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PKC agonism restricts innate immune suppression, promotes antigen cross-presentation and synergizes with agonistic CD40 antibody therapy to activate CD8+ T cells in breast cancer

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Abstract

Myeloid-derived suppressor cells (MDSCs) are an immature innate cell population that expands in pathological conditions such as cancer and suppresses T cells via production of immunosuppressive factors. Conversely, efficient cytotoxic T cell priming is dependent on the ability of antigen-presenting cells (APCs) to cross-present tumor antigens to $CD8^+$ T cells, a process that requires a specific subtype of dendritic cells (DCs) called conventional DC1 (cDC1) which are often dysfunctional in cancer. One way to activate cDC1 is ligation of

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2022.01.017.

CD40 which is abundantly expressed by myeloid cells and its agonism leads to myeloid cell activation. Thus, targeting MDSCs while simultaneously expanding cross-presenting DCs represents a promising strategy that, when combined with agonistic CD40, may result in long-lasting protective immunity. In this study, we investigated the effect of PKC agonists PEP005 and prostratin on MDSC expansion, differentiation, and recruitment to the tumor microenvironment. Our findings demonstrate that PKC agonists decreased MDSC expansion from hematopoietic progenitors and induced M-MDSC differentiation to an APC-like phenotype that expresses cDC1-related markers *via* activation of the p38 mitogen-activated protein kinase (MAPK) pathway. Simultaneously, PKC agonists favored cDC1 expansion at the expense of cDC2 and plasmacytoid DCs (pDC). Functionally, PKC agonists blunted MDSC suppressive activity and enhanced MDSC cross-priming capacity both *in vitro* and *in vivo*. Finally, combination of PKC agonism with agonistic CD40 mAb resulted in a marked reduction in tumor growth with a significant increase in intratumoral activated $CD8^+$ T cells and tissue-resident memory $CD8^+$ T cells in a syngeneic breast cancer mouse model. In sum, this work proposes a novel promising strategy to simultaneously target MDSCs and promote APC function that may have highly impactful clinical relevance in cancer patients.

Keywords

MDSC; DC; Cross-presentation; CD40; PKC

1. Introduction

Immunotherapies that reinvigorate T cell responses such as immune checkpoint blockade (ICB) have revolutionized cancer therapy showing unprecedented long-term antitumor responses [1–3]. However, most patients do not respond to immunotherapies due at least partly to immunosuppression [4–6]. Immunotherapy non-responders have high levels of circulating myeloid-derived suppressor cells (MDSCs)- an immunosuppressive innate cell population that expands under pathological conditions such as cancer. MDSCs are heterogenous and comprise two subtypes that have been identified in both mice and humans: polymorphonuclear MDSCs (PMN-MDSCs) and monocytic MDSCs (M-MDSCs) [7,8]. Although there are currently no approved therapeutic agents that specifically target MDSCs, novel molecular targets that modulate MDSC biology are under active investigation.

MDSCs suppress T cells in the tumor microenvironment (TME) *via* production of immunosuppressive factors such as inducible nitric oxide synthase (iNOS) and arginase 1 [9]. Although PMN-MDSCs represent more than 70% of total MDSCs in peripheral lymphoid organs, the proportion of M-MDSCs in tumors is substantially higher [10]. In addition, M-MDSCs are more suppressive than PMN-MDSCs when compared on a per cell basis [11] which indicates that specific targeting of M-MDSCs may present high translational relevance in cancer patients.

In concert with effector T cells, conventional dendritic cells (cDCs), among other DC subtypes, perform a preferential activation of T cells [12], which is the foundation of the “cancer-immunity cycle”. cDCs can be divided into two different subsets: cDC1

and cDC2 [13,14]. cDC1s depend on the transcription factors *Irf8*, *Batf3*, and *Id2* for their development and express CD103 in mice or CD141 in humans [15]. cDC1s are essential for $CD8^+$ T cell priming and are often dysfunctional in most solid tumors [16]. Although cDC1s arise from DC precursors, recent studies described novel DC subsets that phenotypically, transcriptionally, and functionally resemble cDC1s but do not arise from DC precursors [17,18]. Instead, these cDC1-like cells differentiate from M-MDSCs or monocytic precursors and efficiently cross-prime $CD8^+$ T cells [18]. Moreover, CD40 is abundantly expressed by myeloid cells and its agonism leads to myeloid cell activation. For example, combination of cDC1 generating agents such as FMS-like tyrosine kinase 3 ligand (FLT3L) and agonistic CD40 monoclonal antibodies (mAb) resulted in improved antitumor immunity [19]. Contrary to MDSCs, cDC1s associate with improved prognosis and responsiveness to immunotherapies in patients with cancer [20]. Hence, inducing MDSC differentiation to DC-like cells while simultaneously expanding cross-presenting DCs represents a promising strategy that if combined with agonistic CD40 mAb as an antigen-presenting cell (APC) activating agent, may result in an effective antitumor immune response.

One pathway that may target both MDSCs and DCs to promote APC function is the agonism of the Protein Kinase C (PKC) family. PKC iso-zymes play a critical role in cell signaling and differentiation [21–23]. PKC family comprises 11 different isoforms: α, βI and βII, γ, δ, ε, η, θ, ζ and ι [24]. PKC agonists activate all conventional (α, βI, βII, γ) and novel (δ, ε, η, θ) PKC isoforms by binding to the commonly shared C1b domain [25]. Previous studies show a differentiating effect of PKC agonists in acute myeloid leukemia (AML) [25–27], but their role in MDSC/DC differentiation is unknown. In this study, using *in vitro*, *ex vivo*, and an *in vivo* breast cancer syngeneic mouse model, we show that pharmacological activation of protein kinase C (PKC) using PEP005 (ingenol mebutate) or prostratin decreased MDSC expansion from hematopoietic progenitors in the bone marrow (BM). PKC agonists also induced M-MDSC differentiation to an APC-like phenotype that expresses cDC1-related markers and the transcription factor *Irf8*, but not *Batf3*. Simultaneously, PKC agonists favored cDC1 expansion at the expense of cDC2 and plasmacytoid DCs (pDC). Functionally, PKC agonists blunted MDSC suppressive function of T cells and promoted MDSC cross-priming capacity. Finally, combination of PKC agonism with agonistic CD40 mAb resulted in a marked reduction in tumor growth. Combination therapy synergistically increased intratumoral activated $CD8^+$ T cells and tissue-resident memory $CD8^+$ T cells. In sum, we propose a novel promising strategy to simultaneously target MDSCs and promote APC function that may have potential clinical relevance in cancer patients.

2. Material and methods

2.1. Mice

Animal studies were performed with approval and in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) at the University of Tennessee Health Science Center and in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All animals were housed in a temperature-controlled facility with a 12-h light/dark cycle and *ad libitium* access to food and water. C57BL/6J

(CD45.2$^+$, Stock No: 000664) and congenic CD45.1$^+$ C57BL/6J (Stock No: 002014) mice were purchased from Jackson Laboratories or bred in-house. For OT-I CD8$^+$ T cell studies, spleens from transgenic mice expressing the MHC-I restricted T cell receptor specific for the octamer SIINFEKL peptide ovalbumin$_{257–264}$ (OT-I mice) were a kind gift from Dr. Hongbo Chi at St Jude Children's Research Hospital, Memphis TN. See Supplementary Methods for additional information.

2.2. Orthotopic tumor cell implantation and treatments

E0771-luciferase (luc), a kind gift from Dr. Hasan Korkaya, Augusta University, murine adenocarcinoma breast cancer cell line was originally isolated from a spontaneous tumor from C57BL/6 mouse. Cells were cultured and injected as we previously described [28]. Briefly, cells were cultured in RPMI containing 10% FBS, 100 UI/mL of penicillin, and (100 μg/ml) streptomycin in a humidified chamber at 37 °C under 5% CO_2. E0771-luc cells were injected in the left fourth mammary fat pad of 8-week-old C57BL/6J females at 250,000 cells in 100 μl of 25% Matrigel. Tumor growth was monitored by measuring the length and width of the tumor using digital calipers. Tumor volume was calculated using the following formula: Volume = $(\text{width})^2 \times (\text{length})/2$ [29]. At endpoint, excised tumor mass was determined.

