The Role of PKA Signaling in Dendritic Cells that Regulates Treg Homeostasis and Its Applications

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DEDICATION

This document is dedicated to the graduate students of the McGill University.

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ABSTRACT

The protein kinase A (PKA) signaling pathway has been extensively studied for its role in physiology; however, its role in the immune system remains largely unclear. In this study, we demonstrate that the PKA signaling pathway plays an important role in the regulation of dendritic cell suppressive function by regulating the homeostasis of FoxP3+ regulatory T cells (Tregs). We find that conditional activation of PKA in DCs results in a global expansion of peripheral Foxp3+CD4+ Treg cells. The altered Treg homeostasis is not caused by an increased thymic output but rather the increased proliferation and survival of peripheral mature Treg cells. The PKA activated DCs produced significantly more IL-2 and supported the survival and proliferation of Treg cells. As a consequence, inoculated tumor grew much faster in DC-specific PKA knockout mice than in wildtype controls, indicating that immune suppression was enhanced in vivo. Our findings may provide a new strategy for clinical therapies of immune system related diseases involving disturbed Treg homeostasis, including autoimmune diseases and tumor.

ABRÉGÉ

Le rôle physiologique des voies de signalisation de la protéine kinase A (PKA) a été largement étudié, mais leur rôle dans le système immunitaire reste largement obscur. Dans cette étude, nous démontrons que les voies de signalisation de la PKA jouent un rôle important dans la régulation des fonctions suppressives des cellules dendritiques, en régulant l'homéostasie des cellules T régulatrices Foxp3+ (Tregs). Nous avons découvert que l'activation conditionnelle de la PKA dans les cellules dendritiques entraine une expansion globale des cellules Treg CD4+Foxp3+ périphériques. L'altération de l'homéostasie des Tregs n'est pas causée par une augmentation de la production thymique des Treg mais par une augmentation de la prolifération et de la survie des cellules Treg matures en périphérie. Les cellules dendritiques dont la PKA est activée produisent significativement plus d'interleukine 2 et favorisent la survie et la prolifération des Tregs. En conséquence, les tumeurs grossissent beaucoup plus rapidement lorsqu'elles sont inoculées dans des souris dont la PKA est conditionnellement activée dans les cellules dendritiques, ce qui indique que le système immunitaire de ces souris est inhibé. Nos découvertes pourraient ouvrir la voie à de nouvelles stratégies thérapeutiques pour des maladies reliées au système immunitaire impliquant une homéostasie perturbée des Treg, comme par exemple les maladies auto-immunes et les tumeurs.

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CHAPTER 1
Introduction

1.1 Overview

Since their discovery in 1972, dendritic cells (DCs) have been well characterized for their immunogenic role in the initiation of immunity [1, 2], mainly through the recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) that are expressed on DCs [3, 4].

Recently, there is growing evidence showing that DCs, especially after capturing innocuous self antigens that are released during cell death, can also induce tolerance, either by deleting self-reactive T cells or by expanding regulatory T cells (Treg cells) [5–7]. However, the underlying mechanism still remains to be fully elucidated.

In addition to self antigens, massive cell death may also release a large quantity of ATP, which will be quickly degraded into adenosine in extracellular space by the combined enzymatic action of membrane-bound enzymes ecto-nucleoside triphosphate diphosphohydrolase CD39 and ecto-5'-nucleotidase CD73 [8, 9], thus finally lead to a dramatic increase of extracellular adenosine concentration [10]. Adenosine is a purine nucleoside that has been reported to regulate immune system via adenosine receptors that are expressed on various types of immune cells [11, 12]. Therefore, the increase of extracellular adenosine is not merely the by-product of tissue injury and cell death; it also has an impact on the consequent immune responses. There are four types of adenosine receptors, A1, A2a, A2b and A3, among which A2a receptor is considered as a predominant mediator of immunosuppressive responses [13–15]. A2a receptor is a Gs protein-coupled receptor that delivers signals through stimulation of adenylyl cyclase that generates second messenger cyclic AMP (cAMP), which can then activate various downstream effectors, including protein kinase A (PKA) [16].

Meanwhile, in addition to PRRs, DCs also express non-PRR receptors including adenosine receptor A2a [17]. However, while adenosine-A2a receptor signaling axis has been well recognized as a negative regulator in T cells [18], it is largely unexplored in DCs.

Here we demonstrate that PKA signaling pathway, as the potential downstream effector of adenosine-A2a receptor signaling axis in DCs, may modulate DCs' functionality in the regulation of Treg homeostasis. PKA activation in DCs in response to elevated extracellular adenosine may expand Treg cells, providing DCs a critical mechanism to induce immune tolerance especially in situations involving massive cell death such as tissue damages, hypoxia or inflammation, where the expanded Treg cells can limit over-exaggerated inflammatory responses. This mechanism may also provide a new strategy for clinical therapies of immunological diseases that are related to disturbed Treg homeostasis, including autoimmune diseases and tumor.

1.2 Protein kinase A (PKA) signaling

Protein kinase A (PKA) is also known as cAMP-dependent protein kinase, as its activity is dependent on the intracellular level of cAMP. When cAMP level is low, PKA remains inactive as a tetrameric holoenzyme composed of two catalytic subunits and two regulatory subunits [19]. However, when cAMP level rises as a consequence of signaling transduction through G protein coupled receptors such as adenosine receptors, cAMP binds to PKA regulatory subunits, which changes the confirmation of the regulatory subunits of PKA, finally leading to release and activation of the catalytic subunits of PKA, which is the active form of PKA [20, 21].

PKA can be classified into either type 1 PKA or type 2 PKA based on the two types of regulatory subunits, namely R1 subunits and R2 subunits [22]. Generally speaking, R2 subunits are expressed at a lower level in most tissues and have a lower binding affinity for cAMP compared to their R1 counterparts [22–24]. Nevertheless, both types of regulatory subunits have two variants, resulting in total four regulatory subunits, R1α, R1β, R2α and

R2β [22]. While R1α and R2α are expressed ubiquitously, R1β and R2β are limited to adrenal, adipose and neural tissues [24]. For catalytic subunits of PKA, two isoforms Cα and Cβ have been identified in mice, and similar to the expression pattern of regulatory subunits, Cβ isoform is predominantly located in the brain while Cα is considered as the major PKA catalytic component in non-neural tissues [24, 25]. Thus, in hematopoietic cells, type 1 PKA, which is composed of two R1α subunits and two Cα subunits, may act as the major player of PKA signaling in response to various G protein coupled receptor triggering.

Type 1 PKA is expressed in DCs, but it remains unclear how PKA signaling is activated in DCs. It has been reported that 4 types of adenosine receptors (A1, A2a, A2b and A3) are differentially expressed in different maturation stages of human DCs, where A2a receptor is the predominant form in mature DCs [26]. A2a receptor delivers signals through Gs proteins, which activate adenylyl cyclase to synthesize cAMP, a second messenger for PKA signaling [16]. In vivo, the natural ligand for adenosine receptors is extracellular adenosine, which is converted from ATPs that are abundantly released from dead cells, inflammatory tissues and tumors [27]. In addition to adenosine receptors, PKA can also be activated by EP2/EP4 receptors through PGE2 stimulation [28, 29], whose synthesis can be increased as a consequence of tissue damage. Thus, in addition to other stimuli, adenosine and PGE2 that are generated from dead cells, inflammatory tissues and tumor environment may potentially activate PKA signaling in DCs.

PKA signaling has been reported to interact with BCR signaling in B cells [30], and suppress T cell activation [31], indicating that PKA is an important modulator in various immune responses of both T and B cells. Since DCs play a central role in immune regulation, it is conceivable that PKA signaling could also influence DCs' functionality in the regulation of immune system, however, the role of PKA signaling in DCs and the impact of PKA activation in DCs on immune system are not yet clear.

