Dendritic cell Piezo1 stimulated by mechanical stiffness or inflammatory signals directs the differentiation of T\textsubscript{H}1 and T\textsubscript{reg} cells in cancer

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**Running title:** DC Piezo1 directs T cell differentiation

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Abstract

Dendritic cells (DCs) play an important role in anti-tumor immunity by inducing T cell differentiation. Herein, we found that the DC mechanical sensor Piezo1 stimulated by mechanical stiffness or inflammatory signals directs the reciprocal differentiation of $T_\text{H}1$ and regulatory T ($T_{\text{reg}}$) cells in cancer. Genetic deletion of Piezo1 in DCs inhibited the generation of $T_\text{H}1$ cells while driving the development of $T_{\text{reg}}$ cells in promoting cancer growth in mice. Mechanistically, Piezo1-deficient DCs regulated the secretion of the polarizing cytokines TGFβ1 and IL-12, leading to increased TGFβR2-p-Smad3 activity and decreased IL-12Rβ2-p-STAT4 activity while inducing the reciprocal differentiation of $T_{\text{reg}}$ and $T_\text{H}1$ cells. In addition, Piezo1 integrated the SIRT1-hypoxia-inducible factor-1 alpha (HIF1α)-dependent metabolic pathway and calcium-calcineurin-NFAT signaling pathway to orchestrate reciprocal $T_\text{H}1$ and $T_{\text{reg}}$ lineage commitment through DC-derived IL-12 and TGFβ1. Our studies provide critical insight for understanding the role of the DC-based mechanical regulation of immunopathology in directing T cell lineage commitment in tumor microenvironments.
Introduction

CD4+ T helper cells play a central role in cancer by differentiating into different T cell subsets including T_H1 cells, T_H2 cells, T_H9 cells, T_H17 cells and regulatory T (T_reg) cells, in specific cytokine environments (1, 2). Foreign antigens and all kinds of innate stimuli are often presented by antigen-presenting cells (APCs) to further direct the development and differentiation of different subsets of CD4+ T cells in tumor (3, 4). Inflammatory stimuli, such as bacterial lipopolysaccharide (LPS), cytokine or other innate stimuli, such as oxygen, nutrient availability, and even force and pressure, can alter the immune responses. In particular, the tumor microenvironment often integrates different innate physiological or pathological stimuli to develop a complex stimulation microenvironment (5, 6). As professional APCs, DCs response to various exogenous and endogenous stimuli mainly through three key signal pathways, including costimulatory molecule expression, T cell receptor (TCR) signaling and cytokine production, bridging innate and adaptive immunity, and regulating the differentiation of different T cell subsets (7-14). DC-derived cytokines and chemokines exert proinflammatory or anti-inflammatory effects and are involved in the shaping of distinct T cell subset lineage programs to determine the prognosis of cancer patients. But, how the differentiation of CD4+ T cells is modulated and regulated by innate immune signaling pathways in DCs in the tumor microenvironment remains unclear.
Piezo1 was originally identified as a mechanically activated non-selective cation ion channel with significant permeability to calcium ions, is evolutionally conserved and is involved in the proliferation and development of various types of cells in the context of various types of mechanical or innate stimuli. It has been reported that innate inflammatory stimulation or mechanical changes, such as changes in stiffness, can activate Piezo1 and trigger an inflammatory response (6, 15). Piezo1 exerts significant regulatory effects on many kinds of immune cell functions including macrophages, DCs and T cells in inflammation and cancer (5, 6, 16-19). However, it is still unclear whether Piezo1-targeted DCs affect the differentiation of different subsets of T cells in cancer.

Herein, we found that the DC mechanical sensor Piezo1 stimulated by mechanical stiffness or inflammatory signals directs the reciprocal differentiation of $T_{H1}$ and $T_{reg}$ cells in inhibiting tumor growth.
Results

Inflammatory and stiffness stimuli alter Piezo1 expressions by DCs

As reported(18, 20), environmental stiffness can alter the secretion of inflammatory factors by DCs and the mechanical force receptor Piezo1 may be involved in this regulation. We first examined the effect of environmental stiffness on the expression of Piezo1 in DCs. We used a cell culture system consisting of a poly two methyl siloxane (PDMS) hydrogel-coated plate, as described(15, 20). The mechanical properties of the PDMS hydrogel can be changed by adjusting the matrix/curing agent ratio, and this ratio can be precisely adjusted to simulate physiological tension. Consistent with previous reports(15, 20), we used 2 kPa to mimic lymphoid tissue under physiological conditions and 50 kPa to mimic lymphoid tissue under inflammatory conditions.

Sorted splenic DCs cultured on a stiff hydrogel (E=50 kPa) or plastic plates exhibited significantly enhanced expression of the proinflammatory cytokine IL-12 and diminished expression of the anti-inflammatory cytokine TGFβ1 compared with DCs cultured on a pliant hydrogel (E=2 kPa; Fig.1-Fig. supp. 1A). This suggests that substrate stiffness could regulate inflammatory cytokine secretion by DCs. Moreover, the expression of the mechanical force receptor Piezo1 was significantly upregulated in 50 kPa-conditioned hydrogels compared with 2 kPa-conditioned hydrogels, similar to that in DCs stimulated with the inflammatory stimulus LPS (Fig.1-Fig. supp. 1B). Although 50 kPa-conditioned hydrogels significantly caused more IL-12 and less TGFβ1
compared with 2 kPa, mechanical force receptor Piezo1 deficient DC cells
significant rescue them to normal level (Fig.1-Fig. supp. 1C). These data
suggested that mechanical force receptor Piezo1 mediated the inflammatory
cytokine production induced by substrate stiffness in DCs.

**DC-specific Piezo1-deficient mice exhibited altered T cell differentiation**

To investigate the regulatory effect of Piezo1 expressed by DCs on T cell
function, we generated DC-specific Piezo1 conditional knockout (*Piezo1*<sup>−/−</sup>)
mice with *Piezo1*<sup>flox/flox</sup> and *Cd11c-cre*. *Piezo1*<sup>−/−</sup> mice showed no obvious
abnormalities after birth. However, after 40 weeks of age, these mice showed
lower weight loss and less T cell activation in mesenteric lymph nodes (MLNs),
peyer’s patches (PPs), intraepithelial lymphocytes (IELs) and lamina propria
lymphocytes (LPLs) than WT mice (Fig.1-Fig. supp. 2). Importantly, Piezo1
deficiency in DCs results in fewer IFNγ<sup>+</sup> TH1 cells and more Foxp3<sup>+</sup> T<sub>reg</sub> cells but
does not affect the numbers of T<sub>H2</sub> or T<sub>H17</sub> cells (Fig.1-Fig. supp. 3). Thus, we
conclude that DC-specific Piezo1 deficiency alters the differentiation of T<sub>H1</sub> and
T<sub>reg</sub> cells in aged mice, which might be related to clinical manifestations.

**DC-specific Piezo1 regulates T cell differentiation in promoting tumor
growth**

Next, we studied the effect of DC-specific Piezo1 deletion on T cell
differentiation in MC38 mouse colon cancer. We observed changes in tumor
growth in Piezo1−/− and WT mice. The rate of tumor growth was significantly faster and greater in Piezo1−/− than in WT mice (Fig. 1A). Piezo1−/− mice had more Foxp3+ Treg cells, fewer IFNγ+ T\textsubscript{H}1 cells and normal numbers of T\textsubscript{H}2, T\textsubscript{H}17, CD8+T cell and IFNγ+CD8+T cells in tumor tissue compared with WT control (Fig. 1B, Fig.1-Fig. supp. 4A-B). Furthermore, we isolated T cells from draining lymph nodes (dLNs) of tumor-bearing mice at day 20, 30, and 40 and observed dynamic T cell differentiation. Both Tbx21 and Ifng levels were downregulated rapidly, Foxp3 expression in T cells was upregulated gradually, and Il4, Il10, Il17a, and Gata3, Rorgt expressions did not change in Piezo1−/− mice (Fig. 1C, Fig.1-Fig. supp. 4C). Similar tumor growth and T cell differentiation were also observed in B16.F10 melanoma tumor (Fig.1-Fig. supp. 5). Thus, these data suggest that DC-specific Piezo1 deficiency probably directs T\textsubscript{reg} and T\textsubscript{H}1 cell differentiation to promote the tumor growth in the context of tumor microenvironment.

Further, we observed the antigen specific responses of T cells in tumor-bearing mice during tumor growth. MC38-OVA tumor cells were implanted subcutaneously in WT and Piezo1−/− mice. And at Day 20 after tumor cell implantation, naïve CD45.1+ OTII T cells were isolated from OT II-TCR transgenic mice, labeled with CFSE and transferred into WT and Piezo1−/− bearing-tumor recipient mice (Fig. 1D). After 10 days of adoptive transfer of OTII T cells to recipient tumor-bearing mice, the intracellular staining of the
CD45.1+ CFSE+ donor T cells from tumors in recipient WT and Piezo1−/− mice were analyzed showed that the percentage of CFSE+ T cells is similar between WT and Piezo1−/− bearing tumor mice. However, there are more Foxp3+Treg cells and less IFNγ+T_{H}1 cells, but similar T_{H}2 and T_{H}17 cells in Piezo1−/− compared with WT bearing-tumor mice (Fig. 1D-E). These data suggest that Piezo1 in DCs regulates the T_{H}1 and T_{reg} differentiation in tumor microenvironment with the antigen-specific manner.