PEP005 (PEP) was dissolved in DMSO and administered intratumorally at the indicated doses (in 100 μl PBS with final DMSO concentration <0.5%). Mice were sacrificed at times indicated or in survival study (Fig. 1F) allowed to progress until IACUC determined endpoint. Agonistic CD40 mAb (clone FGK45) or its isotype control (IgG2a-clone 2A3) were purchased from BioXCell (West Lebanon, NH) and administered via intraperitoneal (i.p. 100μg/mouse) injection on days 9 and 17 as a monotherapy or in combination with PEP.

2.3. Preparation of single cell suspensions and flow cytometry analysis

Excised tumors (~500 mg) were minced using scissors in RPMI media containing enzyme cocktail mix from Miltenyi Biotec mouse tumor dissociation kit (Miltenyi Biotec, Auburn, CA) and flow cytometry was performed as described in our previous study [28]. Detailed methodology can be found in the methods section in the supplementary file.

2.4. CD8$^+$ T cell ex vivo suppression assay

CD8$^+$ T cells and MDSCs were isolated as described above. Isolated MDSCs were pretreated with PKC agonists for 3h and plated in complete RPMI media in round bottom 96-well plates at the indicated ratios. Isolated CD8$^+$ T cells were labeled with CellTrace Violet Cell Proliferation Dye (CTV, Thermo Fisher) and were added to MDSCs (5×10^4 CD8$^+$ T cells/well). Mouse T-Activator CD3/CD28 Dynabeads (Thermo Fisher) were added to culture wells according to manufacturer's protocol and cultures were incubated for 72h at 37 °C. Control wells consisted of stimulated CD8$^+$ T cells alone (positive control) and unstimulated CD8$^+$ T cells alone (negative control). Four hours prior to the end of the incubation time, Brefeldin A (Sigma) was added to cultures to allow accumulation of intracellular cytokines before cell surface and intracellular flow cytometry staining.

2.5. RNA isolation and real time PCR analysis

Total RNA was isolated using the RNeasy Mini Kit (Qiagen) according to manufacturer's instructions. cDNA synthesis and real time quantitative PCR (qRT-PCR) was performed as previously described [30].

2.6. Statistics

Statistical differences between experimental groups were determined by unpaired Student's t-tests or one-way ANOVA with Tukey correction for multiple comparisons using statistical software within GraphPad Prism (GraphPad Software, Inc., La Jolla, CA). All data are shown as mean ± standard error of the mean (SEM). For survival curves, log-rank (Mantel-Cox) test was performed. P values less than 0.05 were considered statistically significant.

3. Results

3.1. PKC agonism suppresses tumor growth, increases cDC1, and reduces M-MDSC abundance in E0771 tumor-bearing mice

PKC agonists are differentiating agents in acute myeloid leukemia (AML) [25–27]. To determine the functional consequences of PKC agonists on breast cancer tumor immunity, we first characterized the diversity of tumor-infiltrating immune cells in the spleens and tumors of E0771 tumor-bearing and tumor-free mice. Murine breast cancer cell line E0771 was orthotopically implanted into the fourth mammary fat pad of age matched C57BL/6J female mice. Immune cell composition of the spleens or tumors was quantified 14 days after injection. Flow cytometric analysis of tumors revealed that M-MDSCs ($Ly6C^{hi}$ $Ly6G^{-}$) and PMN-MDSCs ($Ly6C^{lo}$ $Ly6G^{+}$) together accounted for more than 55% of tumor-infiltrating $CD45^{+}$ cells (Fig. 1A and B). M-MDSCs were more abundant than PMN-MDSCs representing 48% and 24% of $CD11b^{+}$ tumor-infiltrating cells, respectively (Fig. 1C), suggesting an intratumoral enrichment in M-MDSCs, which is consistent with previous reports [31]. As expected, we observed a significant expansion of both M-MDSCs and PMN-MDSCs in the spleens of tumor-bearing mice compared to tumor-free mice (Figs. S1A–C). Macrophages, DCs, $CD8^{+}$ T cells, $CD4^{+}$ T cells, and natural killer (NK) cells altogether accounted for no more than 15% of tumor infiltrating $CD45^{+}$ cells (Fig. 1A and B).

To test if PKC agonism impacts breast cancer outcomes, E0771 tumor-bearing mice were treated with PKC agonist PEP intratumorally on days 9, 10 and 11 post-cancer cell implantation. PEP induced a significant decrease in tumor volume (Fig. 1D) and tumor weight (Fig. 1E), and a significantly longer survival (Fig. 1F) compared to vehicle (Veh)-treated mice. PEP did not induce significant changes in body weights (Fig. S1D). To investigate PEP impacts on the TME and systemic immunity, flow cytometry analysis of bone marrow, spleen, and tumor revealed a significant decrease in M-MDSC frequencies (Fig. 1G), but not PMN-MDSCs (Fig. S1E). $CD45^{+}$ cells were significantly decreased by PEP in tumors (Fig. S1F), but no significant changes in total macrophages (Fig. S1G), $MHCII^{hi}$, or $MHCII^{lo}$ (Figs. S1H–I) macrophage subtypes were detected. However, PEP significantly increased intratumoral natural killer (NK) cells (Fig. S1J). Last, PEP significantly increased tumor cDC1 but not cDC2 frequencies (Fig. 1H). To investigate

PEP's direct impact on cancer cell viability, E0771 cells were treated with different concentrations of PEP *in vitro*. Although PEP did not have a significant cytotoxic effect on E0771 cells at 24- and 48-h timepoints (Figs. S2A–B), the highest PEP concentration (2 μM) decreased cancer cell viability by 50% (Fig. S2C). Our findings suggest that although PEP's direct effects on cancer cell viability cannot be excluded, PEP may directly affect M-MDSC and cDC1 differentiation, recruitment, and/or function.

3.2. PKC agonism blunts M-MDSC expansion, migration and alters MDSC suppressive function

MDSCs expand from myeloid progenitors in response to granulocyte-macrophage colony-stimulating factor (GM-CSF) and factors released by tumors [32]. To determine the impact of PKC agonists on MDSC expansion *in vitro*, isolated BM cells were cultured with GM-CSF and E0771 conditioned media (CM) in the presence of Veh, PEP, or another PKC agonist prostratin (Pro) at day 0 (Fig. 2A). $CD11b^+$ $Gr\text{-}1^+$ cell frequencies and absolute cell numbers at day 4 were significantly decreased by PEP (Fig. 2B–D) and Pro (Fig. S3A). $CD11b^+$ $Gr\text{-}1^+$ cells generated *in vitro* were confirmed to be $Ly6C^{hi}$ $Ly6G^-$ M-MDSCs (data not shown). PEP and Pro also significantly increased the frequency of $CD11c^+$ cells compared to Veh (Fig. S3B) suggesting that PKC agonists do not appear to broadly impair normal hematopoiesis. M-MDSCs are recruited to the TME when monocyte chemoattractant protein-1 (MCP-1) binds to its receptor *Ccr2* [11]. PEP ablated expression of *Ccr2* in M-MDSCs isolated from the spleens of tumor-bearing mice (sM-MDSCs) *in vitro* suggestive of impaired migration in response to PKC agonism (Fig. 2E).

To examine the suppressive function of PKC agonism on MDSCs directly and to rule out any direct effect of PEP on T cells, sMDSCs were isolated from tumor-bearing mice and pre-treated with PEP for 3h before co-culture with cell trace violet (CTV)-labeled $CD8^+$ T cells. $CD8^+$ T cells were isolated from the spleen of tumor-free mice and were co-cultured with sMDSCs for 72h in the presence of anti-CD3/CD28 Dynabeads. CTV and interferon gamma (IFNγ) [33] were quantified to measure T cell proliferation and activation. sMDSCs treated with Veh efficiently suppressed $CD8^+$ T cell activation compared to stimulated $CD8^+$ T cells cultured alone. PEP-treated sMDSCs significantly decreased MDSC suppression as evidenced by a 3-fold increase in $CD8^+$ T cell activation compared to Veh (Fig. 2F and G). To determine which subset of MDSCs was most affected by PEP, CTV-labeled $CD8^+$ T cells were cocultured with sM-MDSCs and PMN-MDSCs (sPMN-MDSCs) isolated from tumor-bearing mice that were pre-treated *in vitro* with PEP. PEP-treated sPMN-MDSCs showed a marginal increase in $CD8^+$ T cell proliferation compared to Veh-treated sPMN-MDSCs (Fig. 2H). However, PEP-treated sM-MDSCs significantly increased $CD8^+$ T cell proliferation compared to Veh (Fig. 2I). Together, our data suggest that PKC agonism blunts MDSC expansion and migration and by downregulation of *Ccr2* and subverts MDSC suppressive function.