1.3 Development and function of Treg cells

Treg cells are a distinct group of CD4+ T cells, and in the mouse immune system, they are characterized by the expression of the transcription factor Foxp3 [32, 33]. They play a pivotal role in the maintenance of immune tolerance and preventing inflammatory responses [34]. Several mechanisms have been demonstrated how Treg cells achieve their suppressive function, including secretion of inhibitory cytokines such as TGF-β and IL-10, cytolysis of T effector cells and suppression of DCs by LAG-3 and CTLA-4 dependent cell-cell contact [35, 36].

Foxp3+CD4+ Treg cells can be classified into two subsets: naturally occurring thymus-derived Treg cells (nTregs) and induced Treg cells (iTregs), based on whether they are originated in thymus or induced in periphery from conventional CD4+ T cells upon induction with TGF-β or IL-10 [37–39]. Although both subsets of Treg cells share common Treg markers including Foxp3, CD25, GITR and CTLA-4, there are some molecules that are differentially expressed between nTregs and iTregs (e.g., Helios and Nrp1 are expressed in nTregs but not iTregs.) [40], and recently, Nrp1 has been proved by two research groups to be a good marker to distinguish nTregs from iTregs [41, 42].

Given the importance of Treg cells in controlling immune tolerance, it is thus imperative to understand how Treg homeostasis is maintained well-balanced. Similar to conventional CD4+ T cells, nTregs generation in thymus is highly dependent on TCR signaling, and only thymocytes expressing TCR with a higher affinity for self-antigen-MHC II complexes can develop into Treg cells [43]. In addition to TCR signaling, CD28 co-stimulation and IL-2 cytokine have also been shown to collectively function as key factors in the homeostatic maintenance of Treg cells in both thymus and periphery, by using CD28-/- and IL-2-/- mice or IL-2 neutralization [44–47]. Moreover, because of its role in conversion of conventional CD4+ T cells to Treg cells, TGFβ has also been implicated in the control of peripheral Treg homeostasis [48].

Despite the control from TCR/co-stimulatory signaling and cytokines, robust Treg homeostasis, however, also requires appropriate modulations from other immune cells, among which DCs have been indicated to play a role in regulating Treg homeostasis. For example, thymic DCs activated by thymic stromal lymphopoietin (TSLP) support Treg development in thymus [49], and DCs that are derived from bone marrow cultured in vitro with the presence of GM-CSF have also been reported to help natural Treg expansion [50]. Additionally, treatment of DCs with vitamin D receptor agonists induces tolerogenic DCs, which also promote Treg development and immune tolerance [51]. Taking all together, it is apparent that DCs may exploit certain mechanisms or signaling pathways to modulate their functionality in the regulation of Treg homeostasis. Thus, it would be interesting to know whether PKA signaling in DCs also contributes to the maintenance of Treg homeostasis.

1.4 Dendritic cells and Treg cells

As the most potent professional antigen presenting cells (APCs) in immune system, DCs possess unique capacity to stimulate naïve T lymphocytes through antigen presentation. DCs are bone marrow derived cells, patrolling peripheral tissues as immature DCs for antigens. Upon recognition and uptake of antigens, immature DCs migrate to secondary lymphoid tissues where they interact with T lymphocytes, providing the first signaling through major histocompatibility complex (MHC) loaded with processed antigens, meanwhile DCs undergo maturation by up-regulating various co-stimulatory molecules, such as CD40, CD80 and CD86, as the second signaling to fully activate T lymphocytes, thus initiate the immune response.

In addition to their immunogenic role to initiate immunity, DCs have recently been proposed to be also involved in the induction of immune tolerance through various ways, including Treg cell induction and auto-reactive T cell anergy/apoptosis [7, 52]. However, it

is still not fully clear which type of DCs and through which mechanism that DCs tolerize immunity.

DCs are a heterogeneous cell population that can be divided into many different subsets according to their expression of specific markers and locations, generally including plasmacytoid DCs (pDCs) and classical DCs (cDCs) [53]. pDCs express low level of MHC class II and CD11c (in mouse), and they are also well known as Type I Interferon (IFN) producing cells, as they can rapidly secrete large amounts of type I IFN in response to virus infections [54]. pDCs have also been implicated to play tolerogenic functions, for example, both CpG-activated [55] and HIV-stimulated [56] human pDCs have been shown to promote iTreg generation from naive CD4+ T cells. In mouse, it has also been reported that CD8+ pDCs from mesenteric lymph nodes (MLN) are able to induce T regulatory 1-like cells with regulatory properties after CpG activation [57].

In contrast to pDCs, cDCs generally express high level of MHC class II and CD11c. cDCs can be further divided into different subsets based on different characteristics, such as CD11b+CD8- DCs versus CD11b-CD8+ DCs according to the expression of CD11b and CD8, resident cDCs versus migratory DCs based on whether DCs are migratory or resident in lymphoid tissues. Migratory DCs express extremely high level of MHC class II and intermediate level of CD11c, and besides their unique role in antigen transportation from non-lymphoid tissues to draining lymph nodes, they have recently gained much attention for their tolerogenic roles [58, 59]. Moreover, mucosal CD103+ DCs have also been reported to induce Treg cells via a TGFβ and retinoic acid-dependent mechanism [60]. Similarly in cDCs, it has also been shown that CD8+CD205+ DCs in spleen can induce peripheral Treg cells [61]. Therefore, it is undoubted that DCs play an important role in regulating Treg homeostasis, however, the question remains unclear which signal(s) trigger DCs to acquire their tolerogenic functionalities.

1.5 Type 1 diabetes and Treg cells

Type 1 diabetes (T1D) is an autoimmune disease characterized by insulin deficiency and hyperglycemia due to the destruction of insulin producing β cells by auto-reactive cells in pancreatic islets [62, 63]. In the past few decades, the understanding of T1D has been greatly progressed by using animal models including the nonobese diabetic (NOD) strain of mouse, which spontaneously develops autoimmune insulin dependent diabetes mellitus (IDDM) and is the most commonly used animal model for T1D [64].

T1D is generally considered to be mediated primarily by T cells, as the majority of infiltrating lymphocytes in pancreatic islets are found to be CD4+ and CD8+ T cells, and transfer of T cells from sick mice can induce disease in naive recipients [64–66]. In addition to T cells, the infiltrates may also include B cells, macrophages, DCs and NK cells [67], indicating the complexity of T1D development and progression through not only cell-mediated autoimmune responses but also innate immunity and autoantibody-mediated immune responses.

Foxp3+CD4+ Treg cells have been an important focus of T1D research due to their role in preventing destructive autoimmunity. Analysis of Treg cells in T1D-prone mouse models reveals either a reduced frequency or a function defect of Treg cells [68, 69]. It has been shown that depletion of Foxp3+ Treg cells in NOD mice increases T1D incidence and causes earlier onset of T1D compared to wild type NOD mice [70], while on the other side, adoptive transfer of natural CD25+ Treg cells into NOD mice ameliorates ongoing diabetes [71]. Moreover, IL-2, a cytokine that promotes Treg survival and proliferation, has also been reported to induce an increase of pancreatic Treg cells in NOD mice and result in a long-lasting remission of T1D [72]. These data together strongly imply an essential role of Treg cells in the control of T1D pathogenesis, therefore indicating that modulation of Treg homeostasis in vivo can be a powerful approach to prevent and treat T1D.