**DC-specific Piezo1 expression instructs antigen-specific T_{H}1 and T_{reg} differentiation**

Next, we conducted an adoptive transfer experiment to investigate the T cell response induced by DC Piezo1. Naïve T cells (CD45.2+CD4+TCR+CD44^{low}CD62L^{high}) from C57BL/6 mice were transferred into CD45.1+C57BL/6 recipient mice, and then, the recipient mice were immunized with WT and Piezo1−/− splenic DCs and LPS. The donor cells were analyzed on Day 10 after immunization. T cell proliferation was comparable between the WT and Piezo1−/− mice (Fig. 2A). However, the donor mice immunized with Piezo1−/− splenic DCs exhibited more Foxp3+CD4+ T cells and less IFNγ+CD4+ T cells. Both the WT and Piezo1−/− mice showed similar levels of IL-4 and IL-17 expression among donor CD4+ T cells (Fig. 2B-C). Furthermore, the antigen-specific responses of donor T cells were investigated. Naïve OTII T cells were isolated and transferred into CD45.1+ C57BL/6 recipient mice which
immunized with WT and Piezo1\(^{-/-}\) DCs and antigen. Although WT and Piezo1\(^{-/-}\) showed similar T cell proliferation (Fig. 2D), donor cells immunized with Piezo1\(^{-/-}\) DCs exhibited more Foxp3\(^{+}\) cells, fewer IFN\(\gamma\)^{+} cells and similar numbers of IL-4\(^{+}\)T\(_{H2}\) cells and IL-17A\(^{+}\)T\(_{H17}\) cells (Fig. 2E-F). Further, we examined DCs conditioned by 2 kPa or 50 kPa hydrogel in adoptive transfer experiments. Splenic DCs were isolated from WT and Piezo1\(^{-/-}\) mice and plated on 2 kPa or 50 kPa hydrogels. Naïve T cells or OTII T cells were isolated and transferred into CD45.1\(^{+}\) recipient mice, the recipient mice were immunized with 2 kPa or 50 kPa hydrogel-conditioned DCs with or without antigen. Donor T cells from mice immunized with Piezo1\(^{-/-}\) DCs conditioned with 50 kPa, but not 2 kPa hydrogels included more Foxp3\(^{+}\) T\(_{reg}\) cells and less IFN\(\gamma\)^{+} T\(_{H1}\) cells (Fig. 2G-H). Altogether, these data suggest that the mechanical sensor Piezo1 in DCs stimulated by innate inflammatory or stiffness stimuli directs the reciprocal differentiation of T\(_{H1}\) and T\(_{reg}\) cells.

Piezo1 is required for DC-dependent T cell differentiation

Next, we investigated the effects of Piezo1 expression by DCs on T cell subset differentiation in an in vitro system. Polyclonal T cells or antigen-specific OTII T cells cocultured with Piezo1\(^{-/-}\) DCs in the absence or presence of antigen induced more Foxp3\(^{+}\)T\(_{reg}\) cell and less IFN\(\gamma\)^{+} T\(_{H1}\) cell, even with a variety of Piezo1\(^{-/-}\) DCs including splenic CD11b\(^{+}\) DC or CD8\(\alpha\)^{+} DCs, than WT control DCs (Fig.3-Fig. supp. 1). Moreover, Tbx21 and Ifng expression was significantly
downregulated and Foxp3 expression was significantly upregulated in T cells cocultured with Piezo1\(^{-/-}\) DCs. However, Il4, Il10, Il17a and Gata3, Rorgt expressions were similar in T cells cocultured with Piezo1\(^{-/-}\) DCs or WT DCs (Fig.3-Fig. supp. 2A). These data suggest that Piezo1 deficiency in different kinds of DCs including CD11b\(^{+}\) DC and CD8\(^{α}\)\(^{+}\) DC subsets regulates the reciprocal differentiation of Th1 and Treg cells in an antigen-specific manner. Coculture of antigen-specific OTII T cells with Piezo1\(^{ΔDC}\) splenic CD11b\(^{+}\) DCs conditioned by 2 kPa or 50 kPa hydrogels in the presence of antigen induced a higher expression of Foxp3 and a lower expression of IFNγ (Fig.3-Fig. supp. 2B-C). Together, Piezo1 signals are required for reciprocal Th1 and Treg cell differentiation directed by DCs in an antigen-specific manner.

**DC Piezo1 regulates T cell differentiation through IL-12 and TGFβ1**

APCs regulate T cell differentiation by changing costimulatory molecule expression, TCR signaling and polarizing cytokine production(3). Piezo1 deficient does not alter the cell homeostasis level (Fig.3-Fig. supp. 3A), the expression of MHC, the costimulatory molecules CD80, CD86, CD54, PDL1, PDL2 and CCR7 expressions in DCs (Fig.3-Fig. supp. 3B-C). And, the phagocytosis activities of DC to IgG-coated beads is also comparable between WT and Piezo1\(^{-/-}\) DCs (Fig.3-Fig. supp. 3D). Next, we also detected changes in cytokines secreted by splenic DCs, especially polarizing cytokines important for inducing Th1 and Treg cell differentiation. First, we isolated the DC from MLNs at
40 weeks of age mice and found that Piezo1−/− DCs have less IL-12 (p70) and more TGFβ1 expression (Fig.3-Fig. supp. 4A). These suggest the intestinal environment receives continuous antigen stimulation from microorganisms or food, which may lead to changes in intestinal tension caused by intestinal movements such as food digestion and absorption or inflammatory stimuli, and target the Piezo1 signal of DCs to trigger the differentiation of different subsets of T cells in the aged mice. Next, we found that Piezo1−/− caused DCs significantly higher TGFβ1 production and lower IL-12 (p40), IL-12 (p70), but not IL-23 production in the presence of LPS (Fig. 3A, Fig.3-Fig. supp. 4B-C) or 50 kPa, but not 2 kPa hydrogels (Fig. 3B). These results together suggest that the polarizing cytokines TGFβ1 and IL-12 are probably involved in regulating T cell subset differentiation induced by Piezo1 in DCs stimulating by inflammatory stimuli or stiffness signals.

We selected a splenic DC-T coculture system to determine whether DC-specific Piezo1 expression regulates T cell subset differentiation through the polarizing cytokines TGFβ1 and IL-12. Although Piezo1 deficiency in DCs caused a significantly lower IFNγ+ T_h1 cell percentage, adding IL-12 to the coculture system almost completely recovered the proportion of Piezo1−/− DCs during T_h1 cell differentiation (Fig. 3C). Even DCs conditioned by 50 kPa hydrogel treatment caused similar effects (Fig. 3D). Consistently, blocking TGFβ1 signaling with an anti-TGFβ1 antibody in the coculture system almost
completely recovered the effect of Piezo1<sup>−/−</sup> splenic DCs on T<sub>reg</sub> cell differentiation. Similar effects could be observed when DCs were treated with LPS or 50 kPa hydrogels (Fig. 3C-D). Thus, we could conclude that DC-specific Piezo1 expression regulates the reciprocal differentiation of T<sub>H1</sub> and T<sub>reg</sub> cells through the polarizing cytokines IL-12 and TGFβ1.

**DC-specific Piezo1 expression induces T cell differentiation through TGFβR2 and IL-12Rβ2**

T cell differentiation-inducing cytokines often change the corresponding receptors on the surface of T cells and program the differentiation of T cell subsets(1, 21). We further determined the corresponding receptor expression on T cells. Piezo1 deficiency in splenic DCs significantly increased the expression of TGFβR2 and decreased the expression of IL-12Rβ2 but did not affect the expression of TGFβR1/3 or IL-12Rβ1 on T cells in a DC-T coculture system (Fig. 4A, Fig.4-Fig. supp. 1). Interestingly, Piezo1-deficient DCs also exhibited significantly higher levels of phosphorylated Smad3 and lower levels of phosphorylated STAT4 than WT DCs (Fig. 4B). These data suggest that TGFβR2-p-Smad3 or IL-12Rβ2-p-STAT4 signaling is involved in the T<sub>reg</sub> and T<sub>H1</sub> cell differentiation induced by DC-specific Piezo1 expression.

To determine whether TGFβR2-pSmad3 and IL-12Rβ2-pSTAT4 are required for promoting the T cell differentiation induced by DC-specific Piezo1 expression,
we knocked down IL-12Rβ2 and TGFβR2 expression in T cells with shRNA in a DC-T coculture system (Fig.4-Fig. supp. 2). Although DC-specific Piezo1 deficiency resulted in significantly more Foxp3⁺ Treg cells, higher phosphorylation of Smad3, and less IFNγ⁺ TH1 cells, lower phosphorylation of STAT4, knockdown of TGFβR2 or IL-12Rβ2 expression significantly recovered these effects compared with the WT conditions (Fig. 4C-D). These data suggest that TGFβR2-pSmda3 and IL-12Rβ2-pSTAT4 signaling in T cells are required for the T cell differentiation induced by DC Piezo1.

