ARTICLE

CaMKK2 in myeloid cells is a key regulator of the immune-suppressive microenvironment in breast cancer

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Tumor-associated myeloid cells regulate tumor growth and metastasis, and their accumulation is a negative prognostic factor for breast cancer. Here we find calcium/calmodulin-dependent kinase kinase (CaMKK2) to be highly expressed within intratumoral myeloid cells in mouse models of breast cancer, and demonstrate that its inhibition within myeloid cells suppresses tumor growth by increasing intratumoral accumulation of effector CD8⁺ T cells and immune-stimulatory myeloid subsets. Tumor-associated macrophages (TAMs) isolated from Camkk2⁻/⁻ mice expressed higher levels of chemokines involved in the recruitment of effector T cells compared to WT. Similarly, in vitro generated Camkk2⁻/⁻ macrophages recruit more T cells, and have a reduced capability to suppress T cell proliferation, compared to WT. Treatment with CaMKK2 inhibitors blocks tumor growth in a CD8⁺ T cell-dependent manner, and facilitates a favorable reprogramming of the immune cell microenvironment. These data, credential CaMKK2 as a myeloid-selective checkpoint, the inhibition of which may have utility in the immunotherapy of breast cancer.
The recruitment of myeloid cells is an important process in the initial phases of tumor development. In response to cues from the tumor microenvironment, these cells can differentiate into tumor-associated macrophages (TAM), promoting angiogenesis, and supporting both primary tumor growth and distal metastasis1,2. TAMs are also responsible for the establishment of a robust immune-suppressive tumor ecosystem by inhibiting the differentiation and function of effector T cells, and stimulating the intratumoral accumulation of regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs)3-5. Not surprisingly, TAM density in primary tumors is strongly associated with poor outcomes in breast cancer6-8. These findings have driven the search for therapeutic targets, the manipulation of which will reprogram TAMs in a manner that promotes pro-immunogenic activities9,10.

CaMKK2 is a Ca2+/Calmodulin (CaM)-dependent serine-threonine protein kinase that couples calcium transients triggered by a variety of stimuli to processes involved in the regulation of cell proliferation, survival, and metabolism11. For the most part, these activities require CaMKK2-dependent phosphorylation of its primary substrates, CaMKI, CaMKIV, and/or AMPK12,13. The CaMKK–CaMKI pathway regulates the activity of multiple downstream targets, including ERK, RAC1, CREB, and HDAC5, which in turn impacts the cell cycle, cytoskeletal architecture, cell differentiation, as well as, facilitating hormone and cytokine production14,15. CREB is also a downstream target of CaMKIV, and activation of this signaling cascade promotes dendritic cell survival and increases cytokine secretion from T cells16,17. Both CaM and CaMKK2 can form a complex with AMPK subjecting the bound fraction of AMPK to regulation by calcium rather than AMP13. In addition to functioning as a key integrator/regulator of metabolic responses to energetic stress, AMPK has been shown to play a role(s) in the regulation of hematopoiesis, macrophage polarization, and T cell effector function.

The importance of CaMKK2 in the regulation of a number of inflammatory diseases such as obesity and insulin resistance and prostate and hepatocellular cancers has been established11,12. In prostate cancer cells, CaMKK2 expression is induced by androgens18, and in hepatocytes by chemical carcinogens19. CaMKK2 is also expressed in gastric and ovarian cancers where pro-tumorigenic roles have been suggested19,20. To date, most studies of CaMKK2 in cancer have focused on its cancer cell intrinsic activities. However, this enzyme is also expressed within hematopoietic stem and progenitor cells, and in more mature myeloid cells, including monocytes and peritoneal macrophages11,21-23. Furthermore, genetic ablation of Camkk2 in mice revealed an important role for this enzyme in the development of myeloid cells and in regulating their ability to mount inflammatory responses to various stimuli22,24. These activities of CaMKK2 within myeloid cells suggested to us that it may also impact tumor biology in a cancer cell extrinsic manner. The goal of this study, therefore, was to investigate the extent to which CaMKK2 impacts immune cell repertoire and function in the micro-environment of mammary tumors. We find that deletion of CaMKK2 in myeloid cells, or its pharmacological inhibition, attenuates tumor growth in a CD8+ T cell-dependent manner, facilitating a favorable reprogramming of the immune cell microenvironment. These data, credential CaMKK2 as a myeloid-selective checkpoint, the inhibition of which may have utility in the immunotherapy of breast cancer.

Results

CaMKK2 is expressed in tumor-associated stromal cells. To probe the potential significance of CaMKK2 expression in human breast cancer, we analyzed CaMKK2 expression in two well-
higher in tumors propagated in Camkk2−/− mice when compared with WT mice (Fig. 2e).

Gene expression analysis was next undertaken to define how CaMKK2 knockdown impacted immune cell repertoire in tumors. We first confirmed the expression (qPCR and western immunoblot) of CaMKK2 in myeloid cells isolated from E0771 tumors (Supplementary Fig. 2D, E). Analysis of the whole-tumor lysates from Camkk2−/− mice showed a remarkable upregulation of genes expressed in the cytotoxic effector lymphocyte subsets, such as Granzyme B (Gzmb) and Perforin-1 (Prf1) (Fig. 2f, top). Although expressed at very low levels, a significant upregulation of Foxp3 and Pdcd1 was also observed in tumors from Camkk2−/− mice. This may result from an increased accumulation of regulatory T cells in the more fibrotic/necrotic areas of tumors from Camkk2−/− mice. Notably, the expression of mRNAs encoding the CXCR3 receptor ligands (Cxcl9, Cxcl10, and Cxcl11), which have non-redundant roles in tumoricidal T cell trafficking29, were found to be upregulated in tumors from Camkk2−/−

Fig. 1 CaMKK2 is expressed in mammary tumor-associated stromal cells. a Expression of CaMKK2 in human breast cancer. Representative images (×400 magnification) of malignant mammary tissue samples stained with an anti-CaMKK2 antibody. T and S refer to tumor and stromal cells, respectively. Scale bar = 100 μm. b CaMKK2 staining intensity is correlated between cancer and stromal cells of the same human breast tumors. c The Camkk2 promoter is active in myeloid cells associated with mammary tumors. E0771 cells (4 × 10^5 cells/mouse) were inoculated into the mammary fat pad of (Tg)-Camkk2-EGFP reporter mice. Subsequently, tumors were removed and digested and single-cell suspensions were stained for markers of myeloid cells. The gating strategy used to identify myeloid subsets and lymphoid cells is shown in Supplementary Fig. 1B. (Left) FACS profiles report the expression of EGFP reporter in tumor-associated immune cells. The vertical line refers to negative control (EGFP-negative splenocytes). (Right) Mean fluorescence intensity (MFI) of EGFP expression in immune cell subsets. Bar graph reports the mean ± SEM (standard error of the mean; N = 3 independent tumors in each group). *p < 0.05, ***p < 0.005, respectively. A t test was used to calculate p-values. Similar results have been observed in three independent experiments.
mice compared with WT (Fig. 2f, bottom). Those genes found to be upregulated in tumors grown in *Camkk2*−/− mice (e.g., Cxcl9 and Gzmib) were found to associate with a positive prognosis in human breast cancers (Supplementary Fig. 2F).30