1.6 Tumor and Treg cells

Tumor uses multiple mechanisms to escape from immune rejection, and this includes inefficient cytotoxic T lymphocyte–mediated killing due to the lack of co-stimulatory signal or PD-L1/PD-1 inhibition; Additionally, tumor cells can often actively produce suppressive cytokines such as TGFβ to inhibit immune cells [73]. The last but not least mechanism is the accumulation of Foxp3+CD4+ Treg cells, which has been found in a multitude of tumors including pancreatic, hepatocellular, breast, ovarian, colorectal, lung cancers and melanoma [74, 75]. The accumulation of Treg cells in tumor seems to be very critical for the tumor growth, as depletion of Treg cells in tumor masses induces effective anti-tumor immunity and finally eliminates the etablished tumor [76–79].

Although the reasons for the enrichment of Treg cells in tumor microenvironment are not yet completely clear, multiple mechanisms have been proposed to explain this phenomenon [75, 80]. One of the mechanisms is the recruitment of Treg cells to the tumor through chemokines, and it has been reported that tumors such as human ovarian tumor and Hodgkin lymphoma can produce large amounts of CCL22, which is a ligand for chemokine receptor CCR4, to recruit Treg cells [81, 82]. In addition, conversion may also contribute to the increase of Treg cells in tumor, given that TGFβ produced by tumor cells may convert conventional CD4+ T cells into iTregs [83]. Furthermore, the stability of Treg cells in tumor can be enhanced through Nrp1 signaling in Treg cells with the presence of semaphorin-4a (sema4a) produced by tumor tissues [84]. Finally, the expansion of Treg cells in tumor could also be caused by proliferation of Treg cells within local tumor microenvironment, or distantly in tumor draining lymph nodes [85].

Given the importance of Treg homeostasis in tumor protection and the role of DCs in regulating Treg homeostasis, it is highly possible that DCs may also be hijacked by tumors for their own use to expand Treg cells to suppress immune responses against them. An obvious question following such hypothesis is how tumors manipulate DCs to expand Treg cells for their own protection. In addition to the accumulation of Treg

cells, tumor environment is also highly enriched for adenosine, whose receptor A2a has been demonstrated to suppress the function of T effector cells [18, 86], thus may inhibit anti-tumor immunity. However, it remains unclear whether this adenosine-A2a signaling pathway in tumor infiltrating DCs also contributes to the induction of immune tolerance in tumor microenvironment. Hopefully, the understanding of the above questions may help to provide a new strategy for the purpose of clinic tumor immunotherapy.

1.7 Rationale, hypothesis and objectives

Massive cell death, caused by tissue damage, inflammation and tumors, may release a large quantity of self-antigens which can be captured and presented by DCs. In order to avoid autoimmune responses, DCs must have evolved some mechanism(s) to acquire tolerogenic functionalities to suppress the immune system. In addition to self antigens, dead cells may also lead to a substantial increase of extracellular adenosine and PGE2, and through adenosine receptors and prostaglandin receptors (EP2/EP4), both may potentially activate intracellular PKA signaling in various types of immune cells, including DCs. On the other side, recent publication have demonstrated that tissue damage (induced by UV irradiation) can cause a systemic immunosuppression with an increase of Treg cells [87, 88]. Based on the above, we hypothesize that adenosine/PGE2-PKA signaling axis in DCs may modulate DCs' functionality to promote Treg homeostasis. Therefore, my thesis project will address the question whether adenosine-PKA signaling axis in DCs regulates the homeostasis of Treg cells, and also explore the possibility of targeting this signaling axis in DCs to treat autoimmune diseases and tumor.

CHAPTER 2
Materials and Methods

2.1 Animals and cell lines

C57BL/6, BALB/cJ and NOD/ShiLtJ mice were purchased from the Jackson Laboratory. PKAR1α floxed/floxed mice were first generated in former lab at Columbia University, and then crossed to CD11c-Cre Tg mice to obtain DC-PKAR1α ko mice. DC-PKACα-on mice (B6.129X1-Prkaca^{tm3Gsm}) were purchased from MU-MMRRC (The University of Missouri Mutant Mouse Regional Resource Center). DC-PKAR1α ko mice were backcrossed to NOD/ShiLtJ mice by 15 generations to get DC-PKAR1α ko NOD mice. All animal experiments were performed in accordance with the animal protocols approved by the Animal Care Committee of Institut de Recherches Cliniques de Montréal. Flt3L-secreting B16 melanoma cell line were obtained from Boris Reizis (Columbia University Medical Center).

2.2 Antibodies

Anti-CD3e (145-2C11), anti-TCRβ (H57-597) and anti-B220 (RA3-6B2) were purchased from BD Biosciences. Anti-CD4 (RM4.5), anti-CD8α (53-6.7), anti-CD25 (PC61.5), anti-CD44 (IM7), anti-MHC class II I-Ab (AF6-120.1), anti-CD11b (M1/70), anti-CD11c (N418), anti-CD80 (16-10A1), anti-CD86 (GL1), anti-PD-L1 (MIH5), anti-iCOSL (HK5.3), anti-CD40 (1C10), anti-GITRL (eBioYGL386), anti-OX40L (RM134L) and anti-FoxP3 (FJK-16s) were purchased from eBiosciences. Mouse/Rat Neuropilin-1 APC-conjugated Antibody was purchased from R&D Systems. Mouse IL-2 neutralizing antibody (JES6-1A12) was also purchased from eBiosciences.

2.3 Flow cytometry

Single cell suspensions were prepared from spleens, lymph nodes or thymus by passing through 70μM nylon cell strainers. Erythrocytes were lysed with ACK buffer (Lonza). Cells were incubated with appropriate antibodies labeled with fluorescence in FACS buffer (1% BSA and 0.05% sodium azide in PBS) for 30min at 4°C in the dark. After washes with FACS buffer, stained cells were subsequently analyzed using the CyAn ADP analyzer (Beckman Coulter). Acquired flow cytometry data were analyzed with FlowJo software (Tree Star Inc). Intracellular staining for FoxP3 or Ki67 were performed using Foxp3/Transcription Factor Staining Buffer Set (eBiosciences) as outlined by the manufacturer.

2.4 Purification of splenic DCs

To purify splenic DCs, single cell suspensions from spleens were washed and resuspended in PBS supplied with 2% FBS and 1mM EDTA. Splenic DCs were first enriched by depleting T cells, B cells, NK cells, macrophages and erythrocytes using EasySep Mouse Pan-DC Enrichment Kit (STEMCELL Technologies). Pre-enriched DCs were then stained with anti-mouse MHC class II I-Ab (AF6-120.1) and anti-mouse CD11c (N418) for subsequent sorting. Finally, pure DCs were sorted as MHC class II and CD11c double positive cells by MoFlo (Beckman Coulter). The purity of the resulting MHC class II+CD11c+ DC population was >99%.

2.5 In vitro co-cultures of DCs and T cells

DCs were purified as above. To sort T cell subpopulations (conventional T cells and Treg cells), CD4+ T cells were first enriched by using EasySep Mouse CD4 Positive Selection Kit (STEMCELL Technologies). After staining with anti-mouse CD25 (PC61.5) and anti-mouse CD44 (IM7), CD4+CD25-CD44- T conventional cells and CD4+CD25+CD44- Treg cells were sorted by MoFlo (Beckman Coulter). For conversion

assays, CD4+CD25-CD44- T conventional cells were purified from wt BALB/cJ mice, and co-cultured with DCs at the ratio of 3:1 for 3 days. TGFβ was used at a concentration of 5ng/ml. For Treg proliferation assays, CD4+CD25+CD44- Treg cells were purified from wt BALB/cJ mice, and labeled with 10μM cell proliferation dye eFluor® 450 (eBiosciences) according to manufacturer's instructions. Cell proliferation dye incorporated Treg cells were then co-cultured with DCs at the ratio of 3:1 for 3 days. For Treg survival assays, CD4+CD25+CD44- Treg cells were purified from wt C57BL/6 mice, and co-cultured with DCs at the ratio of 2:1 for 16-22 hours. Annexin V positive cells were analyzed by flow cytometry using Annexin V Apoptosis Detection Kit (eBiosciences).

