and gene expression in response to PRR/integrin stimulation. Cytokines released by activated macrophages induce TAB2 phosphorylation and remove the co-repressor N-COR/HDAC from the androgen receptor (AR)-promoter, which in turn converts the new androgen receptor antagonist used in PC from their intended function as inhibitors of androgen receptor-induced gene expression to activators. In prostate cancer cells: androgens bind and activate AR, which moves to the nucleus and enhances *Camkk2* gene expression. CaMKK2 participates in a positive feedback loop by stimulating AR-dependent transcriptional activity. The CaMKK2-AMPK axis controls glucose metabolism, growth and cell migration. In this model, CaMKK2 is an important component of AR signaling, and a pivotal player of networks controlling macrophage activation and prostate cancer biology. Dotted arrows indicate functional interactions not yet supported by experimental evidence. Abbreviations: PAMP, pathogen-associated molecular pattern; DAMP, damage-associated molecular pattern; PRR, pattern recognition receptors; AR, androgen receptor; HDAC, histone deacetylase.