3.3. PKC agonism skews M-MDSC differentiation towards an APC-like phenotype via activation of the p38 MAPK pathway

To determine if PKC agonism drives MDSC differentiation to a more mature APC phenotype, sMDSCs ($CD11b^+$ $Gr\text{-}1^+$) were treated *ex vivo* with Veh, PEP, or Pro in

the presence of GM-CSF and E0771 CM for 6 days. PKC agonist-treated sMDSCs differentiated into CD103-expressing DC-like cells (Fig. S4) as evidenced by a significant decrease in the frequency of M-MDSCs (Fig. S4A) and a significant increase in the frequency of $CD103^+$ DC-like cells (Fig. S4B) compared to Veh. Moreover, MHCII expression was increased in M-MDSCs with both PEP and Pro treatments (Figs. S4C–D). Of note, PMN-MDSCs were not detected after 6 days of culture (data not shown). To investigate if MDSC/DC phenotype shifts detected *in vitro* were evident *in vivo*, sMDSCs were labeled with CTV and were adoptively transferred into tumor-bearing recipient mice treated with Veh or PEP (Fig. 3A). Consistent with *in vitro* findings, a 2.5-fold increase in the frequency of $CD103^+$ DC-like cells (Fig. 3B) and a decrease in M-MDSC frequency (Fig. 3C) was quantified after PEP treatment compared to Veh. There was also a significantly higher $CD103^+$ DC-like to M-MDSC ratio with PEP compared to Veh (Fig. 3F) while no significant changes in $CD11b^+$ (Fig. 3D) or macrophage frequencies (Fig. 3E) were noted. In addition, CTV^+ sMDSCs from the PEP group displayed fewer $CD11b^+$ $Gr\text{-}1^+$ cells (Fig. S5A) with higher levels of MHCII (Fig. S5B) compared to the Veh group. tSNE plots showed that MHCII was mainly expressed by $CD103^+$ DC-like cells, but not $CD11b^+$ $Gr\text{-}1^+$ cells (Figs. S5A–B).

Since PKC agonism increased sMDSC expression of CD103, which is an integrin primarily expressed by cross-presenting cDC1s [34], expression of other DC markers by sMDSCs ($Ly6G^-$ $Ly6C^{hi}$) was next examined after treatment with PEP *ex vivo*. Consistently, decreased expression of the monocytic marker Ly6C in sMDSCs was quantified after PEP (Fig. 3G). Remarkably, there was increased expression of DC markers [34] CD11c, MHCII, co-stimulatory molecules CD86 and CD40, as well as cross-presenting DC related markers XCR1, CCR7 and CD103 [35] with PEP (Fig. 3G). Programmed death ligand 1 (PD-L1) can be upregulated on APCs upon exposure to inflammatory signals and is abundantly expressed by cross-presenting DCs [34]. PD-L1 expression was increased on sMDSCs upon treatment with PEP compared to Veh (Fig. 3G). PEP upregulated cDC1 transcription factor *Irf8* (Fig. 3H), but not *Batf3* (Fig. S4E). Last, both iNOS (Nos2) and arginase 1 (Arg1) mediate immunosuppressive functions of MDSCs [9]. PKC agonism significantly decreased *Nos2*, but not *Arg1* gene expression in sMDSCs (Figs. S4F–G).

PKC agonists have been reported to induce differentiation of AML blasts *via* p38 kinase and c-Jun N—terminal protein kinase (JNK) pathways [27,36]. p38 activation promotes maturation and cross-presentation by cDC1 and monocyte-derived DCs [37–39]. To investigate if p38 activation downstream of PKC mediates M-MDSC differentiation to APC-like cells, sM-MDSCs were treated with PEP *ex vivo* in the presence of p38 inhibitor SB203580 [40]. PEP increased phosphorylation of p38 (Fig. 3I) and increased the expression of CD11c and CD86 but decreased Ly6C expression (Fig. 3J–L). Pretreatment with p38 inhibitor before the addition of PEP partially abrogated PEP-mediated increases in CD11c and CD86 or decrease in Ly6C (Fig. 3J–L). No effect on M-MDSC differentiation to APC-like cells was observed using the JNK inhibitor P600125 [40] (Figs. S6A–C). These data indicate that p38 signaling by PKC agonism mediates skewing of M-MDSC differentiation to an APC-like phenotype that presents some phenotypic and transcriptional similarities with cross-presenting DCs.

3.4. PKC agonist-treated MDSCs mediate efficient CD8+ T cell cross-priming

To test whether PKC agonism could functionally enhance MDSC cross-presenting capacity, sMDSCs were pretreated with Veh or PEP, then loaded with ovalbumin (OVA) conjugated to Alexa Fluor 647. There was a significant increase in OVA uptake by sMDSCs treated with PEP (Fig. 4A). sMDSCs were next co-cultured with CTV-labeled $CD8^+$ T cells isolated from the spleens of OT-I mice to analyze cross-presentation capacity [41]. PEP treated OVA-loaded sMDSCs significantly enhanced $CD8^+$ T cell proliferation (Fig. 4B) and activation (Fig. 4C). To determine PEP's effect on cross-presentation *in vivo*, CTV-labeled OT-I $CD8^+$ T cells isolated from CD45.2 donor mice were adoptively transferred into congenic CD45.1 recipient mice on day −1 to allow the T cells to home in lymphoid organs. On day 0, sMDSCs that were pretreated *ex vivo* with Veh or PEP and loaded with OVA were adoptively transferred into the same recipient mice (schema, Fig. 4D). On day 3, flow cytometry analysis of the spleens showed that the PEP group had significantly higher OT-I $CD8^+$ $CD45.2^+$ T cells compared to the Veh group (Fig. 4E and F). OT-I $CD8^+$ T cells from the PEP-treated group displayed greater proliferation (Fig. 4G). Our findings suggest that PKC agonist-treated MDSCs can efficiently cross-prime $CD8^+$ T cells *in vitro* and *in vivo*.

3.5. PKC agonism favors hematopoietic progenitor commitment to cDC1 at the expense of cDC2 and pDC

In order to investigate whether the increase in cDC1 frequency observed *in vivo* with PEP treatment (Fig. 1G) is a direct effect on cDC1 generation, BM cells were cultured in the presence of FLT3L and either Veh, PEP, or Pro for 10 days (Fig. 5). PKC agonists yielded significantly greater cDC1 frequencies compared to Veh (Fig. 5A and B). In contrast, PKC agonists decreased the frequencies of cDC2 and pDC (Fig. 5C and D). These results suggest that PKC agonism has a direct effect on the generation of cDC1 from hematopoietic progenitors.

3.6. Combination of PKC agonism and agonistic CD40 mAb inhibits tumor growth and significantly increases intratumoral activated and tissue-resident memory CD8+ T cells

Immature APCs display poor priming capacity of T cells [34]. Therefore, to test the full impact of PEP's anti-tumor effects, additional maturation of APC pools induced by PEP is needed. CD40 is expressed on APCs and induces them to mature into highly functional APCs upon contact with its ligand [42]. The rationale for study design was that a single dose of PEP would induce MDSC differentiation to APC-like cells and recruit additional cDC1s to the tumor. Treatment with CD40 mAb would activate those newly developed APCs which subsequently results in the activation of T cells. A second dose of CD40 mAb would re-educate APCs to further activate T cells. Thus, agonistic CD40 mAb combined with PKC agonism was investigated as a therapeutic approach to blunt tumor growth and prime intratumoral $CD8^+$ T cells (schema in Fig. 6A). While CD40 agonism did not impair tumor proliferation or burden (Fig. 6B and C), and PEP impaired tumor growth as determined above, the combination CD40 mAb therapy + PEP resulted in a slight but not significantly improved inhibition of tumor progression (Fig. 6B) with marked reduction in tumor burden at endpoint (Fig. 6C). Single and combination therapies were well-tolerated with no changes in body weights (Fig. 6D). Importantly, agonistic CD40 mAb + PEP combination therapy

significantly and synergistically increased activated $CD8^+$ T cells expressing $IFN\gamma^+$ and $TNF\alpha^+$ in tumors compared to either treatment alone (Fig. 6E and F). Tissue-resident memory T cells (Trm, $CD103^+CD69^+$) associate with favorable clinical outcome and correlate with enhanced response to immunotherapy [43,44]. Intriguingly, combination therapy significantly increased intratumoral frequencies of $CD8^+$ Trm compared to either treatment alone which was significantly greater than CD40 alone (Fig. 6E–G). Altogether, these findings show that PKC agonism synergized with agonistic CD40 mAb to effectively activate $CD8^+$ T cells and induce T cell memory phenotype.