2.6 Cytokine ELISA

To measure the cytokine production of DCs, 1×10^6/ml purified DCs were cultured in flat-bottomed 96-well plate for 22-26 hours with or without stimulus. For stimulus, either 1μg/ml LPS (Sigma-Aldrich) or 1μM CpG (InvivoGen) was added in the culture. Cytokine concentrations of culture supernatants were determined by ELISA. ELISA kits for IL-2, IL-10 and TGFβ were purchased from eBiosciences, and assays were performed as described by the manufacturer.

2.7 Tumor models

Male mice at the age of 8-10 weeks old were chose for tumor inoculations. B16-OVA, EG7 and Flt3L-secreting B16 tumor cells were harvested in the exponential phase of growth and washed with sterile PBS.

For tumor growth assays, 1×10^6 EG7 tumor cells were subcutaneously injected into DC-PKAR1α ko mice or their littermate controls. Tumors were measured every two days with digital calliper, and tumor volumes were calculated by the following formula [86]: volume = (length$\times$width$\times$width)/2. Mice were euthanized when the tumor volume

reached 2,000 mm^3, and the days after tumor challenging were recorded to draw a survival curve.

For the analysis of tumor infiltrating lymphocytes, wt C57BL/6 male mice were injected subcutaneously with 5×10^6 B16-OVA tumor cells. On day 12-14 post injection, tumors were taken for analysis, and both spleens and lymph nodes were also collected for the purpose of comparison. Tumors were minced into small pieces with scissor and digested in RPMI 1640 medium containing collagenase (Life Technologies) and DNase I (Life Technologies) at 37°C for 1 hour. After that, tumor cell suspensions were processed through 70μM cell strainers, washed with PBS, and ready for the following flow cytometry analysis. CD45+ cells were defined as hematopoietic cells. For the analysis of tumor-infiltrating DCs, Flt3L-secreting B16 tumor cells were specifically used instead of B16-OVA tumor cells, and the subsequent procedures after tumor inoculations were the same as described above.

2.8 Statistical analyses

Statistical significance was determined by Student's t tests using Prism software (GraphPad Software Inc). A P value of $\leq$0.05 was considered as statistically significant.

CHAPTER 3
Results

3.1 Enforced PKA activation in DCs results in global expansion of peripheral Foxp3+CD4+ Treg cells

To investigate whether DCs can regulate Treg homeostasis via PKA signaling pathway, we decided to artificially activate PKA specifically in DCs and then examine whether it affects the homeostasis of Treg cells. PKA is consist of two catalytic subunits and two regulatory subunits. Catalytic subunits function as active form of PKA while regulatory subunits are considered to be the repressor of PKA for their binding to catalytic subunits makes PKA inactive. Thus, to activate PKA, we could either delete the repressor or engineer catalytic subunits such that they are not constrained by repressor.

3.1.1 DC-specific ablation of PKA regulatory subunit R1α results in a marked increase in Treg cells

We first generated DC-specific PKAR1α ko (DC-PKAR1α ko) mice, as R1α appears to be the major repressor of PKA in DCs, due to its high binding-affinity for cAMP and expression level in hematopoietic cells [24]. We achieved this by crossing R1α floxed mice to CD11c-Cre Tg mice that specifically express Cre in CD11c+ DCs [89], in this way, PKA was constitutively activated only in DCs. Subsequent qPCR and western blot analysis confirmed that in DC-PKAR1α ko mice, PKAR1α was successfully deleted in DCs but not T and B cells (Figure 3-1.A and B).

The resulted DC-PKAR1α ko mice were fertile, developed normally and did not show any abnormalities within a normal life span. To determine the impact of DC-PKAR1α ko mutation on immune system, we analyzed the cellularity of thymus, spleen and lymph nodes by flow cytometry and found a significant increase of CD4+ T cells in the spleen of DC-PKAR1α ko mice, however, the numbers of CD8+ T cells, B cells and other

immune cells were not significantly affected (Figure 3-1.C). A similar phenomemon was also found in lymph nodes but not in thymus (Data not shown). Among CD4+ T cells, Foxp3+CD4+ Treg cells were dramatically expanded in the mutant mice (Figure 3-1.D), resulting the percentage of Foxp3+CD4+ Treg cells in total CD4+ T cells increased to 3-4 folds in mutant mice than in WT controls (Figure 3-1.E). Given that the PKAR1α deletion occurred only in DCs, we conclude that the Treg expansion in the mutant mice was caused by PKAR1α deficient DCs.