Our IHC analysis revealed that more T cells accumulated in non-necrotic areas of tumors removed from *Camkk2*−/− mice compared with WT. FACS was used next to quantitatively assess activation and inhibitory markers expressed on CD4+ and CD8+ tumor-infiltrating lymphocytes (TILs) (gating strategy outlined in Supplementary Fig. 3A, B). Increased percentages of CD8+ TILs expressing the activation markers GZMB and CD69 were detected in tumors of *Camkk2*−/− mice compared with WT (Fig. 3a, b). In contrast, tumors from WT mice demonstrated an increased accumulation of CD4+ T cells expressing PD-1 and LAG-3 inhibitory receptors (Fig. 3b). Overall, these results indicate that *Camkk2* knockdown promotes a more vigorous T cell-mediated immune response in tumors. Of significance, in breast cancer, the number of CD8+ T cells in tumors positively correlates with better therapeutic response and prognosis.31,32

Analysis of the tumor-associated myeloid cell repertoire revealed that CD11b+ MHC II+ cells accumulated preferentially in tumors from *Camkk2*−/− mice (Fig. 3c). This was also confirmed using tumors of similar sizes from *Camkk2*−/− and WT mice (Supplementary Fig. 2C and 3C). A large fraction of these cells also expressed the Ly6C marker, a phenotype indicative of inflammatory monocytes. A more detailed analysis confirmed an increase in MHC II+ TAMs in tumors from *Camkk2*−/− mice (Supplementary Fig. 3C, top). Of note, MHC II+ TAMs have previously been shown to exhibit M1-like features, and accumulation of this myeloid subset in tumors positively correlates with tumor regression.33,34

Analysis of the tumor-infiltrating conventional dendritic cell compartment (TIDCs) identified MoDC as the largest fraction of cDCs infiltrating E0771 tumors, followed by cDC2 and cDC1 subsets (Supplementary Fig. 3C, bottom). Interestingly, increased numbers of MoDC were found in tumors from *Camkk2*−/− mice compared with WT (Supplementary Fig. 3C, bottom right). The role of TIDCs subsets in the tumor microenvironment is not fully understood18,35,36, as both detrimental or even beneficial effects on CD8+ T cell activation have been reported for this myeloid subset.37,38

**Table 1 CaMKK2 expression by molecular breast cancer subtypes**

|        | Vienna a | RPCI b | Combined c |
|--------|----------|--------|------------|
| CaMKK2 |          |        |            |
| Low    | 32       | 75%    | 86         |
| High   | 15       | 40%    | 29         |
| ORd    | 0.2      | nd     | 0.3        |
| p-valuee | 0.0247  | nd     | 0.0107     |

N: sample numbers, ns: not determined, ns: not significant

*Immunohistochemical analysis of CaMKK2 expression in human breast cancer tissue microarrays, from two independent datasets (a: Roswell Park Cancer Institute, RPCI). CaMKK2 expression was determined to be low (<2) or high (> = 2), and correlated with molecular subtypes: triple negative (TN), luminal A (LA), and luminal B (LB). Fisher’s exact test was used to determine p-values for the likelihood of association.

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Tumor growth attenuation requires CD8+ T cells and CaMKK2 inhibition in myeloid cells. The extent to which the decreased tumor growth observed in *Camkk2*−/− mice could be attributed to the increased number of cytotoxic T cells within these tumors was next evaluated. *Camkk2*−/− mice were treated with an anti-CD8 antibody to deplete CD8+ T cells or a control IgG (Supplementary Fig. 4A, B). This intervention was accompanied by a dramatic increase in tumor growth in the *Camkk2*−/− background, but was without effect in WT mice (Fig. 3d; Supplementary Fig. 4C). These data confirmed that CD8+ T cells are responsible, at least in part, for the impaired tumor growth observed in *Camkk2*−/− mice.

Since *Camkk2* promoter activity is restricted to the myeloid lineage in tumors (Fig. 1c), it seemed likely that *Camkk2* impacted tumor growth through its ability to regulate CD8+ T cell function secondary to activities within myeloid cells. To test this possibility, we developed a LysMCre *Camkk2*fl/fl mouse model and confirmed a reduced expression of CaMKK2 protein in macrophages of LysMCre *Camkk2*fl/fl compared with LysMCre *Camkk2*wt/wt littermates (Supplementary Fig. 4D). As observed in the whole-body knockout of *Camkk2*, the growth of E0771 tumors was significantly attenuated when propagated in LysMCre *Camkk2*fl/fl mice compared with littermate controls, and this activity was associated with an increased accumulation of CD11b+ MHC II+ cells in tumors from LysMCre *Camkk2*fl/fl mice (Fig. 3e; Supplementary Fig. 4E). These data were confirmed using LysMCre *Camkk2*fl/fl mice and LysMCre *Camkk2*fl/fl mice to rule out off-target effects of Cre expression on myeloid cell function (Supplementary Fig. 4F). Thus, we conclude that ablation of *Camkk2* within myeloid cells is sufficient to attenuate the growth of E0771 mammary tumors in immune-competent mice.

**CaMKK2 influences the expression of key genes in BMDM.** Cancer cell-secreted factors can influence myeloid cell differentiation resulting in an increase in the number/activity of TAMs and other immune-suppressive myeloid cell subsets.4,10 Thus, we reasoned that genetic deletion of *Camkk2* might influence macrophage differentiation and/or activity in a manner that increases their immune-stimulatory phenotype. Analysis of the immune-regulatory cytokines produced by E0771 cells confirmed that, absent any provocative stimuli, they secreted high levels of VEGF, G-CSF, and CCL2 among others (Supplementary Fig. 5A, B). The impact of tumor-conditioned media (TCM) on myeloid cell function was next assessed using bone marrow cells isolated from WT and *Camkk2*−/− mice and differentiated in vitro in the presence of (a) regular media (RM) or (b) TCM (E0771) (Supplementary Fig. 5C).

