LKB1 Positively Regulates Dendritic Cell-induced T Cell Immunity and Suppresses Tumor Development

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ABSTRACT

Background: The functions of dendritic cells (DCs) are influenced by their intracellular metabolism, in which liver kinase B1 (LKB1) plays an important role. However, due to the difficulty in isolating the DCs, the roles of LKB1 in DC maturation and functions in tumor settings have been poorly characterized.

Objective: To investigate the roles of LKB1 in DC functions including phagocytosis and presentation of antigens, activation, T cell differentiation, and ultimately tumor eradication.

Methods: Genetic modification of *Lkb1* in the DCs was made by lentiviral transduction, and their impacts on T cell proliferation, differentiation, activity, or B16 melanoma metastasis were examined by flow cytometry, qPCR, or lung tumor nodule counting.

Results: LKB1 did not affect antigen uptake and presentation by the DCs, but facilitated the stimulation of T cell proliferation. Interestingly, following T cell activation, Foxp3-expressing regulatory T cells (Treg) were increased (P=0.0267) or decreased (P=0.0195) in mice injected with *Lkb1* knockdown DCs or overexpressing DCs, respectively. Further exploration revealed that LKB1 inhibited OX40L (P=0.0385) and CD86 (P=0.0111) expression, and these co-stimulatory molecules enhanced Treg proliferation, and downregulated immune suppressive cytokine IL-10 (P=0.0315). Moreover, we found that the injection of the DCs with limited LKB1 expression before tumor inoculation could reduce their production of granzyme B (P<0.0001) and perforin (P=0.0042) from CD8^{+}T cells, thereby impairing their cytotoxicity and promoting tumor growth.

Conclusion: Our data suggest that LKB1 can enhance DC-mediated T cell immunity by restraining Treg development and thereby suppressing tumor growth.

Keywords: Dendritic Cells; LKB1; Treg; Tumor Immunology

INTRODUCTION

As a highly conserved and widely expressed serine-threonine kinase to modulate various AMP-activated protein kinase superfamily members, as well as their downstream kinase, liver kinase B1 (LKB1), is involved in controlling cell growth, metabolism, cellular responses to energy stress and polarization (1-4). Therefore, LKB1 is important for regulating the survival of hematopoietic stem cells, neuromuscular development, polycystic kidneys, and fibrosis (1, 5-7). Not only that, but also the deletion or mutation of the *Lkb1* gene has been frequently reported in various cancers from different tissues (8-11). Therefore, LKB1 is also termed a tumor suppressor. The immune system is an important defense line to inhibit tumor growth. Dendritic cells (DCs) serve as the most effective antigen-presenting cells (APCs) in all immune cells. It has a significant impact on stimulating the immune responses. When DCs are stimulated by antigens, they undergo migration and become mature. Mature DCs highly express co-stimulatory molecules and major histocompatibility complex (MHC) molecules, to initiate, modulate and maintain immune responses (12). Moreover, DCs can secrete a variety of T cell polarizing factors to trigger the differentiation of Th1 (13), Th2 (14), Th17 (15), Treg, etc. (12). Recently, a critical role of intracellular metabolic pathways in DC activation and immunogenicity has been noticed (16, 17). The immunogenicity of DCs relies on a synthetic metabolic process driven by glycolysis, while the resting and tolerogenic DCs are associated with the more catabolic type of metabolism, represented by enhanced fatty acid oxidation and mitochondrial oxidative phosphorylation (18-20). Since LKB1 is a key factor in regulating intracellular energy metabolism, it is vital to study its effects on immune cells, including DCs, the most powerful APC in host immunity.

Recently the regulation of immune cell function by LKB1 has been increasingly reported. For example, the precursors of T cells lacking LKB1 fail to develop normally (21, 22); Tregs with *Lkb1* knockout cannot stably express Foxp3 and cannot play an immunosuppressive role (23-25); LPS-induced inflammation was more severe in myeloid cell-specific *Lkb1* knockout mice than in wild-type mice (26, 27). There are also reports about the effect of LKB1 on DC functions. The specific knockout of *Lkb1* in CD11c+ cells can lead to an increase of CD8+DCs and a decrease of CD11b+DCs (28). Furthermore, specific knockout of *Lkb1* in DC leads to great amplification of Tregs, especially thymus Tregs in vivo (28-31).

Although several studies have investigated this issue in the past, different results have been raised due to the different experimental conditions, some of which are, indeed, contradictory (18, 28, 32). In the present study, we detected the effect of LKB1 on purified DC by using a stable DC cable with the *Lkb1* gene knockdown or overexpress. Except for the in vitro characterization, the antitumor effect of LKB1 on DCs was also assessed in vivo in an antigen-specific tumor model, providing insight into the treatment of tumors.

MATERIALS AND METHODS

Cell Lines and Mice

DC2.4 is a cell line of GM-CSF-derived bone marrow DCs (GMDCs) from C57BL/6 mice with an immature phenotype, a gift from Prof. Dong Yongjun of Tsinghua University, China (33). B16-OVA are OVA-expressing B16 melanoma cells and were kindly provided by Prof. Xuefeng Wang, Suzhou University, China.

Aged 8-11 weeks C57BL/6 and Balb/c mice employed in this research were held in a specific pathogen-free (SPF) animal house in the Model Animal Research Center of Anhui Normal University (AHNU) Animal Facilities. All programs performed on mice were in agreement with the conditions

established and approved by the AHNU Animal Experimentation Ethics Committee.

Generation of Lkb1-modifying Lentivirus

Complete *Lkb1* sequences were obtained from the mice genome and constructed into the pLJM-overexpression vector using the restriction endonuclease EcoRI/NheI. Alternatively, the shRNA of *Lkb1* was inserted into pLKO.1 vector by EcoRI/AgeI. Then, the modified plasmids were transduced with packaging plasmids pSPAX2 and VSVG into 70-80% confluent 293 T cells using LipofiterTM, where an empty vector was used as a control. 48 h later, the supernatant was harvested, centrifuged (2000 xg, 10 min, 4 °C), and filtered through a 0.45 mm filter tip to obtain the complete lentiviral vector.

Construction of Gene-modified DCs

DC2.4 cells ($1×10^5$) were cultured in a 24-well plate with 2 mL of fresh media to grow up to 70-80% confluency before they were infected with competent lentivirus encoding *Lkb1* or shLKB1 or empty vectors for 24 h, and replaced with fresh media afterward. 72 h later, puromycin (2mg/mL) was added to the infected cells to screen for cells transduced with the target gene. The *Lkb1* high-expressed or knockdown DCs were verified by qPCR, and used for subsequent experiments.

Flow Cytometry

Single-cell suspensions were stained with different combinations of anti-mouse MHCII (APC, M5/114.15.2) (Invitrogen, 17-5321-82), anti-mouse CD11c (PE-Cy7, N418) (Biolegend, 117318), anti-mouse CD86 (FITC, GL1) (eBioscience, 11-0862-82), anti-mouse OX40L (PE, RM134L) (eBioscience, 12-5906-82), anti-mouse SIINFEKL/H2kb (APC, 25-D1.16) (eBioscience, 17-5743-82), anti-mouse CD3 (PE/cy7, 145-2C11) (eBioscience, 25-0031-82), anti-mouse CD4 (PE, GK1.5) (eBioscience, 12-0041-82), anti-mouse CD25 (APC, PC61.5) (eBioscience, 17-0251-82), anti-mouse CD69 (APC, H1.2F3) (eBioscience, 17-0691-82) antibodies and

propidium iodide (1 µg/mL) were added to distinguish the dead cells. BD FACS Canto II flow cytometer (BD Biosciences) was used in cell analysis. The software Flow Jo (Tree Star, Ashland, OR, USA) was used to analyze the data. Cells-only or unstained controls were used as criteria to set the markers and regions.