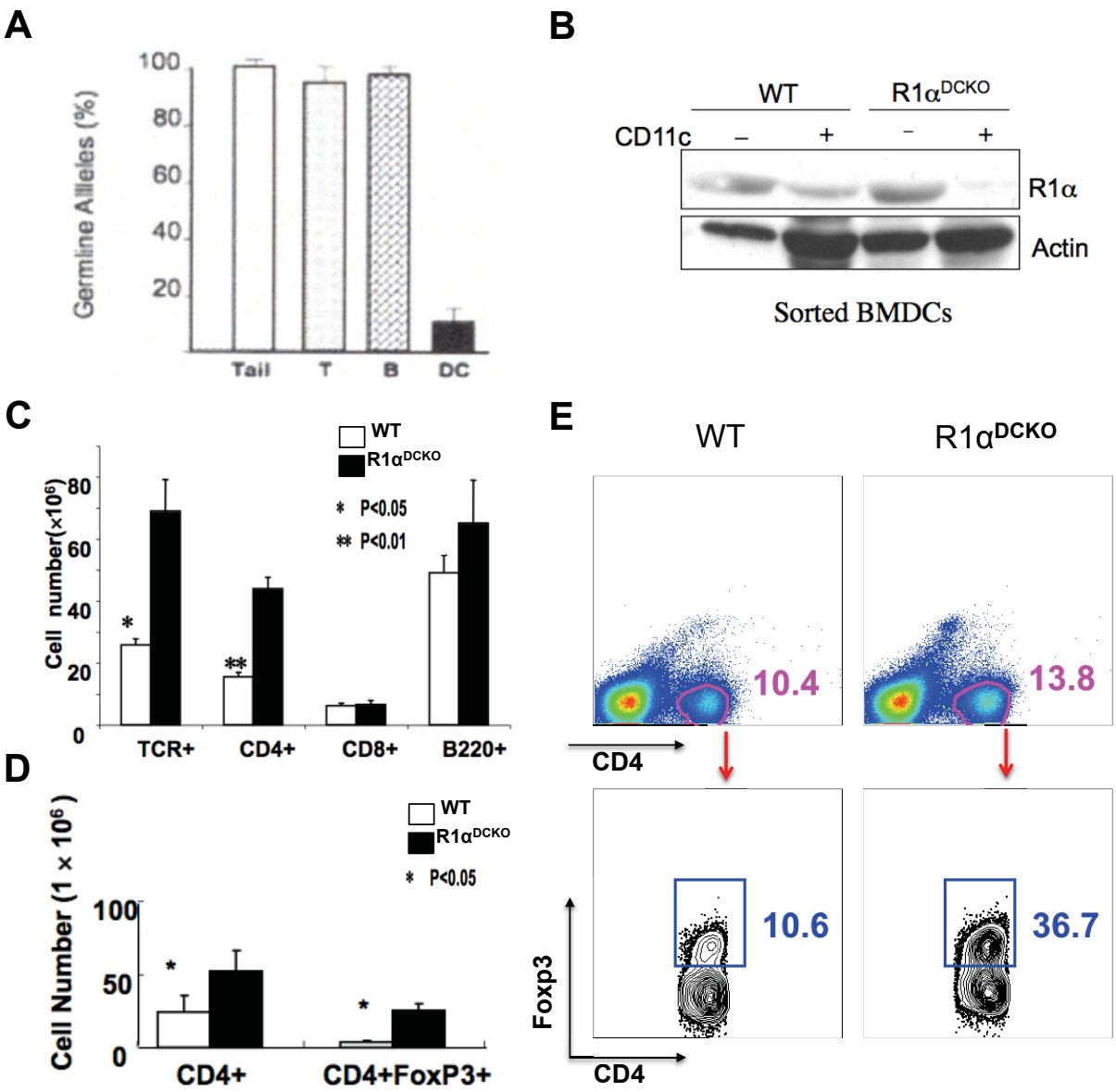

Figure 3–1: **DC specific PKAR1α ko mutation results in an expansion of peripheral Treg cells.** (A) Deletion efficiency of PKAR1α in DC-PKAR1α ko mice. qPCR results of PKAR1α deletion in various types of cells are shown. (B) Western blot analysis of the PKAR1α deletion in DCs. (C and D) Statistical analysis of total numbers of splenic T cells (TCR+), B cells (B220+), CD4+ T cells, CD8+ T cells (C) and Foxp3+CD4+ Treg cells (D) from WT and DC-PKAR1α ko mice by flow cytometry. (E) Flow cytometric analysis to show the percentage of splenic Foxp3+CD4+ Treg cells in total CD4+ T cells from WT and DC-PKAR1α ko mice (results are representative of at least five independent experiments). *P <0.05; **P <0.01. Error bars are SEM. Figure 3-1.A-D are contributed by Hyungjun Oh and Jian Tao.

3.1.2 DC-specific expression of a constitutively active form of PKA catalytic subunit Cα also leads to a global expansion of peripheral Treg cells

In addition to PKA signaling, R1α may also be involved in other signaling pathways. Thus, it is possible that the Treg expansion in DC-PKAR1α ko mice could be a result of some other signaling pathway rather than PKA pathway, but is also regulated by R1α in DCs. To rule out this possibility, we used another mouse strain in which a point mutation was introduced into PKA catalytic subunit Cα (PKACα), creating a constitutively active PKACα that is not repressed by regulatory subunits [90]. Again, we crossed this mouse strain to CD11c-Cre mice to generate new mutant mice that have a DC-specific expression of the constitutively active PKACα (DC-PKACα-on), so that PKA is constitutively activated only in DCs in DC-PKACα-on mice. Similar to what was found in DC-PKAR1α ko mice, DC-PKACα-on mice also had an expansion of Foxp3+CD4+ Treg cells in both spleen and lymph nodes (Figure 3-2). This result thus directly demonstrates that it is the PKA signaling in DCs that boosts the expansion of peripheral Treg cells.

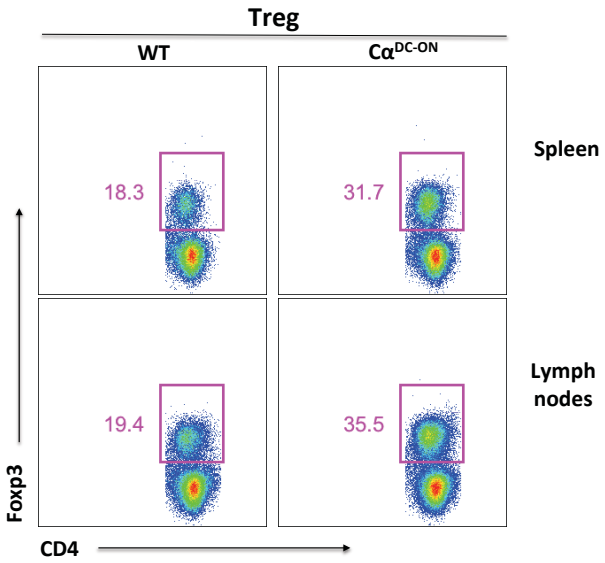

Figure 3–2: **DC-PKACα-on mice have increased proportions of Treg cells.** Flow cytometric analysis of Foxp3+CD4+ Treg cells in spleens and lymph nodes of WT and DC-PKACα-on mice. Cells were gated on CD4+ cells. Data are representative of three independent experiments.

3.2 DC-PKAR1α ko mice possess altered cDC subsets in spleen

DCs can be generally classified into two distinct categories: classical DCs (cDCs) and plasmacytoid DCs (pDCs) [53]. We next sought to determine which type of DCs was affected by PKA activation, therefore could be the possible inducer of Treg expansion in DC-PKAR1α ko mice.

Analysis of different DC subsets in DC-PKAR1α ko mice revealed a normal pDC population in both bone marrow and spleen (Data not shown). In addition, although the total number of cDCs in the spleen of mutant mice was comparable to that of WT cDCs (Figure 3-3.B), their composition was significantly changed. In particular, while WT splenic cDCs were well defined as a single cell population that expressed a high level of CD11c and an intermediate level of MHC II, termed here as subset 1 DCs (S1 DCs), DC-PKAR1α ko mice possessed a new subset 2 DCs (S2 DCs), in addition to S1 DCs (Figure 3-3.A). The S1 DCs were actually resident DCs, but we named them here and in the rest of thesis as S1 DCs in order for them to be compared to S2 DCs.

S2 DCs expressed a high level of MHC II and an intermediate level of CD11c, and similar to S1 DCs, S2 DCs can also be resolved into CD11b+ and CD8+ DCs. Moreover, although the ratio of CD11b+ versus CD8+ DCs in S2 DCs remained the same between DC-PKAR1α ko mice and WT controls, it was almost inverted in S1 DCs, from 63.8% : 25.8% in WT mice to 33.4% : 59.2% in mutant mice (Figure 3-3.C). These results indicate that cDC subsets are affected by PKA activation in DC-PKAR1α ko mice, suggesting that they could be responsible for the expansion of peripheral Treg cells in the mutant mice.