4. Discussion

Evidence suggests that targeting innate immunity-myeloid cells in particular-might improve disease outcome and response to immunotherapy in cancer patients [45]. Unfortunately, some therapeutic strategies that aim to activate innate immunity, such as adjuvant therapies, can also trigger compensatory mechanisms that activate immunosuppressive MDSCs, thus reducing overall treatment efficacy [15]. Therefore, alternative strategies to target myeloid cells in cancer are needed. Our data demonstrate a promising strategy to target MDSCs and induce cDC1s with a single agent that activates PKC. We show that PKC agonism blunts MDSC immunosuppression by inducing their differentiation to an APC-like phenotype while simultaneously directly increasing intratumoral cDC1 pools. These immune changes jointly modify the intratumoral myeloid immune landscape which results in the activation of cytotoxic T cells and induction of tissue-resident T cell memory phenotype when PKC agonism is combined with agonistic CD40 mAb. Our findings support the approach of combining PKC agonists with immunotherapies that activate innate immunity and myeloid cells.

Reprogramming of MDSCs using differentiating agents such as all-trans retinoic acid (ATRA) [46] and adjuvant epigenetic therapy (AET) [47] promoted M-MDSC differentiation into APCs in both mice and humans, which resulted in reduced immunosuppression, improved response to therapy, and disruption of premetastatic niches. PKC agonists have been investigated as potential differentiating agents in AML by promoting myeloblast differentiation to a more mature myeloid phenotype [25–27,48]. PKC agonist PEP005 is currently FDA approved for the treatment of actinic keratosis. In this study, we investigated the effects of PKC agonists prostratin or PEP005 on MDSC differentiation and cDC1 expansion as a novel myeloid modulating approach. Using the E0771 breast cancer orthotopic mouse model, we showed that MDSCs accounted for the majority of tumor infiltrating immune cells. Interestingly, E0771 tumor-bearing mice treated with PEP showed a significant decrease in M-MDSC frequencies in the BM, spleen, and tumor indicating that PEP may not only affect M-MDSCs directly, but also blunt M-MDSC expansion in the marrow, trafficking, and/or recruitment to the TME. Simultaneously, PEP significantly increased intratumoral cDC1s with no significant effects on macrophage or PMN-MDSC frequencies indicating that PEP uniquely affects M-MDSCs and cDC1s, but not other major myeloid subpopulations. In addition, PEP's antitumor effect *in vivo* can also be mediated by NK cells as evidenced by increased intratumoral NK cells. These immune changes correlated with a significant delay in tumor growth after monotherapy PEP administration. However, PEP may have some direct cytotoxic effects on cancer cells at

high doses, which was consistent with previous reports [49–52], but not at lower doses that paralleled the concentration we used in *in vitro* and *ex vivo* assays. Therefore, we cannot exclude that some of PEP's antitumor effects as a monotherapy can be via direct cytotoxicity toward cancer cells.

Given the systemic decreases in M-MDSCs and intratumoral increases in cDC1s with PEP treatment observed *in vivo*, one would expect possible impacts on MDSC/DC myelopoiesis in the BM and/or recruitment to the TME. Indeed, findings show that PKC agonists blunted MDSC expansion from BM progenitors *in vitro*. After 4 days of culture, all $CD11b^+$ $Gr\text{-}1^+$ cells were M-MDSCs which can be explained by the fact that PMN-MDSCs display a very short survival in culture [11]. The fact that PKC agonists increased $CD11c^+$ cells suggests that normal hematopoiesis was not altered but was rather redirected from the MDSC lineage commitment to APC-like lineage commitment. Indeed, *ex vivo* differentiation assays showed that PKC agonists favor cDC1 commitment of BM progenitors at the expense of cDC2 and pDC, which may account for the increase in intratumoral cDC1s observed *in vivo*. A possible explanation of systemic and intratumoral reductions in M-MDSC abundance is alteration of their recruitment to the TME caused by PKC agonists. M-MDSCs are recruited to the TME via the interaction of tumor-secreted C–C Motif Chemokine Ligand 2 (CCL2) activating its receptor CCR2 on M-MDSCs [53]. Accordingly, PEP-treated M-MDSCs showed a significant decrease in *Ccr2* gene expression which may have resulted in decreased recruitment to the TME.

A hallmark of MDSCs is their ability to suppress T cells *via* production of factors such as nitric oxide (NO) or arginase 1 (*Arg1*) which depletes L-arginine that is essential for T cell function [9]. While PMN-MDSCs preferentially express Arg1, M-MDSCs primarily rely upon NO generated by iNOS (*Nos2*) to mediate immune suppression [46]. PKC agonists have been shown to activate T cells directly and reverse T cell exhaustion [54,55]. Therefore, MDSC subsets were pre-treated with PKC agonists before co-culture with T cells to exclude any direct effect on T cell proliferation and activation in *ex vivo* functional assays to isolate impact of PEP on MDSCs. PKC agonist treated MDSCs significantly reduced $CD8^+$ T cell suppression as observed by greater $CD8^+$ T cell proliferation and IFNγ production. Intriguingly, although PKC agonists marginally blunted PMN-MDSC suppressive capacity, this effect was more pronounced in M-MDSCs which correlated with a significant decrease in *Nos2* gene expression, but not *Arg1*, indicating that M-MDSCs represent the primary MDSC subset affected by PKC agonists.

Previous studies showed that a cross-presenting DC-like phenotype can arise from differentiation of immature MDSC precursors or cells of monocytic origin [17,18]. These $CD11c^+$ $CD103^+$ APCs play an important role in antitumor immunity [18]. $CD11c^+$ $CD103^+$ APCs also express cDC1-related cell surface markers and depend on the transcription factor Basic Leucine Zipper ATF-Like Transcription Factor 3 (*Batf3*) [18]. In this study, we showed that PKC agonism induced M-MDSC differentiation into an APC-like phenotype that expresses the integrin CD103 and other cDC1-related cell surface markers using *ex vivo* and adoptive transfer approaches. Interestingly, treatment of M-MDSCs *ex vivo* with PKC agonists significantly increased the cDC1-related transcription factor *Irf8*, but not *Batf3*, suggesting that $CD103^+$ DC-like cells differentiated from M-MDSCs upon PKC

agonism not only differ in ontology from cDC1s but are also at least partly transcriptionally distinct from cDC1s. Thus, PKC-agonist induced DC-like cells are distinct from previously described Ly6C$^+$ CD103$^+$ DCs that also arise from M-MDSCs [18].

Currently, it is unclear which PKC isoform(s) are responsible for this differentiation effect since PKC agonists activate all conventional and novel PKC isoforms [48,56–58]. PKC agonists induce differentiation of myeloblasts in AML *via* the p38 kinase and c-Jun N—terminal protein kinase (JNK) pathways [27,36]. Our results show that the PEP-mediated decrease in Ly6C expression in M-MDSCs and increase in CD11c and CD86 was partly abrogated when co-administered with the p38 inhibitor, but not the JNK inhibitor, indicating that M-MDSC differentiation to an APC-like phenotype is at least partially mediated *via* activation of the p38 pathway. Additional work may elucidate other pathways involved in PKC agonist-induced differentiation of M-MDSCs to APC-like cells.

Cross-priming APCs directly cross-present antigens to CD8$^+$ T cells *via* major histocompatibility complex 1 (MHCI) resulting in a CD8$^+$ T cell-mediated adaptive immune response [59]. In cancer, APC subsets such as cDC1 efficiently cross-present tumor antigen to CD8$^+$ T cells which is crucial for the induction of an efficient antitumor immune response [34]. Herein, PKC agonism significantly increased MDSC antigen uptake, and most importantly, dramatically enhanced MDSC cross-presenting capacity both *in vitro* and *in vivo*. Thus, PKC agonism not only induces MDSC differentiation to APC-like cells that phenotypically resemble cross-presenting DCs, but also functionally enhances MDSC cross-priming capacity.