Analysis of the Phagocytosis of DCs

The uptake ability of DCs was analyzed using the previously described method (34). Briefly, $0.2×10^6$ DCs were cultured with fluorescent OVA-FITC (Bersee, BFR552) (50 µg/mL) in 0.5 mL complete media at 37°C cell culture incubator for 4 h. The cells were washed with PBS before being analyzed by flow cytometry immediately. Negative control cells were incubated at 4°C with OVA-FITC.

Mixed Lymphocyte Reaction

Mixed lymphocyte reaction in vitro was executed by the methods described before (34), with slight modification. Briefly, allogeneic $CD4^+$ T cells from the spleen of Balb/c mice were sorted by flow cytometer and labeled with CFSE (Invitrogen, C34570) at 37 °C for 20 min. Then, $CD4^+$ T cells labeled with CFSE were co-cultured with DCs for 72 h in different proportions (DCs: T cells=1:5,1:10,1:20,1:40) in 96-well U-bottom plate and purified $CD4^+$ T cell were planted in 50000 cells/well. DCs were treated with 25 µg/mL mitomycin C for half an hour in advance. Three days later, $CD4^+$ T cell proliferation was determined by CFSE dilution detected by flow cytometry.

CD4+ T cell Differentiation Assay

For three consecutive weeks, 500 µg of OVA was injected intraperitoneally into C57BL/6 mice once a week. In the fourth week, $1×10^6$ OVA-pulsed DCs were administered intravenously into the OVA-immunized mice. Three days later, the spleens of DC-injected mice were isolated and single-cell suspensions were prepared and stimulated with ionomycin, phorbol 12-myristate 13-acetate, and brefeldin A at 37 °C for 4 h. Then, the cells were washed and

stained with antibodies against CD3 or CD4 before they were perforated by the fixation and permeabilization kit (BD Biosciences, 554722). Subsequently, the cells were stained with antibodies against IFN-γ, IL-17A, IL-4, and IL-10 intracellularly. Differently, in Foxp3 staining, the cells are fixed and permeabilized with Foxp3 buffer. Finally, these cells were detected by flow cytometry.

Cytokines Production in DC Supernatant

The supernatants were collected after incubating 1×10^6/ml DC2.4 in fresh media for 20 h, IL-10 and IL-6 were analyzed by ELISA according to the manufacturer's instructions.

RNA Extraction and qPCR Analysis

Trizol (Sangon Biotech, B511311) was used to extract total RNA at 42°C for five minutes, and genomic DNA was removed at 42 °C for 15 min and reverse transcribed to cDNA at 95°C for 3 min with the PrimeScriptTM RT kit (Accurate Biology, AG11705) following the manufacturer's instructions. The expression of *Lkb1*, granzyme, perforin, and actin on mRNA level was quantified by CFX96 real-time PCR detection system (Bio-Rad), and the data was analyzed by CFX Manager Software. The primer sequences for the indicated genes are: *β-actin* forward: AGC CAT GTA CGT AGC CAT CC, *β-actin* reverse: TCC CTC TCA GCT GTG GTG GTG AA; *Lkb1* forward: TCC TGG AAG AGG AAG TGG GT, *Lkb1* reverse: CCT TCT GGC TTC AC CTT GCT, *granzyme* forward: CCA CTC TCG ACC CTA CAT GG, *granzyme* reverse: GGC CCC CAA AGT GAC ATT TAT; *perforin* forward: AGC ACA AGT TCG TGC CAG G-3′, *perforin* reverse: GCG TCT CTC ATT AGG GAG TTT TT. All the data were expressed relative to *β-actin* as reference genes.

Statistical Analysis

All the data were performed with Graph Pad Prism software and expressed as the mean±SD. All the experiments were repeated at least three times with similar results, and the means and standard deviations of the multiple replicate samples are displayed in the graphs. Differences in statistics between groups have been analyzed by the Student's t-test; P values<0.05 were regarded as statistically significant. (*P<0.05, **P<0.01, ****P<0.0001).

RESULTS

The Successful Construction of Gene-modified DCs

To obtain *Lkb1* gene-modified DCs, we first amplified the coding sequence of the *Lkb1* gene from the spleen cells of a mouse (Figure 1A) and this coding sequence was inserted into the overexpressed lentiviral vector pLJM-empty using the restriction enzymes EcoRI, NheI and DNA ligase. With a similar approach, the synthetic shLkb1 (Figure S1A) was constructed into the lentiviral vector pLKO.1 to make an *Lkb1* knockdown vector. Subsequently, these two recombinant plasmid vectors were transformed into E. coli and positive colonies were verified by PCR (Figures 1B, S1B), and the correct sequence of the target gene was confirmed by sequencing (Figures 1C, S1C). Furthermore, the verified *Lkb1*-modifying plasmids and two packaging plasmids were co-transduced into 293FT cells using liposome transduction reagent to generate a complete lentivirus that could effectively infect immobilized DC line, DC2.4 (33). Finally, the quantitation of *Lkb1* gene expression in transduced DCs was performed by reverse transcription qPCR, where DCs infected with *Lkb1* overexpression recombinant pLJM-LKB1 plasmids (LKB1[Oe] DCs) expressed more LKB1 at the mRNA level than the control DCs transduced with empty plasmids (Vector[Ctrl] DCs) (P=0.0016) (Figure 1D). Similarly, DCs infected with lentiviral vectors with recombinant pLKO.1-shLKB1 plasmids (LKB1[kd] DCs) had significantly lower expression of *Lkb1* transcripts, compared with the DCs infected with lentiviruses containing the control vectors (vector[ctrl] DCs) (P=0.0024) (Figure S1D).
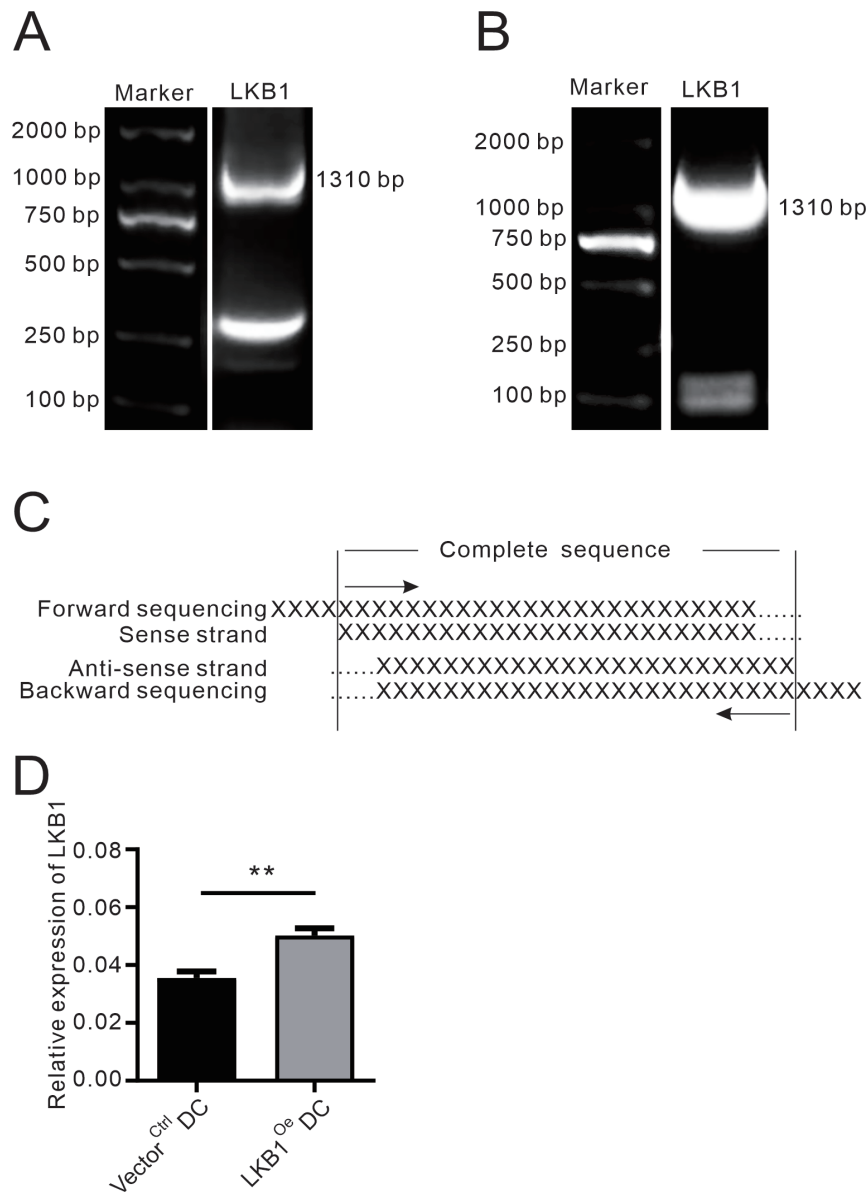
Figure 1. Successful establishment of LKB1-overexpressed DC lines. (A) PCR amplification of the whole *Lkb1* gene fragment derived from mouse spleen. (B) The *Lkb1* overexpressing recombinant plasmids were transformed into active Escherichia coli and amplified. The positive clones were identified by bacteria PCR. (C) Schematic diagram of the comparison between sequencing results and target genes. (D) The relative LKB1 mRNA expression in DC2.4 with *Lkb1* overexpressed DCs (LKB1[Oe] DCs) and their mock-transduced controls (Vector[Ctrl] DCs) were detected by RT-qPCR. The mRNA levels of LKB1 were normalized to β-Actin mRNA. Error bars represent the mean±SD. from three repetitions of an experiment. **P<0.01, unpaired t-test. LKB1: liver kinase B1; DC: Dendritic cell; LKB1[Oe] DCs: *Lkb1* overexpressed DCs