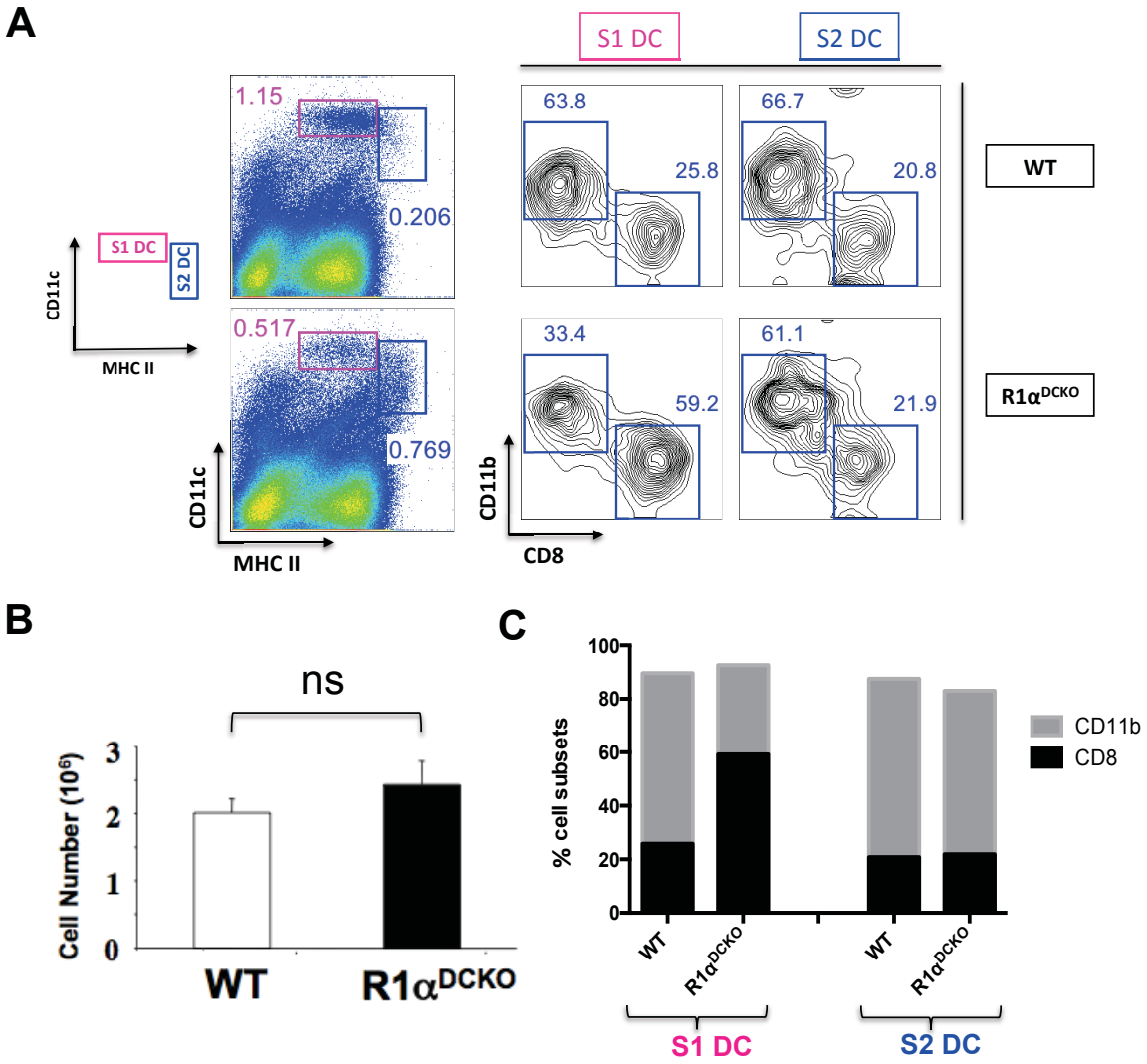

Figure 3–3: **DC specific depletion of PKAR1α alters splenic cDC subsets.** (A) Representative flow cytometric data of cDC subsets in spleens from WT and DC-PKAR1α ko mice. MHC II^{int}CD11c^{high} S1 DCs were shown in red box and MHC II^{high}CD11c^{int} S2 DCs were shown in blue box. (B) Total cell numbers of splenic cDCs in WT and DC-PKAR1α ko mice. (C) Ratios of CD11b+ versus CD8+ DCs in percentage. Each figure is representative of at least ten experiments. Figure 3-3.B is contributed by Hyungjun Oh and Jian Tao.

3.3 Treg expansion in DC-PKAR1α ko mice is primarily due to the proliferation of peripheral Treg cells

The enhanced homeostasis of peripheral Treg cells could be explained by 1) more output from the source, which is the thymus where most Treg cells are generated and developed; 2) enhanced proliferation or survival of mature Treg cells in the periphery; and 3) more conversion of conventional CD4+ T cells into induced Treg cells.

3.3.1 DC-PKAR1α ko mutation does not affect Treg development in thymus of mice at P7

To determine the reason for Treg expansion, we first examined early development of thymic Treg cells in DC-PKAR1α ko mice. Since it is known that thymic Treg cells start their migration to the periphery after day 3 [91], we chose to analyze the mice at day 7 so that thymic Treg cell population could be compared with splenic Treg cell population, using splenic Treg cell population as internal reference. Consistent with our finding in adult DC-PKAR1α ko mice, P7 mutant mice also possessed an increase of Treg cells in spleen, however, in thymus, the percentage of Treg cells in total CD4+ T cells remained unaltered (Figure 3-4), indicating Treg development in thymus is not affected by DC-PKAR1α ko mutation.

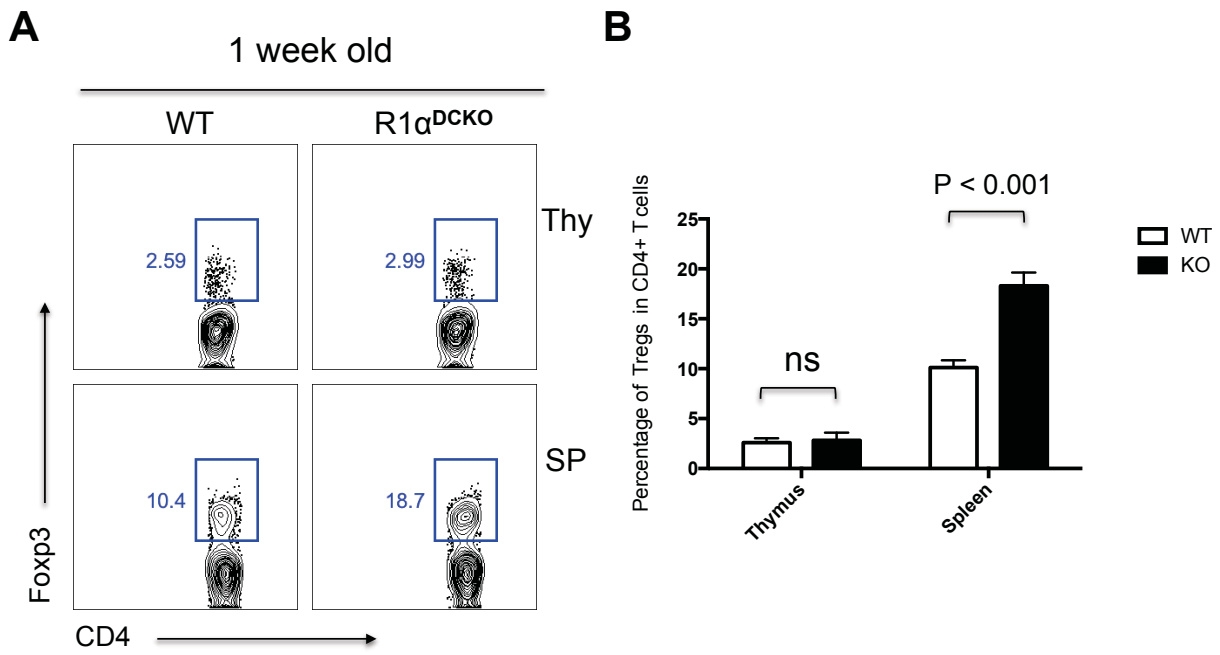

Figure 3–4: **DC specific depletion of PKAR1α does not promote Treg development in thymus.** (A) Representative flow cytometric analysis of thymic Treg cells in WT and DC-PKAR1α ko mice. P7 (1 week old) mice were used for analysis. (B) Statistical analysis of percentages of Treg cells in total CD4+ T cells in thymi and spleens. Data are pooled from 3 WT mice and 4 DC-PKAR1α ko mice.