Immature APCs - DCs in particular - display a poor capacity to prime T cells and often need a secondary activating signal to become fully mature [34]. Once mature, cDC1s up-regulate CD40, CD80, CD86, MHCII, PD-L1, and CCR7 and home in the tumor-draining lymph node wherein they prime T cells [19]. Additionally, APCs express CD40 which induces maturation and activation in response to its ligand [42]. Although we observed an up-regulation of DC markers on PEP-treated sM-MDSCs, some markers such as MHCII and CD40 were not significantly increased. This partial increase in maturation markers was previously described as DC ‘semi-maturation’ which resulted in poor T cell priming in cancer [16]. Therefore, to maximize PKC agonism efficacy, agonistic CD40 mAb combined with PKC agonism *in vivo* was tested to activate APC pools and consequently activate intratumoral cytotoxic T cells. Combination therapy resulted in an improved antitumor benefit compared to either treatment alone. Strikingly, combination therapy synergistically increased intratumoral CD8$^+$ T cell activation and tissue-resident memory CD8$^+$ T cell frequencies compared to either treatment alone. It remains unclear whether this robust activation is driven by ligation of CD40 on certain APCs including cDC1s or CD103$^+$ DC-like cells, or other CD40-expressing cell types, which is to be investigated. Critically, the observed increase in tissue-resident memory CD8$^+$ T cells may be indicative of beneficial long lasting protective antitumor immunity [60]. Intratumoral therapy is a rapidly emerging avenue of cancer treatment as it has the unique capacity of tumor cell lysis followed by the release of tumor-derived antigens which can result in a subsequent activation of tumor-specific effector T cells [61]. T cell activation based on tumor cell lysis not only can control the primary tumor, but also distant metastatic tumors that share

similar antigens and significantly improves response to immune checkpoint blockade in unresectable metastatic advanced cancers [62]. Thus, we administered PEP intratumorally not only to minimize toxicity, but also to induce a robust tumor specific adaptive immune response when combined with CD40 agonism. Altogether, findings presented herein provide a strong rationale for combination of myeloid differentiation therapy - PKC agonism specifically - with agonistic CD40 mAb to blunt immunosuppression and promote antigen-cross presentation to achieve maximum clinical benefit in cancer patients where M-MDSCs are major drivers of a permissive and immunosuppressive microenvironment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Data availability statement

Data are available on reasonable request. All data relevant to the study are included in the article or uploaded as online supplementary information.

Abbreviations

PKC	Protein Kinase C
MDSC	Myeloid-derived Suppressor Cells
cDC	Conventional Dendritic Cells
pDC	Plasmacytoid Dendritic cells
PEP	PEP005, Ingenol Mebutate
APC	Antigen-Presenting Cells
CTV	CellTrace Violet (proliferation dye)
OVA	Chicken Ovalbumin Protein
BM	Bone Marrow

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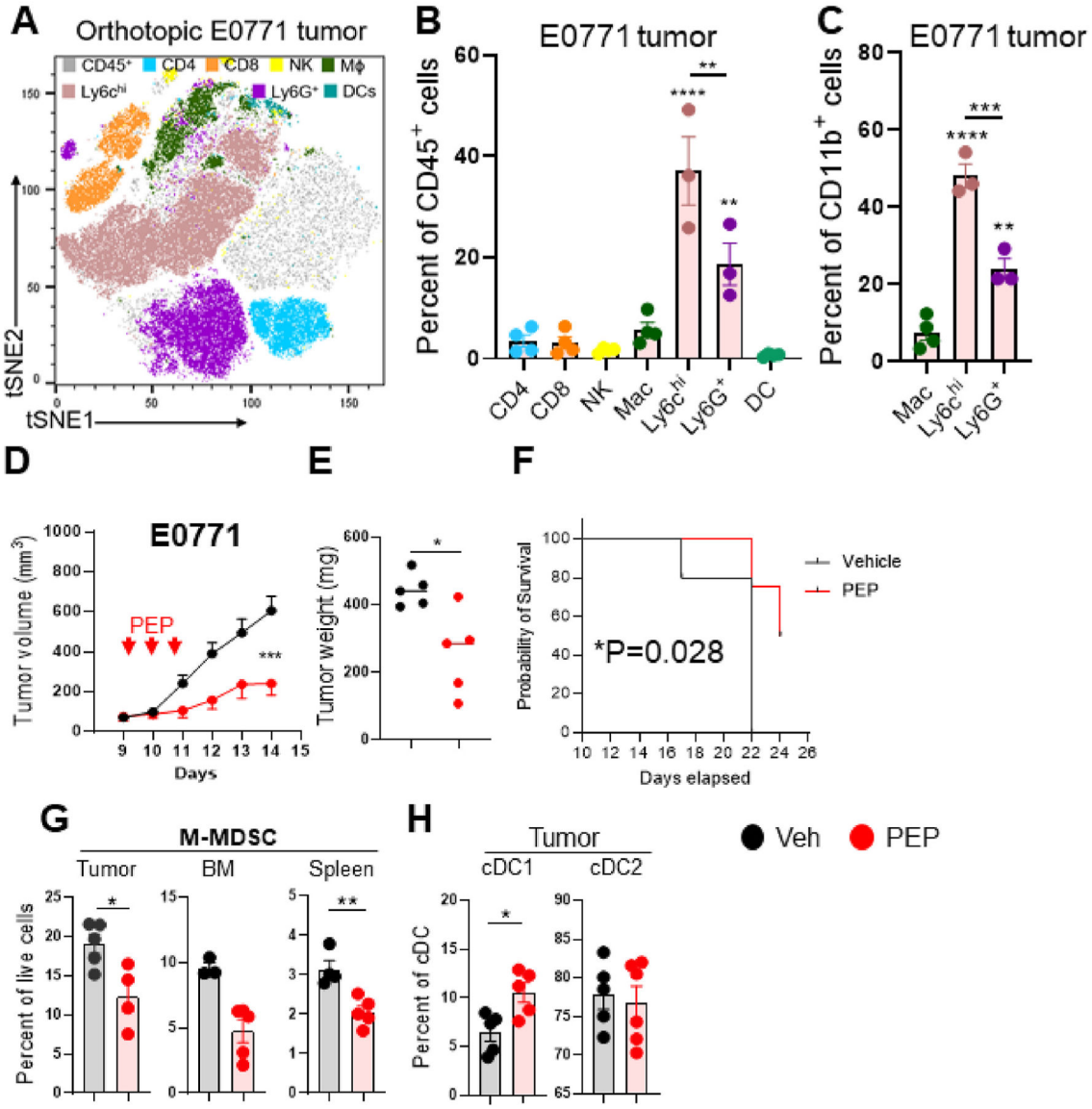

Fig. 1. PKC agonism reduces BC progression, decreases M-MDSCs and increases cDC1s.

E0771 murine BC cells were orthotopically implanted into the fourth mammary fat pad of syngeneic C57BL/6J mice. (**A-C**) Tumors were collected at day 14 post cancer cell injection and single cell suspensions were analyzed by flow cytometry demonstrating high myeloid infiltration. **(A)** tSNE dimensionality reduction showing concatenated flow cytometry analysis of $CD45^+$ tumor-infiltrating immune cell populations. **(B)** Quantification of immune cell populations as percent of $CD45^+$ cells. **(C)** Quantification of myeloid cell content as percent of $CD11b^+$ cells (n = 3–4 mice). One-way ANOVA with multiple comparisons with Tukey correction (**P < 0.01, ***P < 0.005, ****P < 0.001). **(D–G)** E0771 tumor-bearing mice were treated with PEP005 (PEP, 200ng/mouse) or vehicle (Veh) intratumorally (IT) on days 9, 10 and 11 post injection (red arrows). At endpoint on day 14, tumor, bone marrow (BM), and spleen were collected for flow cytometry analysis. **(D)** Tumor growth in mice receiving Veh or PEP. **(E)** Tumor weight at endpoint (day 14). **(F)**

Survival curves of mice. Log-rank (Mantel-Cox) test for survival curves. *P < 0.05. **(G)** Monocytic (M)-MDSC frequencies in tumor, BM, and spleen (n = 3–5 mice). **(H)** cDC1 and cDC2 frequencies of cDCs (n = 5 mice). Shown are means ± SEM. Unpaired *t*-test (*P < 0.05, **P < 0.01, ***P < 0.005).