The effects of TCM on the differentiation program of myeloid cells were evaluated using microarray analysis and confirmed by qPCR. Regardless of genotype, 439 differentially expressed genes (DEGs) were identified in bone marrow-derived myeloid cells (namely, BMDM—the purity assessed by FACS was >90% CD11b+ F4/80+, Ly6C−, CD11cFlmogen) generated in the presence of TCM versus RM. Among these genes were several chemokine genes (*Ccl8, Ccl12, Cxcl10*, and *Ccl11*) (Fig. 4a, b; Supplementary
Furthermore, 455 genes were selectively regulated in Camkk2−/− TCM-BMDM compared with WT TCM-BMDM (Supplementary Fig. 6A), and these DEGs were associated with 24 canonical pathways (Supplementary Data 1). Most of the genes associated with steroid biosynthesis (KEGG 00100; p-value 4.236e-4; Fisher’s method) were downregulated in Camkk2−/− TCM-BMDM compared with WT (Supplementary Fig. 6B). In contrast, several DEGs associated with cytokine–cytokine receptor pathways

**Table 2.**
(KEGG 04060; p-value 4.236e-4; Fisher’s method; Supplementary Fig. 6B, Supplementary Data 2) were upregulated in Camkk2\(^{-/-}\) TCM-BMDM compared with WT. Using gene set enrichment analysis (GSEA)\(^39\), a positive enrichment for interferon response genes and negative enrichment of genes involved in cholesterol biosynthesis were noted in Camkk2\(^{-/-}\) TCM-BMDM compared with WT TCM-BMDM (Supplementary Fig. 6C, D).

We reasoned that ablation of Camkk2 would prompt myeloid progenitors exposed to TCM to develop toward a more immunogenic phenotype compared with those derived from
WT mice. We therefore compared the expression of genes, previously shown by others to be associated with M1, shared by M1 and DCs (M1&DC), or M2 phenotypes, in WT and Camkk2−/− BMDM generated in the presence of RM and TCM. The most remarkable finding in this analysis was that the majority of M1 and M1&DC signature genes were upregulated in Camkk2−/− versus WT BMDM in the presence of TCM (Fig. 4c).

The expression pattern of genes associated with the M2-phenotype was more complex with some genes also being significantly upregulated in Camkk2−/− BMDM cultured in TCM (Fig. 4c). Finally, we used qPCR to evaluate the expression of Nos2, Arg1, Chil3/Ym1 and Retnla/Fizz1 gene signatures (four biological replicates were analyzed for each genotype). A t test was used to calculate p-values. The data are graphed as the mean ± SEM. Asterisks refer to *p < 0.05, **p < 0.01, ***p < 0.005, and ****p < 0.001. This experiment was replicated at least three times with similar results.

**Fig. 4 CaMKK2 regulates gene transcription in BMDM.** Bone marrow-derived macrophages (BMDM) were generated from WT and Camkk2−/− mice in the presence or absence of E0771-conditioned medium (RM and TCM, respectively). Subsequently, BMDM was harvested and analyzed for gene expression. N = 4 biological replicates/group. a, b Volcano plots of differentially expressed genes (DEGs) in WT (left) and Camkk2−/− (right) generated in the presence of RM or TCM. Open circles indicate genes showing comparable expression levels in WT and Camkk2−/− BMDM. DEGs between WT and Camkk2−/− BMDM are shown as green filled circles. Yellow open circles refer to the Camkk2 gene. c Heatmaps of DEGs affiliated with M1, M1 and dendritic cells (M1&DC), or M2 signatures. The color key for the heatmap indicates (row-wise) scaled RPKM values (z-score). d Real-time quantitative PCR (qPCR) analysis of genes associated with M1 (Nos2) and M2 (Arg1, Chil3/Ym1 and Retnla/Fizz1) gene signatures (four biological replicates were analyzed for each genotype). A t test was used to calculate p-values. The data are graphed as the mean ± SEM. Asterisks refer to *p < 0.05, **p < 0.01, ***p < 0.005, and ****p < 0.001. This experiment was replicated at least three times with similar results.
phenotype, when considered in total, these findings indicate that deletion of Camkk2 interfered with the expression of the largely immunosuppressive transcriptional program induced by tumor-derived factors (TCM) in myeloid cells. Further Camkk2 inhibition enhanced the transcription of genes associated with a more immunogenic phenotype, including interferon response genes and chemokines involved in intratumoral T cell trafficking.

**Camkk2 links tumor factor signaling to AMPK activation.** Our data suggest that Camkk2 is required to couple the proximal signaling events induced by tumor-derived factors present in the TCM with downstream molecular effectors. CamkIV was eliminated from this analysis, as we were unable to detect CamkIV/phospho-CamkIV expression within BMDM under the conditions used for these in vitro assays. Comparable levels of phospho-CamkI were detected in RM-BMDM and TCM-BMDM, from both WT and Camkk2−/− mice, indicating that tumor-derived factors are not increasing all aspects of Camkk2 signaling in BMDM (Fig. 5a). Ampkα can be phosphorylated by both Camkk2 and LKB1; however, we found it to be phosphorylated to a lesser degree in Camkk2−/− BMDM compared with WT BMDM when cultured in regular media (RM) (Fig. 5b, c). More importantly, increased Ampkα phosphorylation was observed in WT BMDM when cultured in TCM compared with RM, and this enhanced phosphorylation was attenuated considerably by Camkk2 ablation (Fig. 5b, c). In aggregate, these data indicate that Camkk2 is required for TCM-induced activation of Ampk cascade. Interestingly, in the absence of Camkk2, the exposure to TCM displayed an inhibitory effect on the phosphorylation of Ampk. This finding is suggestive of a wider, yet undefined, function of Camkk2 in processes that link tumor factor-induced signaling to the activation of AMPK. These data are interesting in light of those from others, indicating that Ampkα modulates macrophage polarization toward an M2-like anti-inflammatory phenotype. Previously, it was determined that the PGC1/ERRα complex, a downstream target of AMPK in macrophages, is a key regulator of innate immunity. However, we ruled out the involvement of this complex in Camkk2 biology as myeloid cell-specific knockdown of ERRα failed to impact the growth of mammary tumors in the MMTV model (Supplementary Fig. 7A–D).