Piezo1 regulates IL-12 and TGFβ1 production through the SIRT1-HIF1α-glycolysis pathway

How does Piezo1 regulate IL-12 and TGFβ1 production to direct T cell differentiation? To study the mechanisms underlying the effects of Piezo1, splenic DCs were stimulated by LPS, and we assessed the signaling downstream of LPS stimulation, including Erk, c-jun-NH2-kinase (JNK), p38MAPK, SIRT1, HIF1α and glycolytic molecular signaling.

We investigated the role of glycolysis and oxidative phosphorylation (OXPHOS) signal activities in the functional regulation of DCs induced by Piezo1. LPS treatment led to an increase in the proton production rate (PPR), but splenic DCs treated with the Piezo1 agonist Yoda1 exhibited significantly enhanced PPR values and expression of glycolytic molecules but not oxygen
consumption rates (OCRs) (Fig.5-Fig. supp. 1). Blocking glycolysis with 2-deoxy-D-glucose (2-DG), a prototypical inhibitor of glycolysis pathways, significantly recovered the IL-12 and TGFβ1 production in splenic DCs induced by Yoda1 treatment (Fig.5-Fig. supp. 2). Furthermore, Piezo1 deficiency in DCs significantly decreased the PPR value and the expression of glycolytic molecules but not the OCR value (Fig. 5A-C, Fig.5-Fig. supp. 3A). Blocking glycolysis with 2-DG significantly recovered the productions of IL-12 and TGFβ1 in Piezo1−/− DCs to normal level compared with WT DCs (Fig. 5A-C, Fig.5-Fig. supp. 3B-C). These data altogether suggest that glycolysis activities are required for the polarizing cytokine production in DCs induced by Piezo1.

Additionally, as expected, LPS activated all downstream pathways in WT DCs including p-JNK, p-Erk, p-p38, SIRT1 and HIF1α signal pathway. However, Piezo1 deletion in DCs enhanced the phosphorylation of Erk, p38 and JNK, similar to WT DCs. However, Piezo1−/− DCs exhibited stronger and sustained activation of the histone deacetylase SIRT1 and weaker and shorter activation of the transcription factor HIF1α (Fig. 5D). Thus, Piezo1 is probably associated with SIRT1-HIF1α signaling and glycolysis activation.

To determine whether SIRT1 is involved in this regulation, we used Piezo1 and SIRT1 double knockout (DKO) mice in this investigation. Interestingly, less IL-12 and more TGFβ1 production in Piezo1−/− DCs was significantly reversed in
Piezo1-SIRT1 DKO cells (Fig. 5E, Fig.5-Fig. supp. 4A). Consistently, HIF1α expression and glycolysis activities were significantly recovered to a normal level (Fig.5-Fig. supp. 4B-C). These data suggest SIRT1 is required for the IL-12 and TGFβ1 production in DCs induced by Piezo1 and HIF1α and glycolysis activation is probably related with these alterations.

To determine whether HIF1α is involved in this regulation, we crossed DC HIF1α conditional knockout mice (Hif1a−/−) with Hif1a^floox/floox and Cd11c-Cre mice. Splenic DCs were isolated from WT and Hif1a−/− mice and treated with the Piezo1 agonist Yoda1. More IL-12 and less TGFβ1 in Yoda1 treated DCs was significantly reversed in Yoda1 treated Hif1a−/− DCs (Fig. 5F, Fig.5-Fig. supp. 5A). These data suggest HIF1α is required for the IL-12 and TGFβ1 production in DCs induced by Piezo1.

Moreover, splenic DCs were treated with the Piezo1 agonist Yoda1, which significantly altered glycolysis activity and SIRT1 expression in DCs. SIRT1 expressions cannot be altered in Hif1a−/− DCs (Fig.5-Fig. supp. 5B). However, treatment of Hif1a−/− with Yoda1 significantly reversed the alteration of glycolysis but not SIRT1 expression (Fig.5-Fig. supp. 5B-C). Thus, HIF1α and glycolysis is downstream targets of SIRT1 in regulating the Piezo1-induced cytokine production in DCs.
Piezo1 regulates IL-12 and TGFβ1 production through the calcium-calcineurin-NFAT axis

Mechanically activated ion channel Piezo1 regulates macrophage or DC function by altering calcium permeability (17, 18). Therefore, we assessed the level of calcium influx in Piezo1−/− splenic DCs. Inflammatory or stiffness stimulation caused a significant decrease in the calcium influx in Piezo1−/− DCs compared with the WT control (Fig. 6A). And, Piezo1 agonist Yoda1 treatment significantly enhanced intracellular calcium influx, decreased TGFβ1 secretion and increased IL-12 secretion by DCs exposed to LPS or conditioned by 50 kPa hydrogels (Fig. 6B-D). Importantly, blocking the Ca^{2+} signaling with ruthenium red reversed these alterations. But, another nonspecific ion channel inhibitor, gadolinium, had no effects on calcium influx or cytokine production in DCs (Fig. 6B-D). Consistently, Piezo1−/− DCs showed lower calcium influx and exhibited higher TGFβ1 and lower IL-12 levels, and blocking the Ca^{2+} signaling pathway with ruthenium red, but not gadolinium reversed these alterations in cytokine production (Fig.6-Fig. supp. 1). Thus, calcium signaling pathway is required for the TGFβ1 and IL-12 production in DCs induced by Piezo1.

Previous studies have shown that calcineurin-NFAT are critical molecules of the calcium signaling pathway in regulating the immune response(22-25). These prompted us to investigate whether calcineurin-NFAT signals are necessary for Piezo1 to regulate DC function through calcium signaling pathway. Therefore,
we pharmacologically targeted calcineurin and NFAT to assess their roles in regulating Piezo1-induced TGFβ1 and IL-12 production in DCs. Splenic DCs treated with the Piezo1 agonist Yoda1 caused more IL-12 and less TGFβ1 production, but blocking calcineurin with its inhibitor cyclosporin A (CsA) reversed these alterations in DCs (Fig. 6E, Fig. 6F- Fig. supp. 2A). These data suggest that calcium-calcineurin signaling are probably required for cytokine productions in DCs induced by Piezo1.

As reported (22-25), NFAT is critical transcriptional factor for regulating calcium-calcineurin signaling pathway in mediating immune cell activities. Moreover, upregulation of Piezo1 with Yoda1 treatment enhanced the expression of NFAT, blocking calcineurin with CsA inhibits the expression of NFAT (Fig. 6F). To test the role of NFAT for Piezo1 to regulate DC function, we pharmacologically targeted NFAT with its inhibitor VIVIT to assess the role of NFAT in regulating Piezo1-induced TGFβ1 and IL-12 production by DCs. Piezo1−/− DCs exhibited less IL-12 and more TGFβ1, and blocking calcineurin with CsA and blocking NFAT with VIVIT consistently showed similar cytokine production by DCs (Fig. 6G, Fig. 6F- Fig. supp. 2B). Interestingly, splenic DCs treated with the Piezo1 agonist Yoda1 exhibited more IL-12 and less TGFβ1 production, but blocking NFAT with VIVIT reversed these alterations in DCs (Fig. 6H, Fig. 6F- Fig. supp. 2C). Altogether, these data suggest that the calcium-calcineurin-NFAT axis is required for regulating IL-12 and TGFβ1.
production by DC Piezo1.

**IL-12 and TGFβ1 are critical for DC Piezo1-dependent T cell differentiation in promoting cancer growth**

To test the significance of DC Piezo1-dependent T cell differentiation in ant-tumor immunity, IL-12 and anti-TGFβ1 antibody were locally injected into the tumor once a week to treat tumor-bearing mice. Although the rate of tumor growth was significantly faster and greater in Piezo1−/− than in WT mice, IL-12 or anti-TGFβ1 antibody treatment significantly inhibited the tumor growth caused by Piezo1−/− (Fig. 7A). Consistently, Piezo1−/− tumor-bearing mice had more Foxp3+ Treg cells and fewer IFNγ+ Th1 cells in tumor tissue compared with WT control. However, IL-12 or anti-TGFβ1 antibody treatment reversed these alterations induced by Piezo1−/− (Fig. 7B). Thus, these data suggest IL-12 and TGFβ1 are critical for DC Piezo1-dependent T cell differentiation in regulating cancer growth.