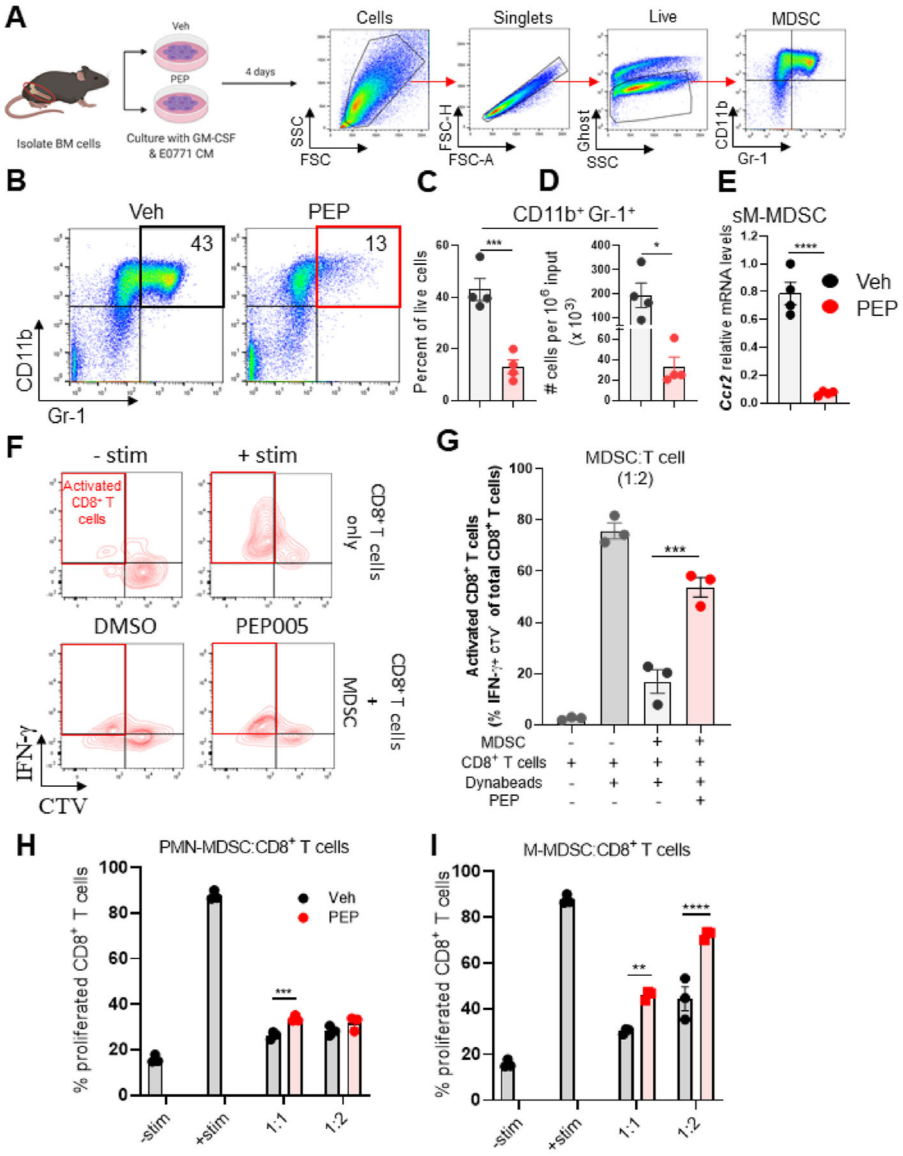

Fig. 2. PKC agonism decreases M-MDSC generation, migration and impairs MDSC suppressive function.

(A–D) BM cells collected from tumor-free C57Bl/6J mice were cultured *in vitro* in the presence of granulocyte-macrophage colony stimulating factor GM-CSF (20 ng/mL) and E0771 conditioned media (CM) (50% V/V) for 4 days in the presence of Veh or PEP to generate MDSCs. **(A)** Experimental outline and gating scheme. **(B)** Representative flow plot of $CD11b^+$ $Gr\text{-}1^+$ cells in Veh- or PEP-treated BM cultures. **(C)** Frequencies and **(D)** absolute cell numbers of $CD11b^+$ $Gr\text{-}1^+$ cells (n = 4 mice). Shown are Means ± SEM. Unpaired t-test (*P < 0.05, ***P < 0.005). **(E)** *Ccr2* gene expression in M-MDSC isolated from the spleens (sM-MDSC) of E0771 tumor-bearing mice and treated *ex vivo* with PEP (20 nM) or Veh for 72h as analyzed by qRT-PCR (n = 4 mice). Shown are Means ± SEM. Unpaired t-test (****P < 0.001). **(F–I)** Splenic $CD11b^+$ $Gr\text{-}1^+$ MDSCs (sMDSCs) were isolated from E0771 tumor-bearing mice and co-cultured with CellTrace Violet (CTV)-labeled $CD8^+$ T cells isolated from the spleens of tumor-free mice at a 1:2 T

cell/MDSC ratio. sMDSCs were pre-treated with PEP (20 nM) or Veh for 3h prior to the addition of T cells and Dynabeads (anti-CD28/CD3). MDSC suppressive properties were assessed by $CD8^+$ T cell activation (IFNγ expression) and proliferation (CTV dilution). **(F)** Representative contour plots and **(G)** IFNγ expression in $CD8^+$ T cells (n = 3 mice). One-way ANOVA with multiple comparisons with Tukey correction (***P < 0.0001). **(H–I)** sPMN-MDSCs and sM-MDSCs were isolated from the spleens of tumor-bearing mice as previously described. Percent of $CD8^+$ T cell proliferation after coculture with Veh- or PEP (20 nM)-pre-treated **(H)** sPMN-MDSCs and **(I)** sM-MDSCs at the indicated T cell:MDSC ratios (n = 3 mice). Shown are Means ± SEM. (**P < 0.01, ***P < 0.005).

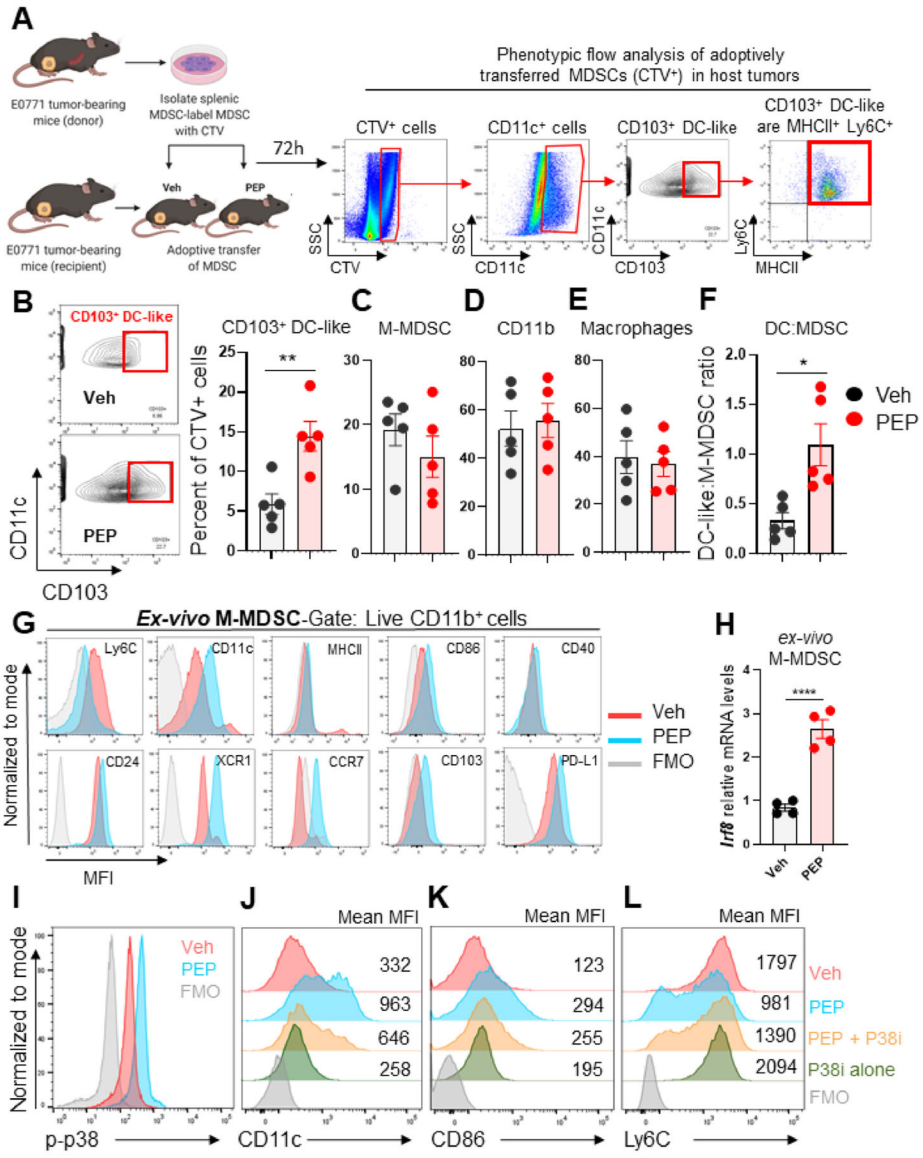

Fig. 3. PKC agonism skews M-MDSC differentiation towards a CD103$^+$ DC-like phenotype.