**Camkk2 controls the immune-stimulatory functions of TCM-BMDM.** The multi-ligand endocytic mannose receptor (CD206/Mrc1) is an established marker of M2-like macrophages, whereas higher levels of MHC II and/or co-stimulatory molecules (e.g., CD80, CD86, and CD40) serve as markers of more immunogenic M1-like macrophages. Thus, we analyzed the expression of these markers in WT and Camkk2−/− BMDM generated in the presence of TCM (Fig. 6a, c; Supplementary Fig. 8A). Regardless of genotype, an increase in the percentage of CD206+ MHC II− (M2-like macrophages) and a decrease in the percentage of CD206− MHC II+ (M1-like macrophages) were detected in BMDM generated in the presence of TCM (Fig. 6a). These changes were attenuated in Camkk2−/− BMDM, such that in the presence of TCM they expressed higher levels of MHC II molecules and have fewer M2-like and more M1-like cells compared with WT (Fig. 6a, b). Camkk2−/− BMDM also expressed significantly higher levels of MHC II and CD40 compared with WT, although its absolute expression level was reduced in response to TCM (Fig. 6b; Supplementary Fig. 8A). BMDM generated in the presence of TCM also expressed higher levels of CD80 and MHC I molecules, although these TCM-induced changes were attenuated in Camkk2−/− BMDM. Finally, CD86 was expressed at comparable levels in WT and Camkk2−/− BMDM in the presence of RM, but higher levels of this co-stimulatory molecule were detected in Camkk2−/− BMDM generated in TCM when compared with WT (Supplementary Fig. 8A). To evaluate the effects of TCM on already differentiated BMDM, cells were first cultured in the presence of RM for 4 days, and then exposed for an additional 48 h to TCM. These experiments confirmed the ability of TCM to increase the percentage of M2-like cells in WT BMDM, but importantly this response was attenuated in Camkk2−/− BMDM (Supplementary Fig. 8B).

The significance of data generated in BMDM was subsequently validated in TAM isolated from E0771 tumors propagated in WT and Camkk2−/− mice. Similar to the results obtained in BMDM, deletion of Camkk2 in the host was associated with a decreased percentage of CD206+ MHC II− TAM, and increased accumulation of CD206− MHC II+ TAMs (Fig. 6d). Of note, increased levels of Cxcl9 and Cxcl14 RNAs were also found in TAM isolated from tumors of Camkk2−/− mice, confirming the results of studies performed in vitro (Fig. 6e; Supplementary Fig. 8D). The ability of WT and Camkk2−/− BMDM to stimulate and recruit T cells was next assessed. Syngeneic T cells were isolated from the spleens of WT mice, stained with the fluorescent dye 5-(and 6)-carboxyfluorescein diacetate succinimimidyl ester (CFSE), and subsequently co-cultured with BMDM, in the presence of an anti-CD3 antibody. After 72 h, cell supernates were collected and assayed for cytokine expression, and T cell proliferation was assessed by FACS (Fig. 7a, b; Supplementary Fig. 8E). IL-2 production was significantly increased in T cells co-cultured with Camkk2−/− BMDM generated in TCM, but not RM (Supplementary Fig. 8E, left panel). Not unexpectedly, IFNγ expression was dramatically suppressed when T cells were cultured with WT BMDM generated in the presence of TCM with Camkk2−/− BMDM generated in TCM, but not RM (Supplementary Fig. 8E, right panel). However, this suppressive effect of TCM was mitigated upon Camkk2 knockdown (Supplementary Fig. 8E, right panel). The functional importance of these differences was highlighted in follow-up T cell proliferation studies, which demonstrated that BMDM derived from WT or Camkk2−/− mice in RM were equally effective at stimulating T cell proliferation. However, T cell proliferation was reduced by ~50% when evaluated in the presence of WT TCM-BMDM, whereas deletion of Camkk2 in BMDM reduced this effect (Fig. 7a, b). We next explored the mechanisms by which Camkk2 regulates the functional interactions between BMDM and T cells. Specifically, the role(s) of physical cell contact vs BMDM-released soluble factors on the proliferation of purified CD4+ and CD8+ T cells was assessed. To this end, purified CFSE-labeled CD4+ and CD8+ T cells were cultured with WT or Camkk2−/− TCM-BMDM, in the presence of an anti-CD3 antibody. In this direct cell contact assay, Camkk2−/− TCM-BMDM showed an increased ability to stimulate both purified T cell subsets, compared with WT TCM-BMDM. CFSE-labeled purified CD4+ and CD8+ T cells were then cultured with anti-CD3/anti-CD28 coated beads in the presence of supernates collected from TCM-BMDM cultures (Supplementary Fig. 8G). Interestingly, WT TCM-BMDM supernates inhibited the proliferation of CD8+ and CD4+ T cells while no inhibitory effect was observed using Camkk2−/− TCM-BMDM supernates (Supplementary Fig. 8G). Taken together these data suggest that the positive effect of Camkk2−/− TCM-BMDM on T cell proliferation likely requires physical contact, and this activity is reinforced by a reduced production of immune-suppressive soluble factors.
Gene expression analysis in whole tumors, isolated TAM, and BMDM identified the CXCR3-ligands (Cxcl9, Cxcl10, and Cxcl11) as relevant targets of CaMKK2 in myeloid cells. Specifically, deletion of Camkk2 resulted in increased expression of the mRNAs encoding Cxcl9 and Cxcl10 in TAMs and BMDM; a finding that could explain the increased accumulation of CD8⁺ T cells in tumors of Camkk2−/− mice. To test this possibility, we developed a transwell assay to evaluate the ability of BMDM to attract in vitro-activated T cells. It was determined that more T cells migrated toward supernates collected from Camkk2−/− TCM-BMDM than those from WT mice (Fig. 7c, left). This conclusion was supported by additional data from a second study using antigen-specific CD8⁺ T cells (OTI T) (Fig. 7c, right).

To further investigate the ability of WT and Camkk2−/− myeloid cells to recruit OVA-specific EGFP-CD8⁺ T cells, we used a validated microfluidic platform to recreate interconnected 3D spaces. This device combines the design of a hydrogel-based microchannel platform with advanced microscopy, allowing single-cell, real-time monitoring, and analysis of antigen-specific CD8⁺ T cell migration. It further allows the monitoring of interactions that occur between effector T cells and cellular components of the tumor microenvironment. To build an on-chip simulation of the tumor microenvironment, BMDMs were co-cultured in a 3D hydrogel-based compartment with E0771 cells in the presence of OVA257–264 peptide. The ability of WT and Camkk2−/− myeloid cells to recruit OVA-specific EGFP-CD8⁺ T cells (EGFP-OTI-T) was tested under competitive conditions. To this end, the two tumor chambers of the microfluidic platforms included WT or Camkk2−/− BMDM (right and left chamber, respectively; Supplementary Fig. 9A, B). Upon hydrogel polymerization, EGFP-OTI-Ts were loaded and uniformly distributed into the immune chamber alongside with OVA257–264-preloaded DsRed bone marrow-generated dendritic cells (DsRed-BMDC). The competitive ability of tumor chambers...
containing WT and Camkk2<sup>−/−</sup> BMDM was evaluated over a period of 72 h by wide-field imaging analysis of the entire microfluidic device, and the total number of OTI T cells within the microchannels and tumor chambers was quantified (Fig. 7d; Supplementary Fig. 9C). OTI T cells initially interacted with antigen-loaded BMDC in the central chamber (1–6 h) and then migrated through micro-channels and 3D tumor compartments at later time points (18–44 h). Under these conditions, a remarkably higher number of OTI T cells migrated through the micro-channels and infiltrated the 3D tumor chamber containing Camkk2<sup>−/−</sup> BMDM, compared with those moving toward the tumor chamber housing WT BMDM (Fig. 7d). Taken together, these studies define an important role for CamKK2 in the regulation of cellular processes involved in the trafficking of T cells into tumors.