LKB1 Promotes the DC-induced Proliferation of CD4+ T cells

Following the successful genetic modification of *Lkb1* in DCs, the effects of LKB1 on DCs were investigated. The presentation of antigens and activation of T cells are the most important functions of DCs Therefore, the effect of LKB1 on the ability of DCs to stimulate T cell proliferation was

the first to be examined. We sorted allogeneic CD4+ T cells and co-cultured these CD4+ T cells with LKB1[Oe] DCs or Vector[Ctrl] DCs in 96-well U-bottom plates after CFSE staining, 72 h later, the expansion of these T cells was examined by flow cytometer. As expected, proliferating CD4+ T cells increased as the proportion of DCs increased in the system, this suggests that the stimulation of T cells

by DCs is dose-dependent (Figures 2A, B). Moreover, regardless of the ratio of DCs to T, the expansion of CD4+ T cells mediated by LKB1Oe DCs was significantly higher than that of the VectorCtrl DCs (Figures 2A, B), although the survival rate was the same for all DCs (data not shown). Furthermore, LKB1kd

DCs were used to verify this result in vivo. OVA-pulsed VectorCtrl DCs or LKB1kd DCs were intravenously injected into mice. We found that more intravenously injected DC2.4 reaches the spleen relative to the lymph nodes (Figure S2). Therefore, three days after inoculation with OVA-pulsed VectorCtrl DCs or
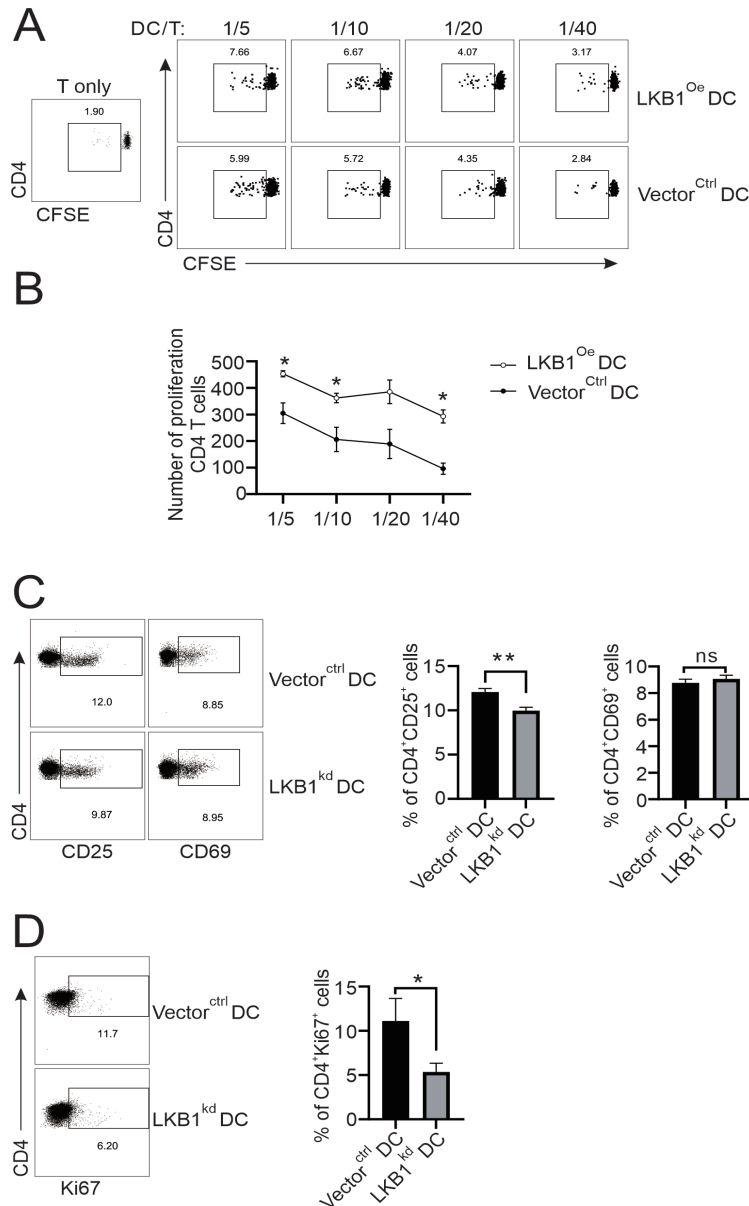