Next, we test to apply a pharmacological approach to target Piezo1 in human DCs and determine whether we can recapitulate our finding in genetic targeting Piezo1. Piezo1 expressions were determined in human DC cells, which is from human peripheral blood monocytes. Inflammatory LPS or 50 kPa-conditioned hydrogels alone or together significantly upregulated Piezo1 expressions (Fig. 7C). Further, we applied Piezo1 agonist Yoda1 to human DC-T cell coculture
system, whereby T cells were isolated from human cord blood. The pharmacological activation of Piezo1 in human DCs largely recapitulated what we observed in genetic mouse DCs in term of the production of IL-12 and TGFβ1 in human DC (Fig. 7D-E) and the alteration of Foxp3+T_{reg} and IFNγ+T_{H1} in human T cells (Fig. 7F-H). Thus, our data demonstrated that Piezo1 mediated an evolutionary conserved signaling pathway in both mouse and human DCs.
Discussion

DCs play a central role in initiating first-line innate immunity and inducing subsequent adaptive immunity in protecting against tumorigenesis (26, 27). As a professional APCs, DCs can efficiently shape antigen-specific adaptive immune responses by presenting various exogenous and endogenous antigen stimuli, regulating cell surface costimulatory molecule expression, and producing cytokines and chemokines (28, 29). Innate inflammatory stimuli include infectious factors, oxygen, nutrient availability, and even force and pressure, often change the DC responses and affect the immune outcome in diseases. Especially, tumor microenvironment usually integrates different innate inflammatory and stiffness stimuli and develop a complex stimulation microenvironment, but how does DC responses to inflammatory and stiffness stimuli and regulates T cell differentiation in tumor remains unclear. Here, our data revealed that the mechanical sensor Piezo1, a signal node, responds to innate inflammatory and/or stiffness stimuli and integrates both the SIRT1-HIF1α-glycolysis metabolic signaling axis and calcium-calcineurin-NFAT signaling in DCs to drive TH1 differentiation while inhibiting Treg lineage commitment in inhibiting tumor growth in the context of complex tumor microenvironment. The changes in IL-12Rβ2/TGFβR2 expression and downstream STAT4/SMAD3 signaling in responding T cells further result in strong DC-T cell crosstalk, indicating the differentiation of Treg and TH1 cells (Fig.7-Fig. supp. 1). Thus, our results contribute to a more comprehensive
understanding of the immunopathological process of DC Piezo1-derived T cell differentiation in the tumor microenvironment.

Recent studies have suggested that Piezo1 is involved in regulating many of diseases, including the infectious inflammation and cancer(30-37). Piezo1 modulates macrophage polarization and stiffness sensing, which are related to calcium influx and the promotion of macrophage activation by actin(17). The Piezo1-mediated response to LPS inflammatory stimulation regulates cell activation (6). Global inhibition of Piezo1 with a peptide inhibitor showed protective effects against both cancer and septic shock(16). In addition to inflammatory stimulation, Piezo1 also showed immune regulatory effects on mechanical signals. Cyclical hydrostatic pressure initiates an inflammatory response via the mechanically activated ion channel Piezo1 (5). Additionally, mechanical stiffness controls DC metabolism and function(18). Although these studies have clearly shown that Piezo1 respond to inflammatory stimulation as well as induce an immune response to mechanical stimulation and regulate immune cell functions, especially in innate immune cells, it is still unclear how to target DCs, to direct T cell differentiation in cancer. Our results showed that Piezo1 responds to inflammatory stimulation or stiffness signals, and subsequently, Piezo1 effectively integrates metabolism signals and ion signals pathway including SIRT1-HIF1α-glycolysis and calcium influx and the calcium-calcineurin-NFAT signaling pathway to direct the differentiation of T_{reg}
and TH1 cells by regulating the production of DC-derived polarizing cytokines, including IL-12 and TGFβ1, in the context of tumor microenvironment (Fig.7-Fig. supp. 1).

Transcriptional factor HIF1α has been implicated as a critical proinflammatory signaling module in myeloid leukocytes(38-43). Consistent with recent findings that SIRT1 is responsible for the deacetylation and destabilization of HIF1α (44, 45). HIF1α is critically involved in regulating Piezo1-induced innate immune cell function(5). The metabolic mechanism is probably critical in regulating DC function(18). HIF1α-dependent glycolysis metabolism is also critical for regulating TH9 differentiation and MDSC development and function(46). The present data showed that Piezo1 could target SIRT1-HIF1α-glycolysis metabolism signaling to modulate DC-derived polarizing cytokine secretion. Ion channel Piezo1 is sensitive to calcium influx in regulating immune cell function (17). These data also showed that blocking calcium influx significantly altered Piezo1-mediated DC-derived cytokine secretion. Previous studies have displayed the roles of calcineurin-NFAT in MDSCs in regulating T_{reg} function(22). Here, we further showed that Piezo1 targeting the calcineurin-NFAT axis modulates DC-derived polarizing cytokine production to direct T_{reg} and TH1 cell differentiation in cancer.

Metabolic regulation and cellular signals are closely and generally associated
with the immune response, but there are still few studies on the regulation of the calcium signaling pathway and immune response (47-49). Our data showed that Piezo1 integrates the metabolic signaling and calcium signaling pathways to modulate DC-derived polarizing cytokine production in the context of cancerous inflammation. Effective immune responses require DCs to function under various conditions, including altered extracellular mechanical tension states, intracellular metabolic states, and ion levels (possibly caused by inflammatory stimulation) or due to migration, nutritional and/or hypoxic environments (tumor microenvironment). The adaptation of DCs to changing metabolic states and calcium signaling results from a mechanism of a ‘mechanical sensor checkpoint’, an active signaling process involved in sensing changes in metabolic and intracellular calcium levels and subsequent signaling transduction and execution (50-53). Our data further suggested that the mechanical sensor Piezo1 in DCs requires the interplay of metabolic and intracellular calcium checkpoints, including the metabolic SIRT1-HIF1α-glycolysis pathway and a sensitive calcium signaling pathway, calcium-calcineurin-NFAT signaling. Therefore, Piezo1 modulator provides a new choice in the research of tumor immune microenvironment intervention, which takes DCs as the target to regulate T cell responses. For example, Yoda1, a Piezo1 activator, can be used to respond to inflammatory and stiffness signals in tumors, and integrate a variety of intracellular signals to effectively direct T cell differentiation in protecting against tumor growth.
### Materials and Methods

#### key resources table

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<td>Jackson Laboratory</td>
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Mice. All animal experiments were approved by the Animal Ethics Committee of Fudan University, Shanghai, China, Beijing Institute of Microbiology and Epidemiology and Beijing Normal University (IACUC-DWZX-2017-003 and CLS-EAW-2017-002) Beijing, China. C57BL/6 Piezo1^{flox/flox}, CD11c-Cre, Piezo1^{-/}, Sirt1^{flox/flox} and Hif1a^{flox/flox} mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). OT-II TCR-transgenic mice were obtained from the Center of Model Animal Research at Nanjing University (Nanjing, China). CD45.1 mouse was obtained from Beijing University Experimental Animal Center (Beijing, China). C57BL/6 mice were obtained from Fudan University Experimental Animal Center or Beijing University Experimental Animal Center (Beijing, China). All the mice had been backcrossed to the C57BL/6 background for at least eight generations and were used at an age of 6–12-week age old. WT control mice were of the same genetic background and, where relevant, included Cre^{+} mice to account for the effects of Cre (no adverse effects due to Cre expression itself were observed in vitro or in vivo).

Tumor model. To establish subcutaneous tumors, 5 x 10^5 MC38 or MC38-OVA tumor cells or 4 x 10^5 B16.F10 melanoma cells were injected into C57BL/6 mice, half male and half female, randomization group. These cells formed a tumor of 1 to 2 cm diameter within 2 to 4 weeks of injection and double blinding detection of mouse tumor size.
**T cell isolation from tumor.** Tumor tissues were cut into pieces and suspended using collagenase D (Worthington, NJ, USA; 400U/ml) and deoxyribonuclease I (DNase I; Beyotime Biotechnology, Shanghai, China; 4U/ml) in 2 ml complete RPMI 1640 medium (Corning). After incubated for 30 mins in a shaker at 37°C, the homogenized tissue was passed through a cell strainer (70 μm; BD Pharmingen). After centrifugation at 200 g for 5 mins, the supernatant was discarded, and the pellet was resuspended with 3 ml of serum-free RPMI 1640. Add 3 ml 70% Percoll (GE Healthcare) to 15 ml centrifuge tube, then gently add 3 ml 40% Percoll to it, and finally slowly add 3 ml cell suspension to it to form a complete interface between the three layers. Centrifugation at 400 g for 25 min with slow acceleration and without breaks created a gradient. The interface cells between two density Perocoll (about 2-4 ml) were collected, washed with PBS for 2 times and stained with T cell antibodies for sorting by flow cytometry and prepared for further analysis.

**Cell isolation from gut-associated lymphatic tissues.** Isolation of lamina propria (LP) lymphocytes was performed as previously(54). The small intestine and large intestine were removed, opened longitudinally, and cut into pieces. After vigorous shaking in HBSS containing EDTA, the supernatants containing epithelial cells and IELs was discarded. The remaining intestinal pieces were digested with collagenase D (Worthington) and pelleted. The pellet was resuspended and placed in a Percoll gradient as described above, and after
centrifugation, the interface containing the LP lymphocytes was collected and
prepared for further analysis.

**Cell adoptive transfer.** Naïve T cells (CD4+TCR+CD62LhiCD44loCD25-) from
C57BL/6 mice or OT-II TCR-transgenic mice were sorted and transferred into
recipient mice. After 24 hrs, the recipient mice were injected s.c. with WT and
*Piezo1*ΔDC DCs mixed with OVA323-339 in the presence of complete Freund’s
adjuvant (CFA; Difco), LPS (Sigma), or 50 kPa hydrogel-conditioned DCs. At
Days 8-9 after immunization, DLN cells were harvested and stimulated with
their cognate peptides for 2-3 days prior to cytokine mRNA expression and
secretion analyses or pulsed with PMA-ionomycin for 5 hrs prior to the
intracellular staining of donor-derived T cells.