**CaMKK2 inhibitors attenuate mammary tumor growth.** STO-609 is a selective CaMKK2 inhibitor that has previously been validated for use in vivo<sup>17,18,49</sup>. Thus, we used this compound to evaluate the impact of CaMKK2 inhibition on the growth of E0771, 4T1, and Met1 cell-derived tumors propagated in syngeneic hosts, as well as in the MMTV- PyMT spontaneous model of mammary carcinoma (Fig. 8a; Supplementary Fig. 10A–C). Given the positive impact of CaMKK2 inhibition by STO-609 on tumor growth, we proceeded to generate novel, chemically distinct, CaMKK2 inhibitors via a discovery campaign that led to the identification of GSK1901320 (denoted GSKi; Supplementary Fig. 10D). This compound is chemically distinct from STO-609, but shows similar antagonist activity to this drug in biochemical assays (Supplementary Fig. 10D). Importantly, GSKi inhibited the growth of E0771-derived tumors in syngeneic hosts to the same degree as STO-609 (Supplementary Fig. 10E). Thus, two chemically distinct, selective CaMKK2 antagonists demonstrated similar inhibitory effects in mouse models of breast cancer. These results justify further exploration/optimization of highly selective CaMKK2 inhibitors as a means to increase tumor immunity.

The impact of CaMKK2 inhibition on immune cell repertoire/ function within tumors was next evaluated. Reflecting what was seen in genetic CaMKK2 knockout models, treatment with STO-609 promoted the accumulation of CD8<sup>+</sup> and NK1.1<sup>+</sup> T cells, CD11b<sup>+</sup> MHC II<sup>+</sup> cells, and MHC II<sup>+</sup> TAMs within E0771 tumors (Fig. 8b, c; Supplementary Fig. 11A–C). STO-609 was without effect in CD8<sup>+</sup> T cell depleted Camkk2<sup>−/−</sup> mice, suggesting that its actions in primary tumor growth are likely limited to immune cells (Fig. 8d).

**Discussion**

Our findings highlight the role(s) of CaMKK2 as a myeloid-expressed regulator that integrates signals controlling activation and differentiation of macrophages in the tumor microenvironment.
Importantly, genetically ablated CaMKK2, or its pharmacological inhibition led to accumulation of less immune-suppressive myeloid cells and more activated CD8+ T cells in the tumor microenvironment, which results in tumor growth inhibition. Mechanistically, our studies reveal that CaMKK2 is required to link tumor-derived factors that act distally on the bone marrow and splenic myeloid progenitors through their ability to secrete AMPK, which likely functions to impair the immune-stimulatory functions of myeloid cells within the TME.

Previously, we reported that the Camkk2 promoter is active in normal circulating monocytes, and that this enzyme is expressed in peritoneal macrophages. Here, we demonstrate that CaMKK2 is also expressed in tumor-associated myeloid cells, as well as in human breast cancer cells. Importantly, our findings indicate that Camkk2 promoter activity is differentially regulated throughout myeloid cell development, with the highest activity observed in Ly6Chigh monocytes, a subset of blood myeloid cells that can differentiate into a heterogeneous group of TAMs. Typically, CD206+ MHC II+ TAMs show an M2-like phenotype, while CD206- MHC II+ cells are M1-like polarized, and have higher anti-tumor activity.

Of note, we found a remarkable increase in the percentage of CD11b+ MHC II+ cells in tumors growing in Camkk2−/− mice and that a large fraction of this heterogeneous population included Ly6Chigh inflammatory monocytes. An increased percentage of CD206+ MHC II+ TAMs and monocyte-derived dendritic cells, and a concomitant decrease in the percentage of CD206- MHC II+ TAMs were also found in tumors of Camkk2−/− mice. Overall, these findings indicate that CaMKK2 plays a critical role in the regulation of processes that determine the fate of myeloid-cell progenitors in the tumor microenvironment and that its deletion/inhibition alters this differentiation program to promote the accumulation of more immunogenic myeloid subsets. The selective expression of CaMKK2 in the myeloid subsets makes this protein an attractive immunotherapeutic target whose inhibition is expected to remodel the tumor microenvironment and stimulate anti-tumor immune responses.

Tumors have a remarkable capacity to modulate the development of myeloid progenitors through their ability to secrete factors that act distally on the bone marrow and splenic myeloid progenitors, or proximally on monocytes recruited to the tumor microenvironment. Our data indicate that in the presence of tumor-derived factors, more CD206+ MHC II+ and less CD206- MHC II+ BMDM are generated in vitro from bone marrow progenitor cells but deletion of Camkk2 in myeloid cells...
myeloid cells within E0771 mammary tumors treated with STO-609 or vehicle. Tumors of comparable size (500–700 mm³) were removed, digested and single-cell suspensions were stained for myeloid and lymphoid markers, and analyzed using the gating strategy reported in Supplementary Fig. 3C and 11. Treatment with STO-609 resulted in the accumulation of CD8⁺ T cells and CD11b⁺ MHC II⁺ myeloid cells (b and c, respectively). Bar graph shows mean ± SEM; N = 5 and 4 tumors in Veh and STO-609 groups, respectively. A t test was used to calculate p-values. d STO-609 failed to affect mammary tumor growth in CD8⁺ T cell-depleted Camkk2⁻/⁻ mice. Camkk2⁻/⁻ mice were treated with anti-CD8 or control isotype antibodies. Subsequently, E0771 cells were orthotopically grafted, and mice treated with STO-609 or vehicle. Tumor volumes were measured (mean ± SEM; N = 3, 7, and 8 in isotype/Veh, CD8/Veh and CD8/STO groups, respectively). Two-way ANOVA test was used to calculate p-values. Asterisks refer to *p < 0.05.