Figure 2. LKB1 promotes the DC-induced proliferation of CD4 T cells. (A) After half an hour of mitomycin C treatment, LKB1Oe DCs and VectorCtrl DCs were co-cultured with CSFE-labelled allogeneic CD4+ T cells for 3 days as described in methods. The CFSE dilution of CD4 T cells was examined by flow cytometry and (B) the proliferated CD4 T cells were plotted into a histogram. (C, D) OVA-pulsed LKB1kd DCs and VectorCtrl DCs were intravenously injected into OVA-sensitized mice. Three days later, a single-cell suspension of the spleen was taken from mice. (C) The expression of CD69 and CD25 on CD4 T cells was detected by flow cytometry. (D) The expression of Ki67 at CD4 T cells was detected by flow cytometry. All experiments were repeated at least two times and similar results were obtained. Bar graphs represent the mean±SD from three repetitions of an experiment.*P<0.05, **P<0.01, unpaired t-test. DC: Dendritic cell; LKB1Oe DCs: *Lkb1* overexpressed DCs; LKB1: liver kinase B1; LKB1kd DCs: *Lkb1* knockdown DCs; CSFE: Carboxyfluorescein Succinimidyl Ester

LKB1[kd] DCs, single-cell suspension of spleen was taken from mice, and the expression of CD25, CD69, and Ki67 at CD4^{+} T cells was measured. We found that CD25, a marker of late activation on the surface T cells, reduced (P=0.0029) in the spleen of mice injected with LKB1[kd] DCs, although CD69 the marker of T cell early activation was not a significant change (P=0.2584) (Figure 2C). Moreover, the expression of Ki67 in CD4^{+} T cells of mice injected with LKB1[kd] DCs also significantly decreased (P=0.0217) compared with the Vector[ctrl] DC-injected mice (Figure 2D). These results suggest that LKB1 in DCs regulates the activation and proliferation of downstream CD4^{+} T cells.

LKB1 Does Not Affect Antigen Uptake by DCs

DCs engulf, process, and finally present antigens to T cells, thereby activating downstream T cell responses. To exclude the possibility that different antigen uptake due to *Lkb1* mutation leads to different DC-induced downstream T cell responses, we further analyzed the capacity of the genetically modified DCs to engulf OVA antigens. DCs were co-cultured with OVA protein conjugated with fluorescence (OVA-FITC) for 4 h and their ability to phagocytose OVA-FITC was tested by flow cytometry. We found that the OVA-FITC expressed in LKB1[Oe] DCs was similar (P=0.9672) to Vector[Ctrl] DCs (Figure 3A). Consistent with this, the same levels (P=0.1255) of OVA-FITC in Vector[ctrl] DCs or LKB1[kd] DCs was found (Figure 3B). These results suggest that the phagocytosis of OVA by DCs was not affected by LKB1.

LKB1 Selectively Inhibits DC-mediated Differentiation of CD4^{+} T cells toward Treg

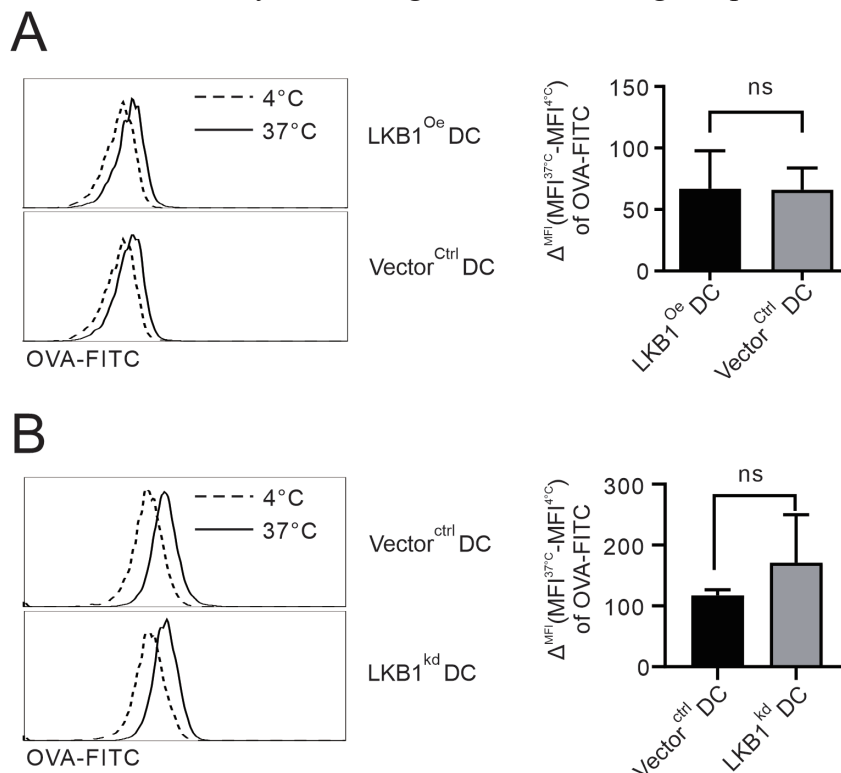Following the proliferation activated by

Figure 3. LKB1 does not affect antigen uptake by DCs. DCs were cocultured with OVA-FITC for 4 h and the phagocytosis was detected by flow cytometry. The endocytosis was assessed by comparing the differences in the mean fluorescence intensity (MFI) between 37°C and 4°C ($\Delta^{MFI}=MFI^{37°C}-MFI^{4°C}$). (A) Phagocytosis of LKB1[Oe] DCs and Vector[Ctrl] DCs was measured by flow cytometry. (B) Phagocytosis of LKB1[kd] DCs and Vector[ctrl] DCs was measured by flow cytometry. Bar graphs represent the mean±SD from three repetitions of an experiment. LKB1[Oe] DCs: *Lkb1* overexpressed DCs; OVA-FITC: Ovalbumin conjugated with fluorescence; LKB1[kd] DCs: *Lkb1* knockdown DCs; MFI: Mean fluorescence intensity; OVA: Ovalbumin

DCs, T cells expand in numbers, during which they are also differentiated into different effector cells. To investigate whether LKB1 affects DC-mediated T cell differentiation, OVA-pulsed DCs was injected into OVA-sensitized mice. Three days later, CD4+ T cell differentiation in the spleen was detected by flow cytometry. Using effector molecules IFNγ, IL-4, and IL-17A as the markers of Th1, Th2, and Th17, respectively, we observed