3.3.2 PKAR1α deficient DCs do not show an enhanced ability to convert conventional CD4+ T cells into Treg cells

To determine whether PKAR1α deficient DCs promote the conversion of conventional CD4+ T cells into induced Treg cells, we sorted out both S1 DCs and S2 DCs from spleens of DC-PKAR1α deficient mice and S1 DCs from WT mice (WT DCs) as controls, and co-cultured them respectively with sorted conventional CD4+ T cells (CD4+CD25-CD44-) in vitro for 3 days, either with or without the presence of TGF-β. In order to induce allogeneic responses, we sorted conventional CD4+ T cells from BALB/c mice while all groups of DCs were from C57BL/6 origin. The percentage of Foxp3+CD4+ Treg cells in total cultured CD4+ T cells in each group was then analyzed on both Day 1 and Day 3 to show the conversion ability of each different group of DCs. To avoid

the possible impact of Treg contamination in sorted conventional CD4+ T cells on the results, another control group was set up where conventional T cells were cultured alone without DCs. We found that all groups of DCs were able to induce the conversion even at day 1, and as expected, TGF-β synergistically promoted the conversion of conventional CD4+ T cells to Treg cells after three day's co-culture, however, S1 and S2 DCs from DC-PKAR1α deficient mice failed to show an enhanced conversion ability (Figure 3-5). In addition, almost no Treg cells were detected in the control group where conventional CD4+ T cells were cultured alone, indicating that the Treg cells found in other groups were indeed resulted from the conversion but not because of Treg contamination in the sorted conventional CD4+ T cells. Our result thus suggests that PKAR1α deficient DCs may promote Treg expansion in DC-PKAR1α ko mice through other ways rather than conversion.

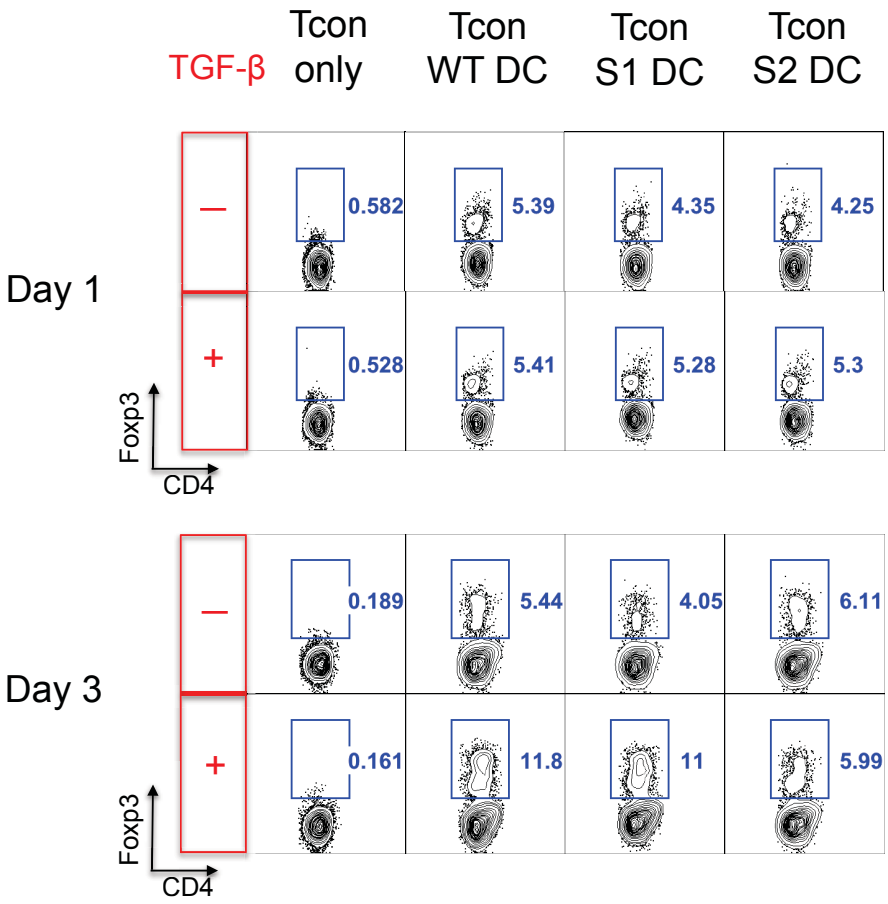

Figure 3–5: **PKAR1α deficient DCs do not enhance the conversion of conventional CD4+ T cells into induced Treg cells in vitro.** Conventional CD4+ T cells (CD4+CD25-CD44-) were sorted out from WT BALB/c mice, and cultured alone, or co-cultured with purified S1 and S2 DCs from DC-PKAR1α ko mice or purified WT DCs from WT C57BL/6 mice in a 3:1 ratio for 3 days in the presence or absence of 5ng/ml TGFβ. The percentage of Foxp3+CD4+ Treg cells were analyzed on day 1 (upper panel) and day 3 (lower panel). The experiment was repeated twice with similar results.

3.3.3 Increased Foxp3+ Treg cells in DC-PKAR1α ko mice are mostly Nrp1+ and express a high level of Ki67

Based on their origin, Treg cells can also be divided into either natural Treg cells (nTregs) or induced Treg cells (iTregs). nTregs are derived from thymus, while iTregs are converted in the periphery from conventional CD4+ T cells upon induction with TGF-β

or IL-10. Since Nrp1 has been reported to be a reliable marker to distinguish nTregs and iTregs [41, 42], we next checked Nrp1 expression on the increased Treg cells in our mutant mice by flow cytometry. Similar to the WT mice, most of the Treg cells in both DC-PKAR1α ko mice and DC-PKACα-on mice were Nrp1+, meaning that they were nTregs (Figure 3-6.A). This finding also supports our previous conclusion that the Treg expansion in the mutant mice is not caused by the enhanced conversion which generates Nrp1 negative iTregs.

Moreover, because our previous data also showed that DC-PKAR1α ko mutation didn't affect Treg development in thymus, we proposed that the increased nTregs are probably expanded through proliferation in the periphery. To test this hypothesis, we examined in Treg cells the expression of Ki67 which is a marker used to indicate cell proliferation, and indeed we found that Treg cells from mutant mice expressed a higher level of Ki67 compared to those from WT mice (Figure 3-6.B), suggesting that they are nTregs undergoing vigorous proliferation.