Deletion of Camkk2 facilitates a significant increase in the expression of genes associated with interferon responses (e.g., Cxcl10) and with an M1-like phenotype (e.g., Nos2 and CD86). Besides its effect on chemokines and cytokine gene expression, genetic deletion of Camkk2 in BMDM is also associated with decreased expression of several genes that regulate cholesterol biosynthesis/homeostasis (Hsd17b7, Dhc24, Sdq1, Cyp51, and Msmo1). This is interesting in light of our previously published work demonstrating that mammary tumor growth was dramatically attenuated by inhibition of the CYP27A1-dependent production of the cholesterol metabolite 27-hydroxycholesterol. Defining the specific links between Camkk2, cholesterol, and tumor biology is a current research priority. Finally, despite the finding that Camkk2 depletion has a positive impact on BMDM (e.g., enhanced T cell attracting ability and reduced capability to inhibit T cell proliferation), other myeloid cells within TME also express Camkk2 and thus may also contribute to the tumor growth inhibition observed in Camkk2-ablated hosts.

Methods
All of the studies involving the use of animals were conducted after prior approval by the Duke or University of Illinois at Urbana-Champaign Institutional Animal Care and Use Committee (IACUC).

Mice. The Tg(Camkk2-EGFP)DF129Gsat reporter mice were originally provided by the Mutant Mouse Regional Resource Center (MMRRC). Transgenic strains contain the coding sequence for enhanced green fluorescent protein (EGFP), followed by a polyadenylation signal, inserted into the mouse genomic
bacterial artificial chromosome RP23-3J124 at the ATG transcription initiation codon of the calcium/calmodulin-dependent protein kinase kinase 2 β (Camkk2) gene so that expression of the reporter gene is driven by the promotor sequences of the mouse gene. Tg(Camkk2-EGFP)/BL6/Gat mice were generated by backcrossing Tg(Camkk2-EGFP)DF129Gat mice to C57BL/6 mice for ten generations. C57BL/6 mice were purchased from the Jackson Laboratory (CA, USA). Wild-type (WT) and Camkk2-EGFP mice have been described previously49. LysMcCre and intermate control LysMcCre + Camkk2loxP mice were generated by crossing B6.129P2-Lyz2tm1(cre)Ifo/J mice from the Jackson Laboratory with Camkk2loxP mice55. Mice were backcrossed to C57BL/6 at least four times during their generation. Genotypes were confirmed by PCR. OTI EGFP mice were generated by crossing OTI EGFP/+ mice with C57BL/6-Tg(EGFP)Oibl (both strains were acquired from The Jackson Laboratories, Bar Harbor, ME, USA). All the mice were used between 8–16 weeks, with gender and age-matched mice used in experimental and control groups. The animals were housed in Duke University animal facilities under a 12-h light/12-h dark cycle with food and water ad libitum. In all, 8–12 week female mice were included in the experiments.

Human tissues. We used tissue microarrays (TMA) that included duplicate 1 mm cores of formalin-fixed, paraffin-embedded primary human breast carcinomas from a group of 47 interpretable tumors from Vienna, Austria46, and 68 samples from Roswell Park Cancer Institute (RPCI), NY47. For all, tumors, grade and ER/PR/HER2 biomarker data were available. Samples were acquired in compliance with ethical regulations for work with human participant and informed consent policy. A set of primary human breast cancers (from the corresponding Institutional Review Boards (Compliance with Hospital Wiener Neustadt, Austria for the Vienna data set and the Roswell Park Cancer Institute for the RPCI data set). Patient consents were obtained before the collection of tissues.

CamKK2 IHC analysis. TMA sections were deparaffinized, treated with sub-boiling antigen retrieval buffer (citrate, pH 6, Abcam, ab93678) for 20 min, and then reacted with previously validated anti-CamKK2 rabbit monoclonal antibody (1:1000 dilution, clones D24 and D25) each at a concentration of 1 mg ml⁻¹. The detection reagent utilized the rabbit Envision kit from Dako (DakoCytomation, cat. no. K4004). Diaminobenzide (DAB) was used as the chromogen, with hematoxylin as the counter-stain. The IHC experiments were performed on an automated immunostainer (Intellipath from Biocare). Paraffin-embedded blocks of THP-1 cells served as external positive controls. Positive cells showed granular cytoplasmic reactivity. Two board certified pathologists (J.G. and A.H.) performed all analyses, including cell-type identification and staining intensity. Macrophages, endothelial cells, and lymphocytes were identified by morphology. Staining intensity in tumor cells was scored as 0 (absent), 0.5 (borderline), 1 (weak), 2 (moderate), or 3 (strong). For statistical analysis, the tumors were categorized as weak (0, 0.5, 1) or expressed (≥2). Ordinal logistic regression was used for binary outcomes, and proportional odds regression was used for grade and molecular class (TN, LA, and LB). There were only a small number of HER2-positive breast cancers in the two cohorts, which were excluded from this analysis. Score was modeled as a binary predictor with less than 1% of cells expressing. For outcomes with low cell counts, each investigator's exact test of association was used. Analyses were conducted in SAS Version 9.3 (SAS Institute, Cary, NC) and the R environment for statistical computing.

Murine mammary tumors. MMTV-PyMT grafts: MMTV-PyMT mice were backcrossed onto a C57BL/6 background61. Resulting mammary tumors were excised, washed in PBS, and diced into equal sized pieces. Tumor pieces were orthografted into the mammary fat pads of WT or Camkk2-/− mice. Resulting tumor growth was assessed by direct caliper measurement.

Flow cytometry. Cell suspension (5 × 10⁶ cells) from dissected tumors, or BMDM (1 × 10⁶), were stained, with antibodies and fixative viability dye listed in Supplementary Table 2, according to the manufacturer’s instructions. The stained cells were analyzed using a BD FACs Canto II (BD, NJ, USA). The data were analyzed using FlowJo (TreeStar, OR, USA).

Microarray analysis. WT and Camkk2-/− BMDMs were generated with L929-conditioned media (30%) in the presence of RM or TC (25%). Four biological replicates for each genotype were generated using long bones of 16 WT and 16 Camkk2-/− female mice (10–12 weeks old). Microarray analysis was performed at Sequencing and Genome Technologies Shared Resource (Duke University). Transcriptome Analysis Console (TAC) Software (TAC Software version 4.0; Advatia Bioinformatics) was used for pathway analysis. PathwayGuide scores pathways using the over-representation of differentially expressed (DE) genes in a given pathway and the perturbation of that pathway computed by propagating the measured expression across the pathway. These algorithms yield two independent probability values, pORA and pAcc, that are then combined to generate the pathway-specific p-value, which is calculated with Fisher’s method with a different investigator using a different batch of STO-609 (Tocris Biosciences, Bristol, UK). Treatment with GSKi was initiated at post-graft day 2.