that the proportion of Th1 (P=0.9499), Th2 (P=0.8750) and Th17 (P=0.4620) in the spleen of mice injected with LKB1[Oe] DCs had no significant change compared with the control group (Figure 4A). Interestingly, when Treg differentiation was inspected, the proportion of Foxp3+ Treg (P=0.0195) significantly decreased (Figure 4B). Functionally, we further examined the levels of IL-10, a cytokine produced by Tregs, and
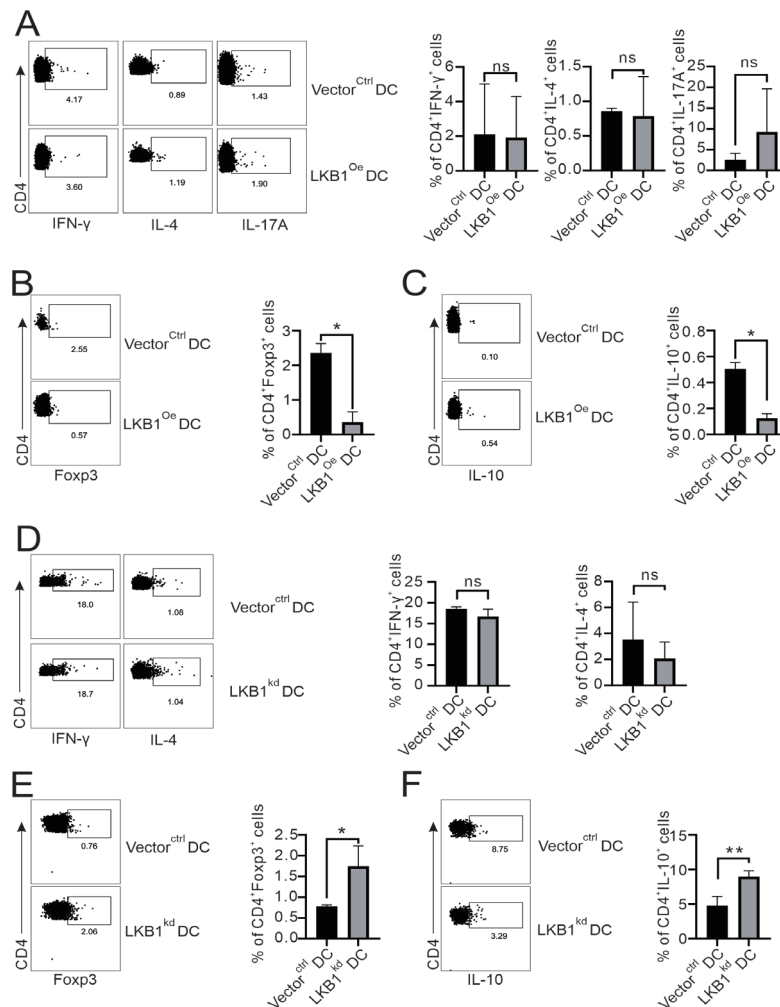

Figure 4. LKB1 selectively inhibits DC-mediated differentiation of CD4 T cells toward Treg. (A-C) OVA-pulsed LKB1[Oe] DCs and Vector[Ctrl] DCs were intravenously injected into OVA-sensitized mice. Three days later, CD4 T cell differentiation in the spleen was detected by flow cytometry. A representative dot plot (left panels) and the summarized results (right panel) are shown. (A) The expression of IFN-γ, IL-4, or IL-17A at CD4 T cells was detected by flow cytometry. (B) The expression of Foxp3 at CD4 T cells were detected by flow cytometry. (C) The expression of IL-10 at CD4 T cells were detected by flow cytometry. (D-F) OVA-pulsed LKB1[kd] DCs and Vector[ctrl] DCs was intravenously injected into OVA-sensitized mice. After 72 h, CD4 T cell differentiation in spleen was detected by flow cytometry. A representative dot plot (left panels) and the summarized results (right panel) are shown. (D) The expression of IFN-γ or IL-4 at CD4 T cells was detected by flow cytometry. (E) The expression of Foxp3 at CD4 T cells was detected by flow cytometry. (F) The expression of IL-10 at CD4 T cells was detected by flow cytometry. Bar graphs represented the mean±SD from 3 independent experiments. *P<0.05, **P<0.01, unpaired T-test. LKB1[Oe] DCs: *Lkb1* overexpressed DCs; IFN: Interferon; IL: Interleukin; DC: Dendritic cell; LKB1: liver kinase B1; LKB1[kd] DCs: *Lkb1* knockdown DCs

found that the proportion of IL-10+ CD4+ T cells (P=0.0126) also significantly reduced (Figure 4C). Finally, reverse outcomes were found in the experiments for Foxp3+CD4+ cells (P=0.0267) and IL-10+ CD4+ cells (P=0.0017) with LKB1kd DCs (Figures 4E, 4F). Collectively, these data suggest that LKB1 could specifically inhibit DC-induced Treg differentiation.

LKB1 Inhibits DCs to Express OX40L, CD86 and IL-10 for Treg Induction

Tregs are the important negative regulators of host immunity. To explore the mechanisms of LKB1 suppressing DC-mediated Treg differentiation, we first inspected the production of Treg-inducing cytokine, IL-10 from the LKB1- overexpressed DCs. Following the examination of the supernatant in VectorCtrl DCs and LKB1Oe DCs cultures by ELISA, we found that the expression of IL-10 (P=0.0315) was significant although

did not dramatically decrease in LKB1-overexpressed DCs, whereas those of Th17 polarizing cytokine, IL-6 (P=0.5642), remain unchanged in the same samples (Figures 5A, B), indicating a specific effect on DC-derived IL-10 by LKB1. This is because in addition to soluble factors, some surface molecules, like OX40L and CD86 on DCs are also involved in Treg induction. Thus, we also investigated the OX40L and CD86 expression in the *Lkb1* gene-modified DCs and found that the *Lkb1* knocked-down DCs had upregulated OX40L (P=0.0385) and CD86 (P=0.0111) expression than the mock-transduced control DCs, whereas the expression of other surfaces co-stimulatory molecule, like CD80 (P=0.6332), was similar between the two samples (Figures 5C, D). Collectively, these data suggest that LKB1 could regulate DC-mediated Treg differentiation via both the surface molecules and secreted factors from DCs.
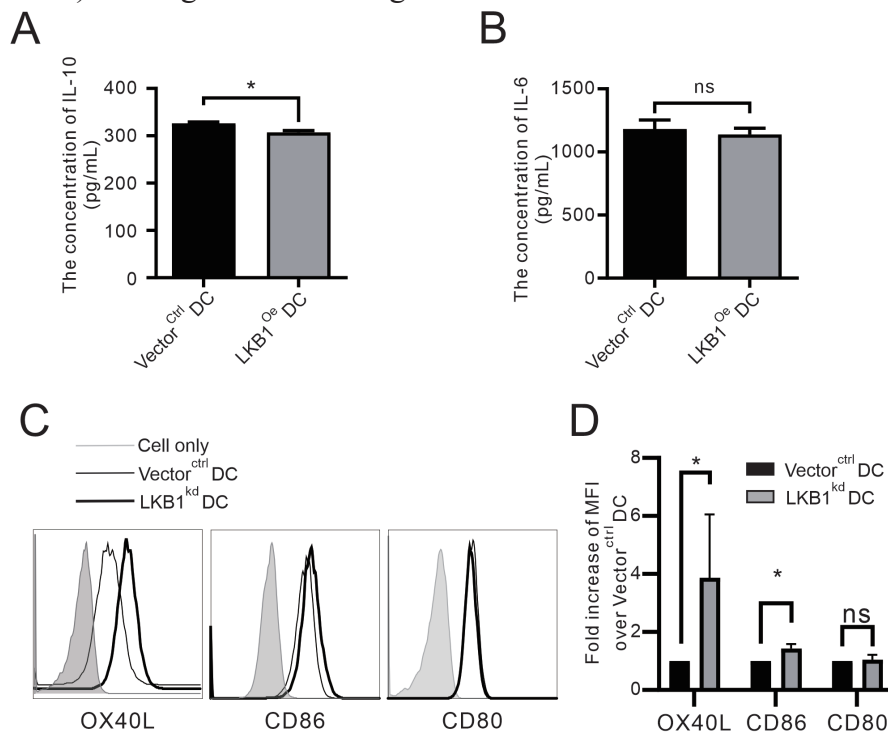