**Cell Cultures and Flow Cytometry.** Spleens were digested with collagenase D,
and DCs (CD11c+TCR’CD19’NK1.1’F4/80’Ly6G’) were sorted with a FACSaria
II (Becton Dickinson, San Diego, CA, USA). Naïve T cells were sorted from
spleen or PLN. For DC-T cell cocultures, DCs and T cells (1:10) were mixed in
the presence of 1 μg/ml OVA323-339 peptide and 100 ng/ml LPS. After 5 days of
culture, live T cells were stimulated with PMA and ionomycin for intracellular
cytokine staining or with plate-bound α-CD3 to measure cytokine secretion and
mRNA expression. T cell proliferation was determined by pulsing cells with
3H-thymidine for the final 12-16 hrs of culture, as previously(55). For drug
treatments, the cells were incubated with vehicle, cyclosporin A (CsA, 10 nM; Sigma), Yoda1 (25 μM, MCE), 11R-VIVIT (100 nM, MCE), ruthenium red (30 μM, Sigma), gadolinium chloride (10 μM, Sigma), 2-deoxy-D-glucose (2-DG, 1 mmol/l, Sigma) or diethyl succinate (1 mmol/l, Sigma) for 0.5-1 hrs before stimulation. For antibody or cytokine treatment, cultures were supplemented with IL-12 (10 μg/ml, Peprotech) and anti-TGFβ1 mAb (20 μg/ml, R&D Systems). Flow cytometry was performed with the following antibodies from eBioscience, BD Biosciences or Abcam: anti-CD11c FITC (N418), anti-CD11c PE (N418), anti-CD11c FITC (N418), anti-CD4 APC-Cy7 (GK1.5; Cat#130-109-536, RRID:AB_2657974), anti-CD8α FITC (53-6.7), anti-CD11b FITC (M1/70), anti-Ly6G PE (RB6-8C5; Cat# ab25378, RRID:AB_470493), anti-F4/80 PE (BM8), anti-CD19 PE (1D3; Cat#340418, RRID:AB_400423), anti-TCR FITC (H57-597), anti-CD44 FITC (IM7), anti-CD62L APC (MEL14), anti-CD80 APC (1C10), anti-CD54 FITC (YN1/1/7.4), anti-MHCII (AF6-120), anti-CD45 APC (30-F11), anti-NK1.1 PE (PK136), anti-CCR7 APC (4B12, Cat#A18389, RRID: AB_2535249), anti-PDL1 PE (MIH5, Cat# 12-5982-82), anti-PDL2 PE (TY25, Cat# 12-5986-82), anti-IFNγ PE (XMG1.2), anti-IL-4 PE (11B11), anti-Foxp3 PE (FJK-16s), anti-mouse IL-12p40 mAb (241812; Cat#BE0051, RRID:AB_1107698) and anti-mouse TGFβ1 mAb (EPR21143). Flow cytometry data were acquired on a FACSCalibur (Becton Dickinson, CA, USA) and the data were analyzed with FlowJo (RRID:SCR_008520; Tree Star, San Carlos, CA, USA).
Phagocytosis Assay. One-micrometer latex microspheres (Polysciences, Inc.) were incubated overnight with 1 mg/ml of mouse IgG-FITC (Jackson ImmunoResearch Laboratories). After rinsing the unbound antibody, the microspheres were added to DCs cultured at a ratio between DC and beads is 1:10 and incubated for 30 min in a humidified incubator at 37°C and 5% CO₂. Unbound or attached noninternalized beads were washed in consecutive rinsing steps with cold PBS. DCs were then stained with anti-CD11c, rinsed in cold PBS. The phagocytosis percentage of DCs were determined with flow cytometry.

Hydrogel-coated plates. Dow Corning Sylgard 527 (Part A and B, Sigma–Aldrich) was used to prepare PDMS hydrogel-coated plates. Part A and Part B of the gel were mixed to achieve the appropriate tension, as described (5, 18, 20). For the 2 kPa gel, the ratio of A:B was 1:2, and for the 50 kPa gel, the ratio of A:B was 0.3. The plates were coated with the hydrogel and incubated overnight at 60 °C. Then, the gels were coated with fibronectin (1 μg/ml, Sigma) for 4 hrs at 37 °C and washed again with PBS.

Oxygen consumption analysis. Cells were plated in 24-well Seahorse plates at 2 x10⁵ cells per well, and a negative control well containing only media without cells was included. A utility plate containing calibrant solution (1 ml/well)
together with the plates containing the injector ports and probes was incubated in a CO\textsubscript{2}-free incubator at 37 °C overnight. The following day, the medium was removed from the cells and replaced with glucose-supplemented XF assay buffer (500 μl/well), and the cell culture plate was incubated in a CO\textsubscript{2}-free incubator for at least 0.5 hrs. Inhibitors (oligomycin, carbonyl cyanide-4-[trifluoromethoxy] phenylhydrazone, 2-DG, and rotenone (70 μl) were added to the appropriate port of the injector plate. This plate, together with the utility plate, was run on the Seahorse for calibration. Then, the utility plate was replaced with the cell culture plate, and the cell culture plate was analyzed on the Seahorse XF-24 instrument.

**Measurement of intracellular Ca\textsuperscript{2+} concentrations.** The intracellular Ca\textsuperscript{2+} concentrations ([Ca\textsuperscript{2+}]) were measured fluorometrically using the fluorescent calcium indicator dye Fura2 AM (Sigma), as previously described (17). Cells were incubated with 5 μM Fura2 AM in HBSS supplemented with 110 mM NaCl, 5 mM KCl, 0.3 mM Na\textsubscript{2}HPO\textsubscript{4}, 0.4 M KH\textsubscript{2}PO\textsubscript{4}, 5.6 mM glucose, 0.8 mM MgSO\textsubscript{4}, 7 mM H\textsubscript{2}O, 4 mM NaHCO\textsubscript{3}, 1.26 mM CaCl\textsubscript{2}, and 15 mM HEPE, at pH 7.4, at room temperature for 60 min.

**RNA and protein expression analysis.** RNA was extracted with a RNeasy kit (QIAGEN, Dusseldorf, Germany), and cDNA was synthesized using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA). An ABI 7900
real-time PCR system was used for quantitative PCR, with primer and probe
sets obtained from Applied Biosystems (Carlsbad, CA). The results were
analyzed using SDS 2.1 software (Applied Biosystems). The cycling threshold
value of the endogenous control gene (Hprt1, which encodes hypoxanthine
guanine phosphoribosyl transferase) was subtracted from the cycling threshold
(ΔC_T). The expression of each target gene is presented as the fold change
relative to that of control samples (2^-ΔΔCT). For the detection of phosphorylated
signaling proteins, purified cells were activated with LPS (Sigma), immediately
fixed with Phosflow Perm buffer (BD Biosciences) and stained with
phycoerythrin or allophycocyanin directly conjugated to antibodies against Erk
phosphorylated at Thr202 and Tyr204 (20A; Cat#612566, RRID:AB_399857;
BD Biosciences), p38MAPK phosphorylated at Thr180 and Thr182 (D3F9; Cell
Signaling Technology), JNK phosphorylated at Thr183 and Tyr185 (G9; Cell
Signaling Technology), STAT4 phosphorylated at Tyr701 and Ser727 (58D6;
Cell Signaling Technology), and SMAD3 phosphorylated at Tyr705 and Ser727
(D3A7; Cell Signaling Technology), as described (55). Intracellular staining
analysis was performed as described(54) using anti-HIF-1α (EPR16897;
Abcam) and anti-SIRT1 (17A7AB4; Abcam) antibodies.

IL-12Rβ2 and TGFβR2 knockdown with RNAi. A gene-knockdown lentiviral
construct was generated by subcloning gene-specific short hairpin RNA
(shRNA) sequences into lentiviral shRNA expression plasmids (pMagic4.1) as
described (46). Lentiviruses were harvested from the culture supernatant of 293T cells (KCB Cat# KCB 200744YJ, RRID: CVCL_0063) transfected with shRNA vector. Sorted OT-II CD4⁺ T cells were infected with the recombinant lentivirus, and green fluorescent protein-expressing cells were isolated using fluorescence sorting 48 hrs later. IL-12Rβ2 and TGFβR2 expression was confirmed using real-time PCR. The sorted T cells expressing either control or shRNA vectors were used for functional assays.

**Human DC and T-Cell Cultures.** For assays of human DC-mediated T-cell differentiation, normal human DCs (CC-2701; Lonza) were cultured and their populations were expanded for 5 days with human granulocyte-macrophage colony-stimulating factor and IL-4 (R&D system), followed by treatment with Yoda1 (25 μM, MCE) and stimulation for 24 hrs with LPS. DCs were washed extensively and cultured with human cord blood CD4⁺ T cells (2C-200; Lonza) at a ratio of 1:10. After 7 day of culture, live T cells were purified and then stimulated either with PMA and ionomycin for intracellular cytokine staining for 5 hrs or with plate-bound anti-CD3 for analysis of mRNA expression.