(A–F) sMDSCs were isolated from E0771 tumor-bearing mice (donor), labeled with CTV, and adoptively transferred into recipient tumor-bearing mice IT with 10^6 cells/mouse. Recipient mice were then treated with PEP (200ng/mouse) or Veh IT once and tumors of recipient mice were analyzed after 3 days via flow. **(A)** Experimental outline and gating scheme of CTV$^+$ cell analysis. Frequencies of **(B)** CD103$^+$ DC-like cells, **(C)** M-MDSCs, **(D)** CD11b$^+$ cells, **(E)** macrophages within gated CTV$^+$ cells, and **(F)** CD103$^+$ DC-like to M-MDSC ratio (n = 5 mice). Shown are Means ± SEM. Unpaired T-test (**P < 0.01). **(G–H)** sM-MDSCs were isolated from E0771 tumor-bearing mice and treated *ex vivo* with Veh or PEP (20 nM) in the presence of GM-CSF (20 ng/mL) for 72h. **(G)** Expression of Ly6C, CD11c, MHCII, CD86, CD40, CD24, XCR1, CCR7, CD103, and PD-L1 upon treatment with Veh (red) or PEP (blue). Peaks in gray represent the fluorescence-minus-one (FMO) controls. Shown are representative histograms (n = 4 mice). **(H)** *Irf8* gene expression

in sM-MDSCs as analyzed by qRT-PCR (n = 4 mice). Shown are means ± SEM. Unpaired T-test (***P < 0.005). (**I**) Expression of phosphorylated p38 (p-p38) in sM-MDSCs treated with Veh (red) or PEP (20 nM) (blue) for 30min. Shown are representative histograms (n = 3 mice). (**J**-**L**) Expression of CD11c, CD86, and Ly6C in sM-MDSCs upon treatment with Veh or PEP (20 nM) and/or pretreatment for 30min with p38 inhibitor SB203580 (10 μM). Shown are representative histograms with mean MFIs (n = 4 mice).

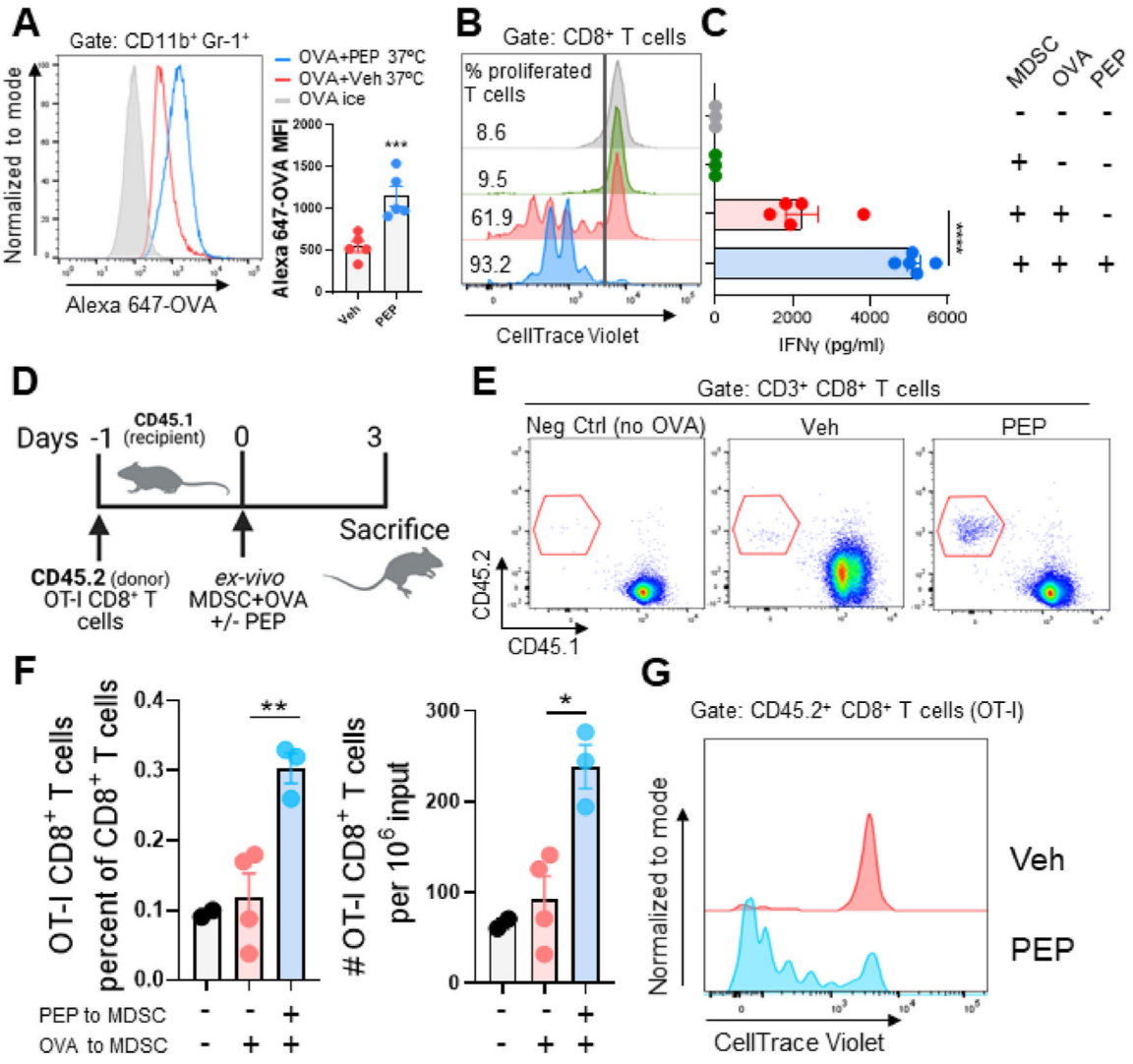

Fig. 4. PKC agonist-treated MDSCs mediate efficient T cell cross-priming *in vitro* and *in vivo*.

(A–C) sMDSCs were pretreated with Veh or PEP (20 nM) overnight, then loaded with Alexa 647-conjugated ovalbumin (Alexa 647-OVA) for 3h before co-culture with CTV-labeled $CD8^+$ T cells isolated from the spleens of OT-I mice for an additional 72h. OT-I $CD8^+$ T cell proliferation and activation were assessed by CTV dilution via flow and IFNγ concentration in supernatants via ELISA, respectively. **(A)** MDSC OVA uptake was evaluated by Alexa 647-OVA MFI upon pretreatment with Veh or PEP (20 nM). Peak in gray represents OVA uptake by MDSCs loaded with Alexa 647-OVA for 3h on ice (negative control). Shown is a representative histogram (left) and Means ± SEM (right). (n = 5 mice). Unpaired *t*-test (***P < 0.005). **(B)** CTV dilution assessing OT-I $CD8^+$ T cell proliferation. Shown is a representative histogram (n = 5 mice). **(C)** Bar graph shows IFNγ concentration in co-culture supernatants (n = 5 mice). One-way ANOVA with multiple comparisons with Tukey correction (****P < 0.001). **(D–G)** 1×10^6 $CD45.2^+$ $CD8^+$ T cells were isolated from the spleens of OT-I mice (donor), labeled with CTV, and adoptively transferred into CD45.1 mice (recipient) via tail vein injection on day −1. On day 1, sMDSCs that were

pretreated with Veh or PEP (20 nM overnight) were loaded with OVA for 3h, then adoptively transferred into the same recipient mice via tail vein injection. Negative control consisted of adoptively transferred MDSCs without OVA. OT-I $CD8^+$ T cells were analyzed via flow. **(D)** Experimental outline. **(E)** Representative flow plots of $CD45.2^+$ OT-I $CD8^+$ T cells in the spleens of recipient mice. **(F)** OT-I $CD8^+$ T cell frequencies (left) and absolute cell numbers (right) in the spleens of recipient mice. **(G)** CTV dilution within gated $CD45.2^+$ OT-I $CD8^+$ T cells. Shown is a representative histogram (n = 3–4 mice). Shown are Means ± SEM. One-way ANOVA with multiple comparisons with Tukey correction (*P < 0.05, **P < 0.01).