Figure 5. The impact of LKB1 on cytokine expression of DCs. (A) The concentrations of IL-10 in the supernatants from LKB1Oe DCs and VectorCtrl DCs were assayed by ELISA. (B) The concentrations of IL-6 in the supernatants from LKB1Oe DCs and VectorCtrl DCs were assayed by ELISA. (C) The expression of OX40L, CD86, and CD80 on LKB1kd DCs and Vectorctrl DCs was detected by flow cytometry. (D) The figure on the right shows the statistical results. All the experiments were repeated at least two times and similar results were obtained. Bar graphs represent the mean±SD from three repetitions of an experiment.*P<0.05, unpaired T-test. DC: Dendritic cell; LKB1: liver kinase B1; LKB1Oe DCs: *Lkb1* overexpressed DCs; LKB1kd DCs: *Lkb1* knockdown DCs; MFI: Mean fluorescence intensity

Antigen-specific Tumor Rejection is Compromised After the Adoptive Transfer of Lkb1-Knockdown DCs

Following the inspection of the impact of LKB1 on DC-induced CD4^{+} T cell immunity, we next investigated its effect on the activity of CD8^{+} T cells, and its anti-tumor efficacy. To check DC-specific immune responses, we primed mice with OVA-pulsed DCs, and then the splenocytes from sensitized mice were co-cultured with OVA for 5 days, and the transcripts of perforin and granzyme from CD8^{+} T cells were measured by qPCR. We found that granzyme (P<0.0001) and perforin (P=0.0042) significantly reduced after the

transfer of OVA pulsed LKB1[kd] DCs (Figure 6A), indicating a killing promotive effect on CD8^{+} T cells by LKB1 in DCs. Finally, to verify the promoting effect of LKB1 in DCs for T cell immunity in an actual tumor-diseased setting, OVA-expressed B16 melanoma cells were used against OVA-pulsed DCs. OVA-pulsed LKB1[kd] DCs or Vector[ctrl] DCs were immunized in the paws of mice, and four days later, B16-OVAs were injected intravenously. Fourteen days after tumor inoculation, the lungs of the mice were taken and tumor nodules were counted. Immunization of OVA pulsed Vector[ctrl] DCs effectively prevented the metathesis of OVA-expressing melanoma
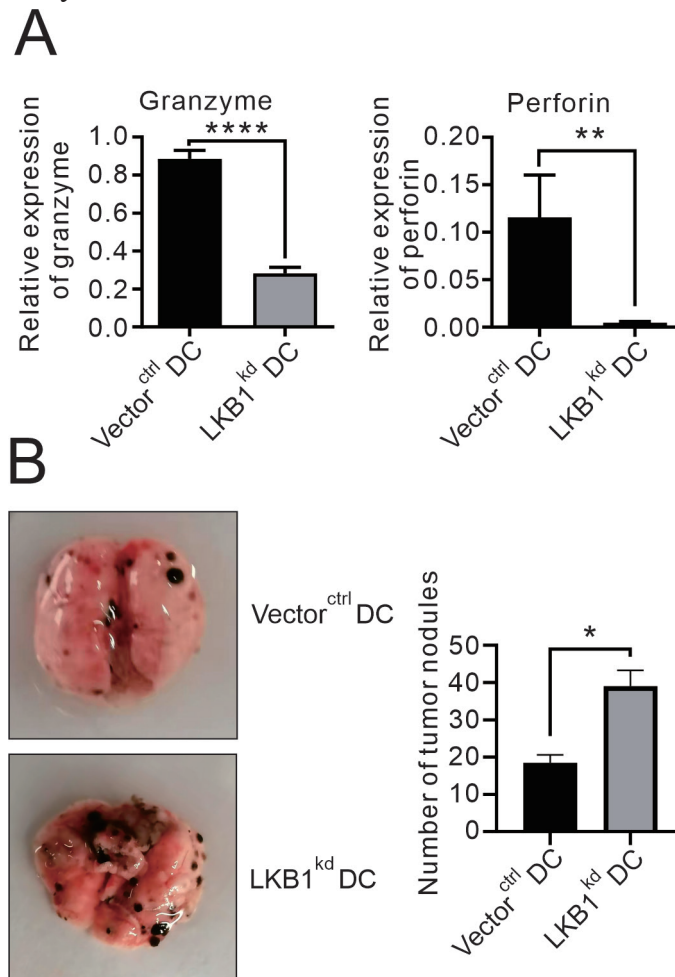

Figure 6. Reduced anti-tumor efficacy of DCs after LKB1 knockdown. (A). The splenocytes from OVA-pulsed LKB1[kd] DCs and Vector[ctrl] DCs sensitized mice were pulsed with 10 µg/mL OVA for five days before cells were examined for their perforin and granzyme expression by RT-qPCR. (B) C57BL/6 mice were immunized s.c. in both right and left heels with 1×10^{6} OVA-pulsed LKB1[kd] DCs or Vector[ctrl] DCs four days before *i.v.* injection of 1×10^{6} B16-OVA melanoma cells. Fourteen days later, the lungs were harvested and tumor nodules were counted. The representative photos (left panel), and summarized results (right panel) are shown. Bar graphs represented the mean±SD from 3 mice. *P<0.05, **P<0.01, ****P<0.0001, unpaired t-test. LKB1[kd] DCs: *Lkb1* knockdown DCs; DC: Dendritic cell; LKB1: liver kinase B1

cells into the lung if not completely (Figure 6B), suggesting an antigen-specific tumor suppressive effect. Noticeably, this antigen-specific tumor suppressive effect was hampered by pre-injection of LKB1[kd] DCs, and resulted in a significant increase in tumor nodules (P=0.0257) (Figure 6B), indicating that LKB1 in DCs plays an indispensable role in DC-mediated immunity. Collectively, these data suggest that DC immunogenicity for T cell responses in vivo was regulated by LKB1, the loss of LKB1 will result in compromised tumor immunity.

DISCUSSION

In the present work, the role of LKB1 in the immune regulation of DCs was systematically investigated using genetically modified myeloid DCs in vivo. Stable *Lkb1* knockeddown or overexpressed DCs were obtained by the delivery of genetically engineered units into the genomic DNA of DC2.4 via lentiviral vectors to probe the effect of LKB1 on DC-mediated T cell immunity and tumor immunity in clinical settings. We demonstrated that LKB1 is required to maintain the immunogenic role of DCs in stimulating T cells in vivo, with unique characteristics of specifically inhibiting CD4^{+} T differentiation into Treg. Furthermore, the positive regulation of T cell stimulation by LKB1 is intrinsic to DCs, rather than enhanced antigen uptake. In addition, the selective suppression of DC-mediated Treg differentiation by LKB1 was co-committed with its inhibiting the expression of Treg-inducing molecules OX40L and IL-10 in DCs. Ultimately, the combined roles of promoting immunogenic and suppressing immunotolerant T cells by LKB1 in DCs greatly enhanced immunological surveillance in the host and inhibited tumor metathesis in vivo.