Murine tumor dissection. Tumors were removed from mice and minced with a surgical scalpel. Minced tissues were added to 5 ml of HBSS containing CaCl₂ and MgCl₂ (ThermoFisherScientific, MA, USA), and supplemented with collagenase 2 mg ml⁻¹ and DNase 1.0 mg ml⁻¹ (Roche, Basel, Switzerland). Subsequently, they were transferred to gentle MACS C tubes, dissected with gentle MACS dissociator (Miltenyi, Bergisch Gladbach, Germany), and incubated for 45 min at 37 °C. At the end of this dissection, tumors were digested again with gentle MACS dissociator, and cell suspensions passed through 70-μm cell strainers (Corning, NY, USA), washed twice in PBS w/2% FBS, and used for FACS analysis.

Flow cytometry. Cell suspension (5 × 10⁶ cells) from dissected tumors, or BMDM (1 × 10⁶), were stained, with antibodies and fixative viability dye listed in Supplementary Table 2, according to the manufacturer’s instructions. The stained cells were analyzed using a BD FACs Canto II (BD, NJ, USA). The data were analyzed using FlowJo (TreeStar, OR, USA).

CD8⁺ cell depletion. To deplete CD8⁺ cells, mice were injected intraperitoneally with 200 μl of anti-mouse CD8 monoclonal antibody (YTS 169.4) or its IgG control isotype (clone LTF-2; catalog #: BE0090, BioXCell, NH, USA) each at a concentration of 1 mg ml⁻¹ according with regime shown in Supplementary Fig. 4A. Tg(MaC) E0771 was generated by M. Weil. The effect of CD8 depletion was monitored by flow cytometry using 50 μl of peripheral blood and stained with APC-anti-mouse CD8 (6F10) and APC-Cy7-anti mouse CD45.2 (clone 104; BioLegend).

L929-differentiation medium and E0771 tumor-conditioned medium (TCM). L929 cells were acquired from Duke Cell Culture Facility and cultured in the DMEM (Gibco, MA, USA) supplemented with 2 mM L-glutamine, 1.0 mM sodium pyruvate, and 10% fetal bovine serum (FBS, Hyclone, MA, USA). Cells were maintained in a humidified 37 °C CO2 incubator and passed at 70% confluence. To generate conditioned medium, media was removed from 70% confluent cultures and replaced with fresh media. After 3 days, supernatates were collected and aliquots stored at −80 °C.

E0771 mouse breast cancer cells were cultured in RPMI-1640 (Gibco, MA, USA) supplemented with 2 mM L-glutamine, 1.5 g L⁻¹ sodium bicarbonate, 1.0 mM sodium pyruvate (all from Gibco, MA, USA), and 8% fetal bovine serum (FBS, Hyclone, MA, USA). Cells were maintained in a humidified 37 °C CO2 incubator and passed at 70% confluence. To generate conditioned medium, media was removed from 70% confluent cultures and replaced with fresh media. After 3 days, culture supernates were collected and aliquots stored at −80 °C. Cytokines in conditioned media were analyzed as described below.
Bonferroni correction. Microarray data are available at GEO (accession number: GSE160357).

**Gene set enrichment analysis.** Gene set enrichment analysis (GSEA) was applied to our microarray data. All genes were ranked by the fold-change between the Camkk2 null and WT control samples. Normalized Enrichment Score (NES) and adjusted q-values were computed using the GSEA method, based on 1000 random permutations of the ranked gene sets. Gene sets collected (MsigDB, http://www.broadinstitute.org/gezi/misgdb/) were used to determine enriched pathways.

**Real-time quantitative RT-PCR assay.** RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Quantitative PCR was performed using iQ SYBR Green Supermix (Bio-Rad, CA, USA) with respective primers and cDNA. The assay was run on the CFX96 Real-Time System (Bio-Rad, CA, USA). The primer sequences are listed in the Supplementary Table 3.

**Immunoblot.** BMDM were washed three times with 2 mL of cold-ice PBS and lysed with 0.15 mL of M-PER mammalian protein extraction reagent with Halt protease and phosphatase inhibitors (Thermo Scientific). Equal amounts of protein per sample/lanes were denatured and resolved by SDS-PAGE. Proteins were transferred to Immobilon-FL membranes (Millipore, Billerica, MA), and quantitative immunoblotting was performed using the Odyssey infrared immunoblotting detection system (LI-COR Biosciences, Lincoln, NE). Primary antibodies used were anti-CamKII (AbCam, Cambridge, MA), anti-CaMKK2 (AbCam, Cambridge, MA), anti-Kv1.3 (AbCam, Cambridge, MA), anti-CaMKK1 (ThermoFisher, catalog # PA5-38343) at 1:1000 dilution, and anti-actin (AC-40, catalog # A4700; Sigma-Aldrich; dilution 1:20000); anti-phospho-CaMKK1 (ThermoFisher; catalog # 2772), anti-phospho-AMPK (ThermoFisher; catalog # 4471) at 1:1000 dilution and AMPKα (F6, catalog # 2793; dilution 1:1000) were from Cell Signaling (Danvers, MA). Secondary antibodies used were anti-mouse IgG (Rockland, catalog # 605-105-121; Invitrogen; dilution 1:2000) and anti-rabbit IgG (Rockland, catalog # 61-112-002; Rockland Immunochemicals, Gilbertsville, PA; dilution 1:5000). All antibodies were used according to the manufacturer’s instructions. Images of uncult and unmunaptated blots are presented in Supplementary Fig. 12.