**Statistical analysis.** All the data are presented as the mean ± SD. Student’s unpaired t test was used for the comparison of means to evaluate differences between groups. A P value (alpha-value) of less than 0.05 was considered statistically significant.
Acknowledgements

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COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.
References


Figure legends

Figure 1. DC-specific Piezo1 regulates T cell differentiation in cancer.

(A) MC38 tumor cells were implanted subcutaneously in WT and Piezo1<sup>−/−</sup> mice (n=10) and tumor size was measured every 5 days for 40 days. (B) Intracellular staining of IFNγ, IL-4, IL-17A and Foxp3 expression by CD4<sup>+</sup> T cells sorting from the tumor of WT and Piezo1<sup>−/−</sup> tumor-bearing mice at Day 40. (C) mRNA expression of the indicated genes by CD4<sup>+</sup> T cells isolated from the draining lymph nodes (dLNs) of tumor from WT and Piezo1<sup>−/−</sup> tumor-bearing mice on the indicated days (the levels in WT mice at Day 20 were set to 1). (D) MC38 OVA tumor cells were implanted subcutaneously in WT and Piezo1<sup>−/−</sup> mice (n=10) and at Day 20, the CD45.1<sup>+</sup> donor CFSE<sup>+</sup>OT II CD4<sup>+</sup>T cells were transferred into WT and Piezo1<sup>−/−</sup> tumor-bearing mice for 10 days. The CD45.1<sup>+</sup> CFSE<sup>+</sup> donor T cells from tumors were analyzed and the intracellular staining of IFNγ, IL-4, IL-17A and Foxp3 expression among CFSE<sub>low</sub> donor T cells. The data are representative of three to four independent experiments (mean ± s.d.; n=4). **P<0.01, and ***P<0.001, compared with the indicated groups.

Figure 1-source data 1. Tumor size in WT and Piezo1<sup>−/−</sup> tumor-bearing mice.

Figure 1-figure supplement 1. Piezo1 expressions of DCs following innate stimuli. Il12b and Tgfb1 mRNA (A & C) and Piezo1 mRNA (B) expression of splenic DCs from C57BL/6 or/and Piezo1<sup>−/−</sup> mice with the indicated treatment (LPS, 10 ng/ml or conditioned with 2 kPa or 50 kPa-hydrogels plate). Levels in the vehicle group were set to 1. Data are representative three to four
independent experiments (mean ± s.d.; n=3-4). ***P<0.001, compared with the indicated groups.

**Figure 1-figure supplement 2. DC-specific Piezo1 deficiency reduced T cell activities in aged mice.** (A) Body weights of WT and Piezo1<sup>+/−</sup> mice are shown (n=20). (B-C) Piezo1 deficiency in DCs resulted in less CD44<sup>high</sup>CD62<sup>low</sup> cells in the CD4<sup>+</sup> T cell population than WT DCs. A typical figure shown in B and data summarized in C. The data are representative of three independent experiments (mean ± s.d.; n=4-20). *P<0.05 and ***P<0.001, compared with the indicated groups.

**Figure 1-figure supplement 3. DC-specific Piezo1 deficiency alters T cell differentiation in the aged mice.** Intracellular IFNγ, IL-4, IL-17A and Foxp3 expression by CD4<sup>+</sup> T cells from the MLN, PP, IEL and LPL in WT and Piezo1<sup>+/−</sup> mice. A representative figure shown (A), and the percentage of positive cells summarized (B). The data are representative of three independent experiments (mean ± s.d.; n=4-5). ***P<0.001, compared with the indicated groups.

**Figure 1-figure supplement 4. DC-specific Piezo1 regulates T cell differentiation in cancer.** MC38 tumor cells were implanted subcutaneously in WT and Piezo1<sup>+/−</sup> mice (n=10). (A) CD8<sup>+</sup>T cell number in tumor of WT and Piezo1<sup>+/−</sup> tumor-bearing mice at Day 40. (B) Intracellular staining of IFNγ expression of CD8<sup>+</sup> T cells from the tumor of WT and Piezo1<sup>+/−</sup> tumor-bearing mice at Day 40. The representative data from flow cytometry analysis shown (left), and statistical results summarized (right). (C) mRNA expression of the
indicated genes by CD4+ T cells isolated from the draining lymph nodes (dLNs) of tumor from WT and Piezo1−/− tumor-bearing mice on the indicated days (the levels in WT mice at Day 20 were set to 1). The data are representative of three independent experiments (mean ± s.d.; n=4). n.s., not significant.

**Figure 1-figure supplement 5. DC-specific Piezo1 regulates T cell differentiation in cancer.** (A) B16.F10 tumor cells were implanted subcutaneously in WT and Piezo1−/− mice (n=10) and tumor size was measured every 5 days for 30 days. (B) Intracellular staining of IFNγ, IL-4, IL-17A and Foxp3 expression of CD4+ T cells sorted from the tumor of WT and Piezo1−/− tumor-bearing mice at Day 30. The representative data from flow cytometry analysis shown (left), and statistical results summarized (right). The data are representative of three to four independent experiments (mean ± s.d.; n=4).

***P<0.001, compared with the indicated groups.

**Figure 2.** DC-specific Piezo1 expression directs the differentiation of TH1 and Treg cells in vivo.

(A-C) Naïve CD45.2+ T cells were transferred into CD45.1+ C57BL/6 WT mice, and the mice were immunized with WT and Piezo1−/− splenic DCs and LPS. DLN cells were analyzed at Day 7 after immunization. (A) Donor CD45.2+ T cell proliferation after stimulation with anti-CD3 (2 μg/ml) and anti-CD28 (2 μg/ml) antibodies. (B-C) Intracellular staining of IFNγ, IL-4, IL-17A and Foxp3 expression in donor-derived (CD45.2+) CD4+ T cells after PMA and ionomycin
stimulation. A representative figure shown in B, and the data summarized in C. 

(D-F) Naïve OT-II T cells were transferred into CD45.1+ C57BL/6 WT mice, and the mice were immunized with WT and Piezo1<sup>−/−</sup> splenic DCs and OVA+CFA. DLN cells were analyzed at Day 7 after immunization. (D) Donor CD45.2+ T cell proliferation after stimulation with OVA. (E-F) Intracellular staining of IFNγ, IL-4, IL-17A and Foxp3 in donor-derived (CD45.2+) CD4<sup>+</sup> T cells after OVA stimulation. A representative figure shown in E, and the data summarized in F. 

(G-H) Splenic DCs isolated from WT and Piezo1<sup>−/−</sup> mice were plated on 2 kPa and 50 kPa hydrogels and incubated for 24 hrs. (G) Naïve T cells were transferred into CD45.1+ C57BL/6 WT mice, and the mice were immunized with 2 kPa and 50 kPa hydrogel-conditioned DCs. DLN cells were analyzed at Day 7 after immunization. Intracellular staining of IFNγ, IL-4 and IL-17A in donor-derived (CD45.2+) CD4<sup>+</sup> T cells after PMA and ionomycin stimulation. A representative figure shown on the left, and the data summarized on the right. 

(H) Naïve OT-II T cells were transferred into CD45.1+ C57BL/6 WT mice, and the mice were immunized with 2 kPa or 50 kPa hydrogel-conditioned DCs and OVA+CFA. DLN cells were analyzed at Day 7 after immunization. Intracellular staining of IFNγ, IL-4 and IL-17A in donor-derived (CD45.2+) CD4<sup>+</sup> T cells after OVA stimulation. A representative figure shown on the left, and the data summarized on the right. The data are representative of three to four independent experiments (mean ± s.d.; n=3-4). ***P<0.001, compared with the indicated groups.
Figure 3. **Piezo1 regulates IL-12 and TGFβ1 production by DCs to direct TH1 and Treg cell differentiation.**

(A-B) Intracellular staining of IL-12p40 and TGFβ1 expression in WT and *Piezo1*\(^{-/-}\) splenic DCs after 5 hrs of treatment with LPS (A; 10 ng/ml) or culture on 2 kPa and 50 kPa hydrogels (B). A representative figure shown on the left, and the data summarized on the right. (C) Intracellular staining of IFNγ and Foxp3 in T cells cocultured with WT and *Piezo1*\(^{-/-}\) splenic DCs in the presence of the indicated treatments (IL-12, Peprotech, 10 μg/ml or anti-TGFβ1, R&D Systems, 20 μg/ml) for 5 days. A representative figure shown on the left, and the data summarized on the right. (D) Intracellular staining of IFNγ (upper panel) and Foxp3 (lower panel) in T cells cocultured with WT and *Piezo1*\(^{ ADC}\) splenic DCs conditioned with 50 kPa hydrogel and the indicated treatments for 5 days and data are summarized. The data are representative of three independent experiments (mean ± s.d.; n=3-5). \(* * *P<0.001,\) compared with the indicated groups.

Figure 3-figure supplement 1. DC-specific Piezo1 expression directs TH1 and Treg differentiation in vitro. (A-B) C57BL/6 naïve CD4\(^+\) T cells were cocultured with LPS-pulsed splenic CD11b\(^+\) DCs (A) or CD8\(^+\) DCs (B) from WT and *Piezo1*\(^{-/-}\) mice for 5 days. Intracellular staining of IFNγ and Foxp3 in T cells. A representative figure shown on the left, and the data summarized on the right. (C-D) Naïve CD4\(^+\) T cells from OT II mice were cocultured with LPS-pulsed
splenic CD11b⁺ DCs (C) or CD8⁺ DCs (D) from WT and Piezo1⁻/⁻ mice for 5 days. Intracellular staining of IFNγ and Foxp3 in T cells. A representative figure shown on the left, and the data summarized on the right. The data are representative of three independent experiments (mean ± s.d.; n=3-6). ***P<0.001, compared with the indicated groups.