As the most powerful APC, the activated DCs can effectively induce T cell proliferation. However, the role of LKB1 on T cell proliferation induced by DC is controversial. Leonard et al. found that LKB1-deficient

GMDCs had a stronger ability to promote T cell activation and proliferation after LPS stimulation in vivo (28). However, OVA-specific T cell proliferation assays by Chen et al. showed that LKB1 did not affect the ability of DCs to prime antigen-specific T cell responses (30). In the experimental system adopted by Leonard et al., GMDC was not effectively purified. Since macrophages can significantly promote inflammation after LKB1 deletion (26, 27), the large number of macrophages in bone marrow-derived DCs by GM-CSF (35) may affect the accuracy of the experiment. In addition, the effect of LKB1 on the migration of GMDC also impacts the stimulation of T cells by GMDC in vivo. Also problematic is the different composition of DC subtypes in CD11c^{+}MHCII^{+} cells from *Lkb1*[f/f] and CD11c[Cre]*Lkb1*[f/f] mice used by Chen et al. It was found that the deletion of LKB1 in CD11c^{+} cells resulted in increased CD8^{+} DCs and decreased CD11b^{+} DCs, although it did not affect the change of total DC. These experimental defects may be the main reason for the contradictory results. To avoid the experimental error caused by the disorderly cells and the influence of LKB1 on cell migration, we constructed *Lkb1*-overexpressed DC cell lines and performed a mixed lymphocyte reaction in vitro to measure the ability of DCs to prime T cell responses (Figure 2A). Although *Lkb1* knockdown led to a higher expression of CCR7 (data not show), the chemokine receptor associated with DC migration, our results showed that *Lkb1* knockdown DCs did not enhance T cell response in vivo, but instead damaged DC-induced T cell activation and proliferation (Figures 2C, D). Taken together, these results suggest that LKB1 in DCs can promote DC-induced T cell responses.

Consistent with our results, multiple studies have shown that the deletion of LKB1 in CD11c^{+} cells leads to a significant expansion of Treg, but these studies do not have a unified view on how LKB1 in DC inhibits Treg differentiation (28-31). B7/CD28 co-stimulation represents a primary pathway

for the promotion of the Treg homeostasis (36-40). Several studies have shown that CD80/CD86 is essential for the DC-induced Treg proliferation (37, 41, 42). OX40L on DCs has also been reported to promote Treg proliferation (43, 44). Therefore, to investigate the mechanism by which LKB1 affects DC-induced Treg amplification, we examined the expression of OX40L, CD86, and CD80 on DCs after *Lkb1* modification. Higher expression of OX40L and CD86 was found on *Lkb1* - knockdown DCs (Figure 5). Consistent with our results, a study found that OX40L is concerned with the regulation of the performance of LKB1 on DCs-induced Treg proliferation, and a following study additionally found that *Lkb1*-deficient DC-induced Treg proliferation may well be effectively eliminated once neutralizing CD86 with anti-CD86 antibodies (28, 30). In addition, we also found that LKB1 inhibited the secretion of IL-10 in DCs. These results suggest that LKB1 can regulate Treg proliferation not only by affecting the expression of OX40L and CD86 on DCs, but also by influencing the secretion of IL-10 in DCs to affect the differentiation of CD4^{+} T cells to Treg.

In addition to the effect on CD4^{+} T cells, we further explored the effect of LKB1 in DCs on CD8^{+} T cells. We found that the expression of granzyme and perforin considerably reduced within the spleen of mice injected with *Lkb1*-knockdown DCs. Combined with the data shown in Figure 2, it is straightforward to visualize the positive impact of LKB1 on DC-mediated host immunity. These findings promoted us to test the therapeutic translation of LKB1 in the setting of the tumor. To check DCs' specific effect, the mice were inoculated with B16-OVA, and the DCs with *Lkb1* knockdown or not were adoptively transferred to compare the difference in tumor eradication. Previous studies have shown that mice with specific knockout *Lkb1* in CD11c^{+} cells have significantly impaired their ability to inhibit tumor growth (28-30). Consistent with this, in our study, the knockdown of

Lkb1 also reduced the therapeutic effect of exogenous DC on tumors (Figure 6).

Altogether, the contribution of LKB1 in DCs to T cell immunity makes this molecule a particularly appealing target for the inhibition of tumor growth. Given that DCs are consistently being investigated as vaccines to improve host immunity, genetic targeting of this potent immunogenic molecule in DCs would be readily translated in the clinic to restore the impaired anti-tumor immunity of patients with cancer.

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REFERENCES

1. Zhang Y, Meng Q, Sun Q, Xu ZX, Zhou H, Wang Y. LKB1 deficiency-induced metabolic reprogramming in tumorigenesis and non-neoplastic diseases. Mol Metab. 2021;44:101131.
2. Ciccarese F, Zulato E, Indraccolo S. LKB1/AMPK Pathway and Drug Response in Cancer: A Therapeutic Perspective. Oxid Med Cell Longev. 2019;2019:8730816.
3. Shackelford DB, Shaw RJ. The LKB1-AMPK pathway: metabolism and growth control in tumour suppression. Nat Rev Cancer. 2009;9(8):563-575.

4. Shaw RJ, Kosmatka M, Bardeesy N, Hurley RL, Witters LA, DePinho RA, et al. The tumor suppressor LKB1 kinase directly activates AMP-activated kinase and regulates apoptosis in response to energy stress. Proc Natl Acad Sci U S A. 2004;101(10):3329-3335.

5. Flowers EM, Sudderth J, Zacharias L, Mernaugh G, Zent R, DeBerardinis RJ, et al. Lkb1 deficiency confers glutamine dependency in polycystic kidney disease. Nat Commun. 2018;9(1):814.

6. Han SH, Malaga-Dieguez L, Chinga F, Kang HM, Tao J, Reidy K, et al. Deletion of Lkb1 in Renal Tubular Epithelial Cells Leads to CKD by Altering Metabolism. J Am Soc Nephrol. 2016;27(2):439-453.

7. Shan T, Xu Z, Liu J, Wu W, Wang Y. Lkb1 regulation of skeletal muscle development, metabolism and muscle progenitor cell homeostasis. J Cell Physiol. 2017;232(10):2653-2656.

8. Jiang S, Chen R, Yu J, Li N, Ke R, Luo L, et al. Clinical significance and role of LKB1 in gastric cancer. Mol Med Rep. 2016;13(1):249-256.

9. Chen IC, Chang YC, Lu YS, Chung KP, Huang CS, Lu TP, et al. Clinical Relevance of Liver Kinase B1(LKB1) Protein and Gene Expression in Breast Cancer. Sci Rep. 2016;6:21374.

10. Nagaraj AS, Lahtela J, Hemmes A, Pellinen T, Blom S, Devlin JR, et al. Cell of Origin Links Histotype Spectrum to Immune Microenvironment Diversity in Non-small-Cell Lung Cancer Driven by Mutant Kras and Loss of Lkb1. Cell Rep. 2017;18(3):673-684.

11. Jia C, Medina V, Liu C, He L, Qian D, Taojian T, et al. Crosstalk of LKB1- and PTEN-regulated signals in liver morphogenesis and tumor development. Hepatol Commun. 2017;1(2):153-167.

12. Durai V, Murphy KM. Functions of Murine Dendritic Cells. Immunity. 2016;45(4):719-736.

13. Liu CH, Fan YT, Dias A, Esper L, Corn RA, Bafica A, et al. Cutting edge: dendritic cells are essential for in vivo IL-12 production and development of resistance against Toxoplasma gondii infection in mice. J Immunol. 2006;177(1):31-35.

14. Hammad H, Plantinga M, Deswarte K, Pouliot P, Willart MA, Kool M, et al. Inflammatory dendritic cells--not basophils--are necessary and sufficient for induction of Th2 immunity to inhaled house dust mite allergen. J Exp Med. 2010;207(10):2097-2111.

15. Linehan JL, Dileepan T, Kashem SW, Kaplan DH, Cleary P, Jenkins MK. Generation of Th17 cells in response to intranasal infection requires TGF-beta1 from dendritic cells and IL-6 from CD301b+ dendritic cells. Proc Natl Acad Sci U S A. 2015;112(41):12782-12787.