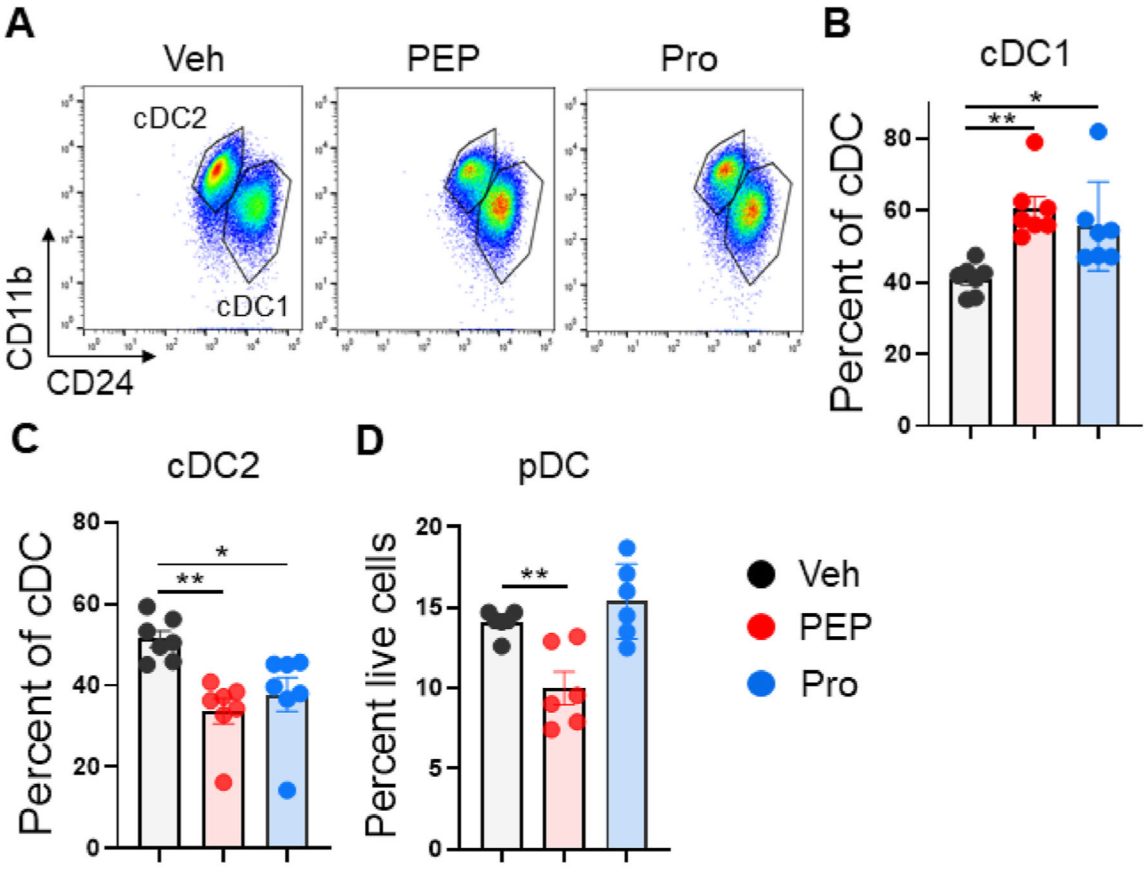

Fig. 5. PKC agonism skews BM progenitor differentiation towards cDC1 at the expense of cDC2 and pDC.

BM cells were cultured *in vitro* in the presence of DC generator FMS-like tyrosine kinase 3 ligand (FLT3L) (100 ng/mL) and treated with Veh, PEP (20 nM) or prostratin (Pro, 1 μM) on day 0. Cells were collected on day 10 to analyze different DC subsets via flow (n = 6–7 mice). **(A)** Representative flow plots showing cDC1 and cDC2 populations. Frequencies of **(B)** cDC1, **(C)** cDC2 and **(D)** pDC in BM cells treated with Veh, PEP, or Pro on day 10 are shown. Shown are Means ± SEM. One-way ANOVA with multiple comparisons with Tukey correction (*P < 0.05, **P < 0.01).

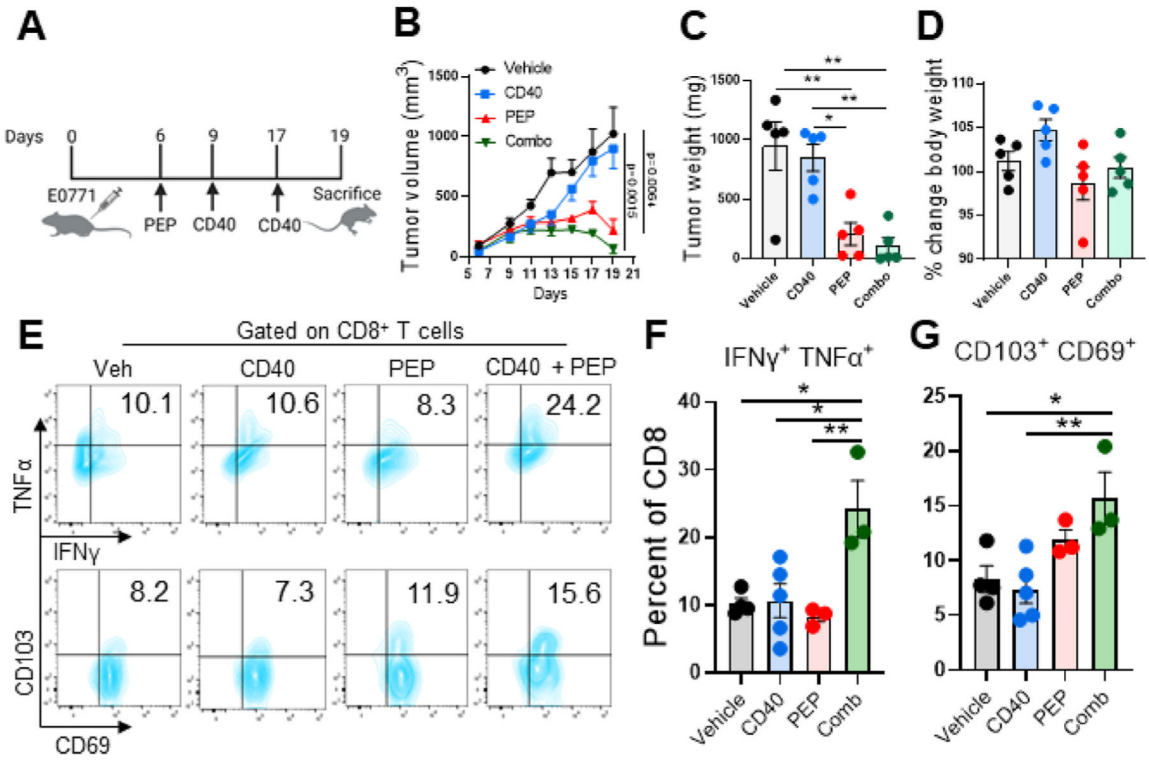

Fig. 6. Combination of PKC agonism and agonistic CD40 mAb promotes CD8$^+$ T cell activation, tissue-resident memory CD8$^+$ T cells and tumor regression in E0771 tumor-bearing mice.

E0771 tumor-bearing mice were treated with Veh, PEP (1 μg/mouse-IT), CD40 agonist (100μg/mouse-i.p.) or PEP-CD40 agonist combination on days indicated in A. Mice were sacrificed on day 19 and spleens and tumors were harvested for flow analysis. **(A)** Experimental outline. Tumor burden was assessed by **(B)** tumor volume as a function of time and **(C)** tumor weight at endpoint. **(D)** Percent change in body weights reported between days 6 (before treatment) and 19 (endpoint) (n = 5 mice). **(E)** Representative flow plots showing tumor IFNγ^+ TNFα^+ and CD103$^+$ CD69$^+$ CD8$^+$ T cells. Frequencies of tumor **(F)** IFNγ^+ TNFα^+ CD8$^+$ T cells and **(G)** CD103+ CD69$^+$ CD8$^+$ T cells (n = 3–5 mice). Shown are Means ± SEM. One-way ANOVA with multiple comparisons with Tukey correction (*P < 0.05, **P < 0.01).