**Figure 3-figure supplement 2. DC-specific Piezo1 expression directs Th1 and Treg differentiation in vitro.** (A) Naïve T cells from C57BL/6 mice were cocultured with splenic DCs from WT and Piezo1⁻/⁻ mice for 5 days in the presence of anti-CD3 (2 ng/ml) and LPS (10 ng/ml). The relative mRNA expression of the indicated genes in T cells was determined with qPCR. The levels in the WT group and on Day 0 were set to 1. (B) splenic CD11b⁺ DCs isolated from WT and Piezo1⁻/⁻ mice were plated on 2 kPa and 50 kPa hydrogels (1 x 10⁵ cells/well) for 24 hrs. Naïve CD4⁺ T cells from OT II mice were cocultured with 2 kPa and 50 kPa hydrogel-conditioned DCs in the presence of OVA (5 μg/ml) for 5 days. Intracellular staining of IFNγ, IL-4, IL-17A and Foxp3 in T cells. (C) Naïve T cells from OTII mice were cocultured with 2 kPa and 50 kPa hydrogel-conditioned splenic CD11b⁺ DCs in the presence of OVA (5 μg/ml) for 5 days. The relative mRNA expression of the indicated genes in T cells was determined with qPCR. The levels in the WT group with 2 kPa were set to 1. The data are representative of three independent experiments (mean ± s.d.; n=4-6). ***P<0.001, compared with the indicated groups.

**Figure 3-figure supplement 3. Piezo1 regulates DC homeostasis and**
function. (A) 7-AAD and Annexin V staining in WT and Piezo1\(^{-/-}\) splenic DCs. The representative data from flow cytometry analysis shown (left), and data summarized (right). (B-C) Expression of indicated molecules in WT and Piezo1\(^{-/-}\) splenic DCs in the presence of LPS (10 ng/ml) from flow cytometry analysis. (D) Phagocytosis of WT and Piezo1\(^{-/-}\) splenic DCs to IgG-coated beads in the presence of LPS (10 ng/ml). The representative data from flow cytometry shown (left), and data summarized (right). Data are representative three independent experiments (mean ± s.d.; n=3). n.s., not significant.

**Figure 3-figure supplement 4. Piezo1 regulates DC cytokine production.**

(A) mRNA expressions of \(\text{Il12}\) (p70) and \(\text{Tgfb1}\) expression in WT and Piezo1\(^{-/-}\) MLN DCs. (B) mRNA expressions of \(\text{Il12}\) (p70), \(\text{Il23}\) and \(\text{Tgfb1}\) expression in WT and Piezo1\(^{-/-}\) splenic DCs treated with LPS (10 ng/ml) for 5 hrs. (C) Production of IL-12p70 (left panel) and TGF\(\beta\)1 (right panel) in culture supernatant in WT and Piezo1\(^{-/-}\) splenic DCs treated with LPS (10 ng/ml) for 5 hrs. The data are representative of three independent experiments (mean ± s.d.; n=3). ***\(P<0.001\), compared with the indicated groups.

**Figure 4. TGF\(\beta\)R2-Smad3 and IL-12R\(\beta\)2-pSTAT4 are required for the T cell differentiation induced by DC-specific Piezo1 expression.**

(A) Expression of TGF\(\beta\)R2 and IL-12R\(\beta\)2 in T cells cocultured with WT or Piezo1\(^{-/-}\) splenic DCs for 5 days. A representative figure shown on the left, and the data summarized on the right. (B) Intracellular staining of p-Smad3 and
p-STAT4 in T cells cocultured with WT or Piezo1⁻/- splenic DCs for 5 days. A representative figure shown on the left, and the data summarized on the right. (C-D) Sorted naïve T cells were transfected with control, Tgfbr2 shRNA vector or Il12rb2 shRNA vector and cocultured with WT or Piezo1⁻/- DCs for 5 days. Intracellular staining of IFNγ (C; upper panel) and Foxp3 (C; lower panel) in T cells. A representative figure shown on the left, and the data summarized on the right. (D) Intracellular staining of p-Smad3 and p-STAT4 in T cells and data summarized. The data are representative of three independent experiments (mean ± s.d.; n=4). ***P<0.001, compared with the indicated groups.

Figure 4-figure supplement 1. Expressions of TGFβR1/2 and IL-12Rβ1 in T cells induced by Piezo1⁻/- DCs. Expression of TGFβR1, TGFβR3 (A) and IL-12Rβ1 (B) in T cells cocultured with WT or Piezo1⁻/- splenic DCs for 5 days. Data are representative three independent experiments (mean ± s.d.; n=4).

Figure 4-figure supplement 2. Knockdown of Tgfbr2 shRNA and Il12rb2 shRNA in T cells. Sorted CD4⁺ T cells were transfected with control or Tgfbr2 shRNA vector (A) or Il12rb2 shRNA vector (B) and stimulated with WT or Piezo1⁻/- splenic DCs for 5 days in the presence of LPS (10 ng/ml). Expression of indicated mRNA (A or B) were determined with qPCR (Levels of Ctrl siRNA were set to 1). Data are representative of three independent experiments (mean ± s.d.; n=3). ***P<0.001 compared with the indicated groups.

Figure 5. Piezo1 regulates TGFβ1 and IL-12 production through the
**SIRT1-HIF1α-glycolysis pathway**

(A-B) Sorted splenic DCs from WT or *Piezo1*−/− mice were stimulated with LPS (10 ng/ml; A) or with 2 kPa or 50 kPa hydrogels (B) for 24 hrs in the presence or absence of 2-DG (1 mmol/l). The PPR was analyzed as a readout for glycolysis.

(C) mRNA expression of glycolytic molecules in splenic DCs from WT or *Piezo1*−/− mice treated with LPS (10 ng/ml) for 12 hrs. The levels in the WT control group were set to 1.

(D) Intracellular staining of p38, Erk, and JNK phosphorylation and SIRT1 and HIF1α expression in splenic DCs from WT or *Piezo1*−/− mice. A representative figure shown in the upper panel, and the data summarized in the lower panel.

(E) Splenic DCs from WT, *Piezo1*−/− (*Piezo1*−/−), *Sirt1*−/−, and *Piezo1/Sirt1* double knockout (DKO; *Piezo1*−/−*Sirt1*−/−) mice were stimulated with LPS (10 ng/ml). Intracellular staining of IL-12p40. A representative figure shown on the left, and the data summarized on the right.

(F) Splenic DCs from WT or *Hif1a*−/− mice were stimulated with LPS (10 ng/ml) in the presence or absence of Yoda1 (25 μM). Intracellular staining of IL-12p40. A representative figure shown on the left, and the data summarized on the right.

The data are representative of three independent experiments (mean ± s.d.; n=3-4). **P<0.01 and ***P<0.001, compared with the indicated groups.

**Figure 5-figure supplement 1. Piezo1 alters glycolytic metabolic signaling activities of DCs.** Sorted splenic DCs from WT mice were stimulated with LPS (10 ng/ml) or with 2 kPa or 50 kPa-hydrogels conditions for 24 hrs in the presence or absence of 2-DG (1 mM) or Yoda1 (25 μM). (A) The PPR was...
analyzed as a readout for glycolysis. (B) The OCR was analyzed as a readout for oxidative phosphorylation (OXPHOS). (C) mRNA expression of glycolytic molecules in splenic DCs from WT mice in the presence of LPS (10 ng/ml) and Yoda1 (25 μM). Levels in the vehicle treatment at Glut1 group were set to 1. Data are representative three independent experiments (mean ± s.d.; n=4). ***P<0.001, compared with the indicated groups.

Figure 5-figure supplement 2. Glycolysis activities are required for IL-12 and TGFβ1 production in DCs induced by Yoda1 treatment. (A-B) Sorted splenic DCs from WT mice were stimulated with LPS (10 ng/ml) or with 2 kPa or 50 kPa-hydrogels conditions for 24 hrs in the presence or absence of 2-DG (1 mM) or Yoda1 (25 μM). Intracellular staining of IL-12p40 (A) and TGFβ1 (B) expression with indicated treatment. Data are representative three independent experiments (mean ± s.d.; n=3). *P<0.05 and ***P<0.001, compared with the indicated groups.

Figure 5-figure supplement 3. Glycolytic metabolism activities are required for IL-12 and TGFβ1 production in DCs induced by Piezo1 deficiency. (A) Sorted splenic DCs from WT or Piezo1−/− mice were stimulated with LPS (10 ng/ml) or 2 kPa or 50 kPa hydrogels for 24 hrs in the presence or absence of succinate (5 mM). The OCR was analyzed as a readout for OXPHOS. (B-C) Sorted splenic DCs from WT or Piezo1−/− mice were stimulated with LPS (10 ng/ml) or with 50 kPa-hydrogels conditions for 24 hrs in the presence or absence of 2-DG (1 mM). Intracellular staining of IL-12p40 (B) and
TGFβ1 (C) expression with indicated treatment. Data are representative three
independent experiments (mean ± s.d.; n=3). ***P<0.001, compared with the
indicated groups.