16. Giovanelli P, Sandoval TA, Cubillos-Ruiz JR. Dendritic Cell Metabolism and Function in Tumors. Trends Immunol. 2019;40(8):699-718.

17. Buck MD, Sowell RT, Kaech SM, Pearce EL. Metabolic Instruction of Immunity. Cell. 2017;169(4):570-586.

18. Everts B, Amiel E, Huang SC, Smith AM, Chang CH, Lam WY, et al. TLR-driven early glycolytic reprogramming via the kinases TBK1-IKKvarepsilon supports the anabolic demands of dendritic cell activation. Nat Immunol. 2014;15(4):323-332.

19. Malinarich F, Duan K, Hamid RA, Bijin A, Lin WX, Poidinger M, et al. High mitochondrial respiration and glycolytic capacity represent a metabolic phenotype of human tolerogenic dendritic cells. J Immunol. 2015;194(11):5174-5186.

20. Ferreira GB, Vanherwegen AS, Eelen G, Gutierrez ACF, Van Lommel L, Marchal K, et al. Vitamin D3 Induces Tolerance in Human Dendritic Cells by Activation of Intracellular Metabolic Pathways. Cell Rep. 2015;10(5):711-725.

21. Cao Y, Li H, Liu H, Zhang M, Hua Z, Ji H, et al. LKB1 regulates TCR-mediated PLCgamma1 activation and thymocyte positive selection. EMBO J. 2011;30(10):2083-2093.

22. MacIver NJ, Blagih J, Saucillo DC, Tonelli L, Griss T, Rathmell JC, et al. The liver kinase B1 is a central regulator of T cell development, activation, and metabolism. J Immunol. 2011;187(8):4187-4198.

23. Yang K, Blanco DB, Neale G, Vogel P, Avila J, Clish CB, et al. Homeostatic control of metabolic and functional fitness of Treg cells by LKB1 signalling. Nature. 2017;548(7669):602-606.

24. Su X, Wang Q, Guo W, Pei X, Niu Q, Liu M, et al. Loss of Lkb1 impairs Treg function and stability to aggravate graft-versus-host disease after bone marrow transplantation. Cell Mol Immunol. 2020;17(5):483-495.

25. Wu D, Luo Y, Guo W, Niu Q, Xue T, Yang F, et al. Lkb1 maintains Treg cell lineage identity. Nat Commun. 2017;8:15876.

26. Liu Z, Zhang W, Zhang M, Zhu H, Moriasi C, Zou MH. Liver kinase B1 suppresses lipopolysaccharide-induced nuclear factor kappaB (NF-kappaB) activation in macrophages. J Biol Chem. 2015;290(4):2312-2320.

27. Deng J, Wen C, Ding X, Zhang X, Hou G, Liu A, et al. LKB1-MARK2 signalling mediates lipopolysaccharide-induced production of cytokines in mouse macrophages. J Cell Mol Med. 2020;24(19):11307-11317.

28. Pelgrom LR, Patente TA, Sergushichev A, Esaulova E, Otto F, Ozir-Fazalalikhan A, et al. LKB1 expressed in dendritic cells governs the development and expansion of thymus-derived regulatory T cells. Cell Res. 2019;29(5):406-419.

29. Wang Y, Du X, Wei J, Long L, Tan H, Guy C, et

al. LKB1 orchestrates dendritic cell metabolic quiescence and anti-tumor immunity. Cell Res. 2019;29(5):391-405.

30. Chen S, Fang L, Guo W, Zhou Y, Yu G, Li W, et al. Control of Treg cell homeostasis and immune equilibrium by Lkb1 in dendritic cells. Nat Commun. 2018;9(1):5298.

31. Chen J, Zhou Y, Chen S, Liu M, Guo W, Wang Q, et al. Lkb1 in dendritic cells restricts CD8(+) Foxp3(+)regulatory T cells expansion in vivo. Exp Cell Res. 2019;384(2):111650.

32. Krawczyk CM, Holowka T, Sun J, Blagih J, Amiel E, DeBerardinis RJ, et al. Toll-like receptor-induced changes in glycolytic metabolism regulate dendritic cell activation. Blood. 2010;115(23):4742-4749.

33. Shen Z, Reznikoff G, Dranoff G, Rock KL. Cloned dendritic cells can present exogenous antigens on both MHC class I and class II molecules. J Immunol. 1997;158(6):2723-2730.

34. Zhang W, Ding Y, Sun L, Hong Q, Sun Y, Han L, et al. Bone marrow-derived inflammatory and steady state DCs are different in both functions and survival. Cell Immunol. 2018;331:100-109.

35. Helft J, Bottcher J, Chakravarty P, Zelenay S, Huotari J, Schraml BU, et al. GM-CSF Mouse Bone Marrow Cultures Comprise a Heterogeneous Population of CD11c(+)MHCII(+) Macrophages and Dendritic Cells. Immunity. 2015;42(6):1197-1211.

36. Salomon B, Lenschow DJ, Rhee L, Ashourian N, Singh B, Sharpe A, et al. B7/CD28 costimulation is essential for the homeostasis of the CD4+CD25+ immunoregulatory T cells that control autoimmune diabetes. Immunity. 2000;12(4):431-440.

37. Tang Q, Henriksen KJ, Boden EK, Tooley AJ, Ye J, Subudhi SK, et al. Cutting edge: CD28 controls peripheral homeostasis of CD4+CD25+ regulatory T cells. J Immunol. 2003;171(7):3348-3352.

38. Tai X, Cowan M, Feigenbaum L, Singer A. CD28 costimulation of developing thymocytes induces Foxp3 expression and regulatory T cell differentiation independently of interleukin 2. Nat Immunol. 2005;6(2):152-162.

39. Lio CW, Dodson LF, Deppong CM, Hsieh CS, Green JM. CD28 facilitates the generation of Foxp3(-) cytokine responsive regulatory T cell precursors. J Immunol. 2010;184(11):6007-6013.

40. Williams JA, Zhang J, Jeon H, Nitta T, Ohigashi I, Klug D, et al. Thymic medullary epithelium and thymocyte self-tolerance require cooperation between CD28-CD80/86 and CD40-CD40L costimulatory pathways. J Immunol. 2014;192(2):630-640.

41. Zheng Y, Manzotti CN, Liu M, Burke F, Mead KI, Sansom DM. CD86 and CD80 differentially modulate the suppressive function of human regulatory T cells. J Immunol. 2004;172(5):2778-2784.

42. Bar-On L, Birnberg T, Kim KW, Jung S. Dendritic cell-restricted CD80/86 deficiency results in peripheral regulatory T-cell reduction but is not associated with lymphocyte hyperactivation. Eur J Immunol. 2011;41(2):291-298.

43. Ruby CE, Yates MA, Hirschhorn-Cymerman D, Chlebeck P, Wolchok JD, Houghton AN, et al. Cutting Edge: OX40 agonists can drive regulatory T cell expansion if the cytokine milieu is right. J Immunol. 2009;183(8):4853-4857.

44. Bhattacharya P, Gopisetty A, Ganesh BB, Sheng JR, Prabhakar BS. GM-CSF-induced, bone-marrow-derived dendritic cells can expand natural Tregs and induce adaptive Tregs by different mechanisms. J Leukoc Biol. 2011;89(2):235-249.