**T cell functional assays.** T cells were enriched from the spleen of C57BL/6J mice using the CDS® T Cell Isolation Kit II (Miltenyi, Bergisch Gladbach, Germany; catalog # 130-095-130). CD4+ and CD8+ T cells were purified by negative selection using EasySep CD4 and CD8 isolation kits (Stemcell Technology, Cambridge, MA; catalog # 19752 and 19753, respectively). Flow cytometry was used to establish degree of purity. After two washings in PBS with 2% FBS and 2 mM EDTA, 1 × 107 T cells were re-suspended in 1 mL of PBS with 2 μg/mL CFSE (CellTrace CFSE Cell Proliferation Kit, Invitrogen; catalog # C34554) and incubated at 37 °C, 5% CO2 for 20 min. In all, 35 μL of RPMI with 10% FBS were subsequently added to the cells and incubated for an additional 10 min. Subsequently, T cells were washed twice in PBS with 2% FBS and 2 mM EDTA. Cell numbers were determined and 1 × 106 CFSE-labeled T cells in 100 μL were added to 18-well plates (Corning). The plates were incubated with 200 μL of complete media in the presence or absence of 10 ng/ml purified NA/LE hamster anti-mouse CD3e (clone 145-2C11; BD Biosciences). T cell proliferation was measured at 72 h by flow cytometry. This experiment was replicated using purified CD4+ and CD8+ T cells from WT and Camkk2−/− BMDM were generated in the presence of TCM. The immunodulatory activity of these supernatants was tested on CD4+ and CD8+ T cells. To this purpose, 1 × 106 CFSE-labeled CD4+ or CD8+ T cells were seeded into flat-bottom 96-well plates (Corning) with 200 μL of complete media in the presence or absence of 10 ng/ml purified NA/LE hamster anti-mouse CD3e (clone 145-2C11; BD Biosciences). After 24 h, supernates were collected for cytokine detection. T cell proliferation was measured at 72 h by flow cytometry. This experiment was replicated using purified CD4+ and CD8+ T cells from WT and Camkk2−/− BMDM were generated in the presence of TCM. The immune-modulatory activity of these supernatants was tested on CD4+ and CD8+ T cells. To this purpose, 1 × 106 CFSE-labeled CD4+ or CD8+ T cells were seeded into 96-well plates (Corning) with 200 μL of complete media with an optimal number of Dynabeads Mouse T-Activator CD3/CD28 (ThermoFisher; catalog # 114564; bead/T cell ratio 1:1) in the presence or absence of TCM-BMDM supernates. T cell proliferation was measured at 72 h by flow cytometry. T cells isolated from lymph nodes of WT mice were activated for 48 days using a用人 (IHC)-type (3,3′-diaminobenzidine (DAB) substrate) vector (Vector Laboratories, catalog # SK-4010). To identify macrophages, sections were stained with an antibody against the macrophage marker iNOS (Abcam; catalog # ab2696; dilution 1:500) or anti-CD31 antibody (Abcam; catalog # ab134168; dilution 1:150) at 4 °C overnight. Sections were then washed and incubated with biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA; catalog # BA-1000; dilution 1:200) for 1 h, and subsequently with avidin–biotin peroxidase (Vectorstain Elite ABC kit; Vector Laboratories; Burlingame, CA; catalog # PK-4010) for 1 h. Color was developed using 3,3′-diaminobenzidine (DAB) substrate (Vector Laboratories, catalog # SK-4010). To identify macrophages, sections were stained with a rabbit polyclonal antibody against mouse iNOS (Abcam; catalog # ab1514; dilution 1:2000) at 4 °C overnight. Sections were then washed and incubated with biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA; catalog # BA-1000; dilution 1:200) for 1 h, and subsequently with avidin–biotin peroxidase (Vectorstain Elite ABC kit; Vector Laboratories; Burlingame, CA; catalog # PK-4010) for 1 h. Color was developed using 3,3′-diaminobenzidine (DAB) substrate (Vector Laboratories, catalog # SK-4010). Hematoxylin and eosin stain (Vector Laboratories; catalog # H-3502) and Trichrome Stain (Electron Microscopy Sciences, Hatfield, PA, USA; cat# 1137) were used to the recall stained sections. Images were acquired using Olympus BX51WI upright microscope using a x4, x20, or x40 objective. Images were quantified with ITCN plug-in for the ImageJ software.

**Statistics.** Values were assessed for normality and where appropriate either in-transformed or a non-parametric test was selected. Tests used to calculate p-values are reported in the figure legends. Graphpad Prism was used for analysis unless otherwise stated. Statistical approaches for Table 1 and Supplementary Table 1 have been described elsewhere. NATURE COMMUNICATIONS | https://doi.org/10.1038/s41467-019-10424-5 | www.nature.com/naturecommunications
treated mice/samples and single-blind assessment of the experimental results were used.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

The Microarray data have been deposited in the GEO database under the accession code GSE106357. All the other data supporting the findings of this study are available within the article and its supplementary information files and from the corresponding authors [LR, DPM] upon reasonable request.

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**Tables**

1. **Table 1.** Summary of references.

**Figures**

1. **Figure 1.** Schematic representation of the experimental design.

**Supplementary information**

1. **Supplementary Figure 1.** Representative images of tumor sections stained with anti-CD8 antibody.

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**Declaration of interests**

1. The authors declare that they have no competing interests.

**Conflict of interest**

1. The authors declare that there is no conflict of interest.

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**Abbreviations**

1. **AMPK**: 5′-AMP-activated protein kinase
2. **CaMKK2**: Calcium/calmodulin-dependent protein kinase kinase 2
3. **CaMK**: Calcium/calmodulin-dependent protein kinase
4. **CaM**: Calmodulin
5. **IL-10**: Interleukin-10
6. **CD8+**: CD8-positive
7. **CaM**: Calcium/calmodulin
8. **CaM**: Calcium/calmodulin
9. **CaM**: Calcium/calmodulin
10. **CaM**: Calcium/calmodulin
11. **CaM**: Calcium/calmodulin
12. **CaM**: Calcium/calmodulin
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Author contributions
L.R., E.R.N., W.H., N.C., D.M., C.y.C. and D.P.M. conceived and designed the experiments; L.R., E.R.N., W.H., S.A.L., W.L., Y.J., S.P., A.E.B., D.M., Y.L., A.M.M. and F.R.B. carried out the experiments; D.H.D., W.J.Z., A.R.M. and B.Y. developed and characterized CaMKK2 inhibitors; A.M.M. carried out the bioinformatics analysis; J.G. and A.H. performed analysis on IHC; L.R., E.R.N., D.M., C.y.C., N.C., A.R.M. and D.P.M. analyzed and interpreted the data; L.R., D.M., C.y.C. and D.P.M. wrote the paper.

Additional information
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Competing interests: The authors declare the following competing interests: L.R., E.R.N., W.H., N.C. and D.P.M. have applied for a patent covering the use of CaMKK2 inhibitors alone or in combination with immunotherapy for the treatment of cancer. Title: “CaMKK2 inhibitor compositions and methods of using the same.” Racioppi, L., Nelson, E.R., Huang, W., Chao, N. and McDonnell, D.P. Provisional Patent Application No.: 62/ 371,309; August 5, 2016. The remaining authors declare no competing financial interests.

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