Figure 5-figure supplement 4. TGFβ1, HIF1α and glycolysis activity
alteration in DCs induced by Piezo1 and SIRT1 deficiency. Splenic DCs
from WT, Piezo1−/−, Sirt1−/−, and Piezo1/Sirt1 double knockout (DKO) mice were
stimulated with LPS (10 ng/ml). (A) Intracellular staining of TGFβ1. (B)
Intracellular staining of HIF1α. (C) PPR was analyzed as a readout for
glycolysis. Data are representative three independent experiments (mean ±
s.d.; n=3-4). ***P<0.001, compared with the indicated groups.

Figure 5-figure supplement 5. TGFβ1, SIRT1 and glycolysis activity
alteration in DCs induced by Piezo1-HIF1α signaling. Splenic DCs from WT
or Hif1a−/− mice were stimulated with LPS (10 ng/ml) in the presence or absence
of Yoda1 (25 μM). (A) Intracellular staining of TGFβ1. (B) Intracellular staining
of SIRT1. (C) PPR was analyzed as a readout for glycolysis. Data are
representative three independent experiments (mean ± s.d.; n=3-4). ***P<0.001,
compared with the indicated groups.

Figure 6. Piezo1 regulates TGFβ1 and IL-12 production through the
calcium-calcineurin-NFAT axis

(A) Measurement of intracellular Ca^{2+} concentrations with Fura2 dye in splenic
DCs from WT or Piezo1−/− mice treated with LPS (10 ng/ml) or cultured on plates
containing 50 kPa hydrogels. (B) Intracellular Ca\(^{2+}\) concentrations measured with Fura2 in splenic DCs from WT mice after the indicated treatment (Yoda1, 25 μM, MCE; ruthenium red, 30 μM, Sigma; gadolinium chloride, 10 μM, Sigma).

(C-D) Intracellular staining of TGFβ1 (C) and IL-12p40 (D) in splenic DCs from WT mice after the indicated treatments. (E) Intracellular staining of IL-12p40 in splenic DCs from WT mice after the indicated treatments. A representative figure shown on the left, and the data summarized on the right. (F) Intracellular staining of NFATc1 in splenic DCs from WT mice after the indicated treatments (CsA, 10 nM). A representative figure shown on the left, and data summarized on the right. (G-H) Intracellular staining of IL-12p40 in splenic DCs from WT or Piezo1\(^{-/-}\) mice after the indicated treatments (Yoda1, 25 μM, MCE; 11R-VIVIT, 100 nM, MCE; CsA, 10 nM, Sigma). A representative figure shown on the left and data summarized on the right. The data are representative of three to four independent experiments (mean ± s.d.; n=3-4). ***P<0.001, compared with the indicated groups.

**Figure 6-figure supplement 1.** Piezo1 regulates TGFβ1 and IL-12 production through calcium signaling. (A) Intracellular Ca\(^{2+}\) using Fura2 in splenic DCs from WT or Piezo1\(^{-/-}\) mice with indicated treatment (Yoda1, 25 μM; Ruthenium red, 30 μM; Gadolinium chloride, 10 μM). (B-C) Intracellular staining of TGFβ1 (B) and IL-12p40 (C) in splenic DCs from WT or Piezo1\(^{-/-}\) mice with indicated treatments. Data are representative three independent experiments.
Figure 6-figure supplement 2. Piezo1 regulates TGFβ1 production through calcium-calcineurin-NFAT axis. (A or C) Intracellular staining of TGFβ1 in splenic DCs from WT mice with indicated treatments. (B) Intracellular staining of TGFβ1 in splenic DCs from WT or Piezo1<sup>−/−</sup> mice with indicated treatments (Yoda1, 25 μM; 11R-VIVIT, 100 nM; CsA, 10 nM). Data are representative three to four independent experiments (mean ± s.d.; n=3-5). ***P<0.001, compared with the indicated groups.

Figure 7. IL-12 and TGFβ1 are critical for DC piezo1-dependent T cell differentiation in promoting cancer growth.

(A) MC38 tumor cells were implanted subcutaneously in WT and Piezo1<sup>−/−</sup> mice (n=10), IL-12 100 ng or anti-TGFβ1 mAb 200 ng per mouse in 50 μl volume or vehicle (PBS) was locally injected into tumor once a week and tumor size was measured every 5 days for 40 days. (B) Intracellular staining of IFNγ, IL-17A and Foxp3 expression in CD4<sup>+</sup> T cells from the tumor of WT and Piezo1<sup>−/−</sup> tumor-bearing mice at Day 40. (C) Piezo1 mRNA expression of human DCs with the indicated treatment (LPS, 10 ng/mL or conditioned with 2 kPa or 50 kPa-hydrogels plate or LPS + 50 kPa-hydrogels plate). Levels in the vehicle group were set to 1. (D-E) IL-12p70 (D) and TGFβ1 (E) production of human DCs treated by LPS (10 ng/ml) for 5 hrs. (F-H) Human DCs pulsed with LPS (10 ng/ml) and LPS + 10 μM Yoda1.
ng/ml) were cocultured with human T cells for 5 days, in the absence or presence of Yoda1 (25 μM). The intracellular staining of IFNγ and Foxp3 in T cells. ***P<0.001 compared with the indicated groups. Data are representative of three independent experiments (mean ± s.d.; n=3-4). ***P<0.001, compared with the indicated groups.

**Figure 7-figure supplement 1. DC Piezo1 controls the differentiation of T\(^\text{H}1\) and T\(\text{reg}\) cells in cancer.** Proposed model of how Piezo1 in DCs responses to inflammatory stimuli or stiffness signals to regulate the differentiation of T\(^\text{H}1\) and T\(\text{reg}\) cell populations in regulating cancer growth.
Figure 1-figure supplement 1

A

B

C

ll12p40 mRNA (relative value)

Tgfβ1 mRNA (relative value)

Piezo1 mRNA (relative value)

mRNA (relative value)

ll12p40  

Tgfβ1

Vehicle  LPS  2 kPa  50 kPa  Plastic

Vehicle  LPS  2 kPa  50 kPa  Plastic

Vehicle  2 kPa  50 kPa

Vehicle  2 kPa  50 kPa
Figure 1-figure supplement 5

A

B16.F10 melanoma

- WT
- Piezo1⁻/⁻

Tumor size (mm²)

Time (days)

B

WT

Piezo1⁻/⁻

IFNγ
IL-17A

Cells (%)

IFNγ IL-4 IL-17A Foxp3

***
Figure 3-figure supplement 1

A

LPS Stimulation
CD11b+DC + C57BL/6 T cells

WT

8

11

IFNγ

FoXP3

IL-17A

CD4

Piezo1−/−

18

21.1

B

LPS stimulation
CD8+DC + C57BL/6 T

WT

IFNγ

FoXP3

Piezo1−/−

Cells (%)

***

***

C

LPS Stimulation
CD11b+DC + OTII T cells

WT

26

13.4

IFNγ

FoXP3

IL-17A

CD4

Piezo1−/−

17

28.2

D

LPS stimulation
CD8+DC + OTII T

WT

IFNγ

FoXP3

Piezo1−/−

Cells (%)

***

***

***

***
Figure 4-figure supplement 1

A

- WT
- Piezo1-/-

TGFβR1

MFI

B

- WT
- Piezo1-/-

IL-12Rβ1

MFI
Figure 5-figure supplement 1

A

Vehicle
Yoda1

PPR (pmoles/min)

LPS  LPS + 2-DG  2 kPa  2 kPa + 2-DG  50 kPa  50 kPa + 2-DG

***

B

Vehicle
Yoda1

OCR (pmoles/min)

WT  Yoda1

C

Vehicle
Yoda1

mRNA

Glut1  Gpi  Enol  LdhA  Mct4

***
Figure 5-figure supplement 4

A

Graph showing TGFβ1 (MFI) levels for WT, Piezo1−/−, Sirt1−/−, and DKO.

B

Graph showing HF1α (MFI) levels for WT, Piezo1−/−, Sirt1−/−, and DKO.

C

Graph showing PPR (pmoles/min) for WT, Piezo1−/−, Sirt1−/−, and DKO.
Figure 5-figure supplement 5

A

B

C

\( \text{TGF}\beta_1 \) (MFI)

\( \text{SIRT1} \) (MFI)

\( \text{PPR} \) (pmoles/min)

WT  Yoda1  Hif1a\(^{-} \)  Yoda1; Hif1a\(^{-} \)

WT  Yoda1  Hif1a\(^{-} \)  Yoda1; Hif1a\(^{-} \)

WT  Yoda1  Hif1a\(^{-} \)  Yoda1; Hif1a\(^{-} \)

***

n.s.
Figure 6-figure supplement 2

A

B

C

TGFβ1 (MFI)

Veh Yoda1 CsA Yoda1+ CsA

WT Piezo1−/− CsA VIVIT

WT Yoda1 VIVIT Yoda1+ VIVIT

***

***