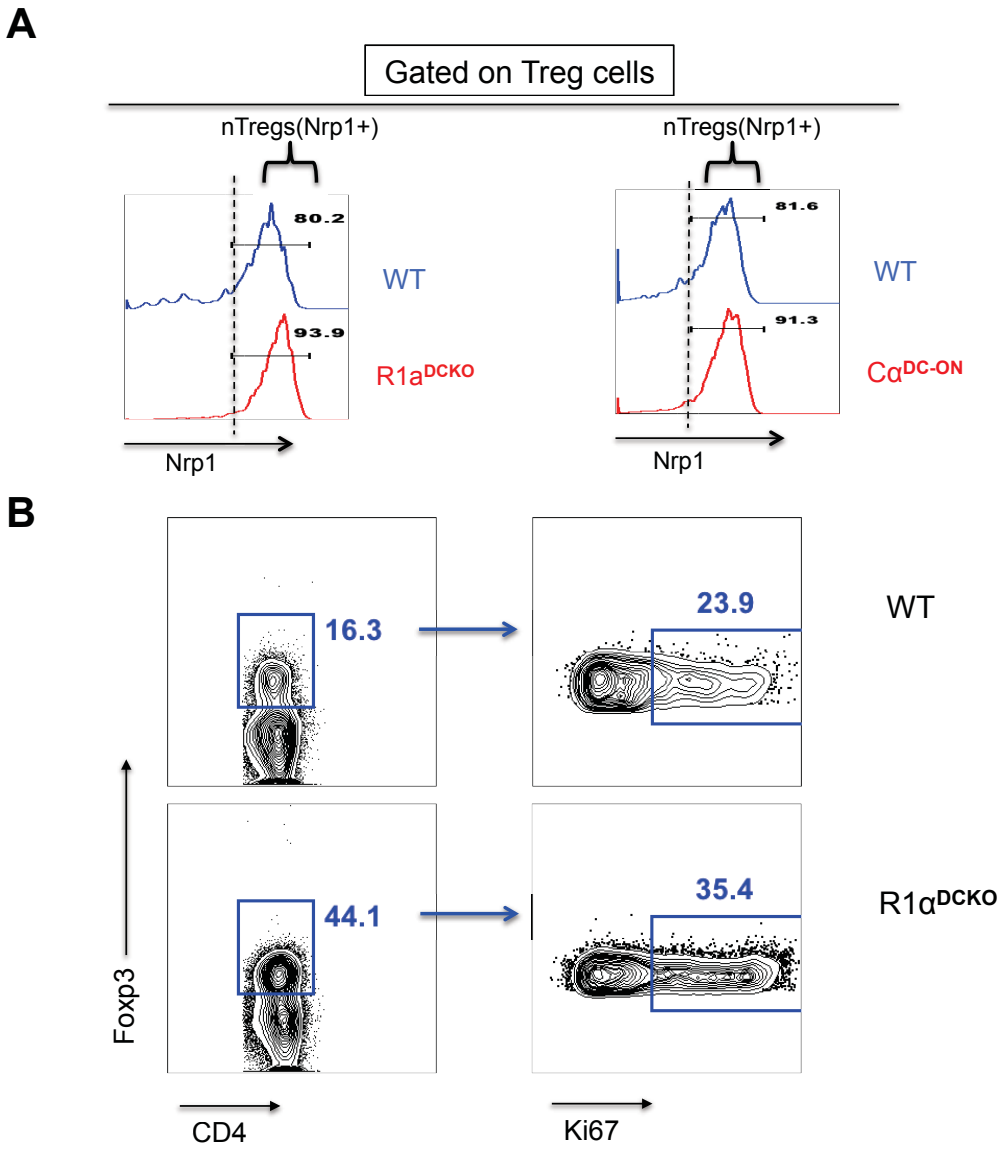

Figure 3–6: **Increased Treg cells in DC-PKAR1α ko mice are mostly Nrp1+ nTregs and express a high level of Ki67.** (A) Nrp1 expression on Treg cells in WT, DC-PKAR1α ko (left panel) and DC-PKACα-on (right panel) mice. (B) Treg cells from WT and DC-PKAR1α ko mice were analyzed for the expression of Ki67. Results are representative of at least three independent experiments.

3.3.4 PKAR1α deficient DCs promote proliferation of Treg cells in vitro

To further confirm our hypothesis that the increased Treg cells in mutant mice are expanded through proliferation by PKAR1α deficient DCs, we then sorted out again

different groups of DCs, including S1 DCs and S2 DCs from DC-PKAR1α deficient mice and S1 DCs from WT mice (WT DCs) as controls. Treg cells from WT BALB/c mice were also purified by sorting based on their surface markers (CD4+CD25+CD44-) and then labeled with cell proliferation dye eFluor® 450. After 3 day's co-culture of Treg cells with different groups of DCs, we found that Treg cells from both S1 and S2 DCs' group were more proliferative than those from WT DCs' group, indicated by that the percentage of Treg cells in proliferation was almost doubled (Figure 3-7). This result demonstrates that PKAR1α deficient DCs promote the proliferation of Treg cells in vitro. Taken all results together, we therefore conclude that Treg expansion in DC-PKAR1α ko mice is primarily caused by the proliferation of peripheral Treg cells.

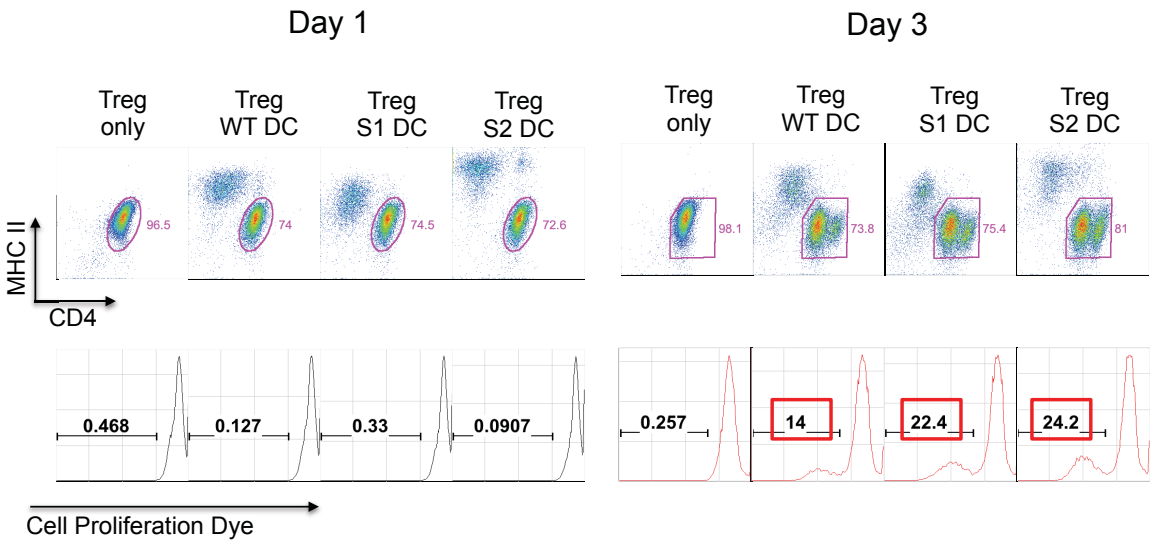

Figure 3–7: **PKAR1α deficient DCs promote the proliferation of Treg cells in vitro.** Treg cells (CD4+CD25+CD44-) sorted from WT BALB/c mice were labeled with 10μM cell proliferation dye eFluor® 450, and co-cultured with purified S1 and S2 DCs from DC-PKAR1α ko mice or with purified WT DCs in a 3:1 ratio for 3 days. Proliferation of cultured Treg cells were analyzed on day1 (left panel) and day3 (right panel) by intracellular staining of cell proliferation dye eFluor® 450(lower panel, gated on CD4+ cells). The experiment was repeated twice with similar results.

3.4 PKAR1α ko mutation in DCs alters the phenotype and function of DCs

3.4.1 PKAR1α deficient DCs show an activated phenotype of various co-stimulatory molecules including PD-L1

DCs are well known for their capability of antigen presentation, which requires co-stimulation. It is thus possible that change of the expression pattern of co-stimulatory molecules on DCs can not only affect DCs' phenotypes but also affect DCs' functionality in immune regulation, including the regulation of Treg homeostasis.

To determine whether DC-PKAR1α ko mutation changes the phenotypes of cDCs, we examined various co-stimulatory molecules together with MHC II that are expressed on DCs by flow cytometry. Although the expression levels of TNF-TNFR family members such as CD40, GITRL and OX40L in mutant cDC subsets remained the same as their WT counterparts, B7 family members were differentially expressed (Figure 3-8.A). In particular, CD86 was up-regulated in both S1 DCs and S2 DCs from mutant mice, and more interestingly, the expression pattern of PD-L1 was dramatically changed in DC-PKAR1α ko mice (Figure 3-8.A). Further analysis revealed that PD-L1 was co-expressed with CD11b in S1 DCs, but in S2 DCs, both CD11b and CD8 DCs started to express PD-L1, and in mutant S2 DCs, PD-L1 was also greatly up-regulated (Figure 3-8.B). These data suggest that DC-PKAR1α ko mutation changes the phenotypes of cDCs, which might be responsible for the Treg expansion in DC-PKAR1α ko mice. However, whether it is true, and more specifically, whether it is because of PD-L1 expression on DCs that causes the expansion of Treg cells in DC-PKAR1α ko mice, further studies must be done before we can draw a conclusion.

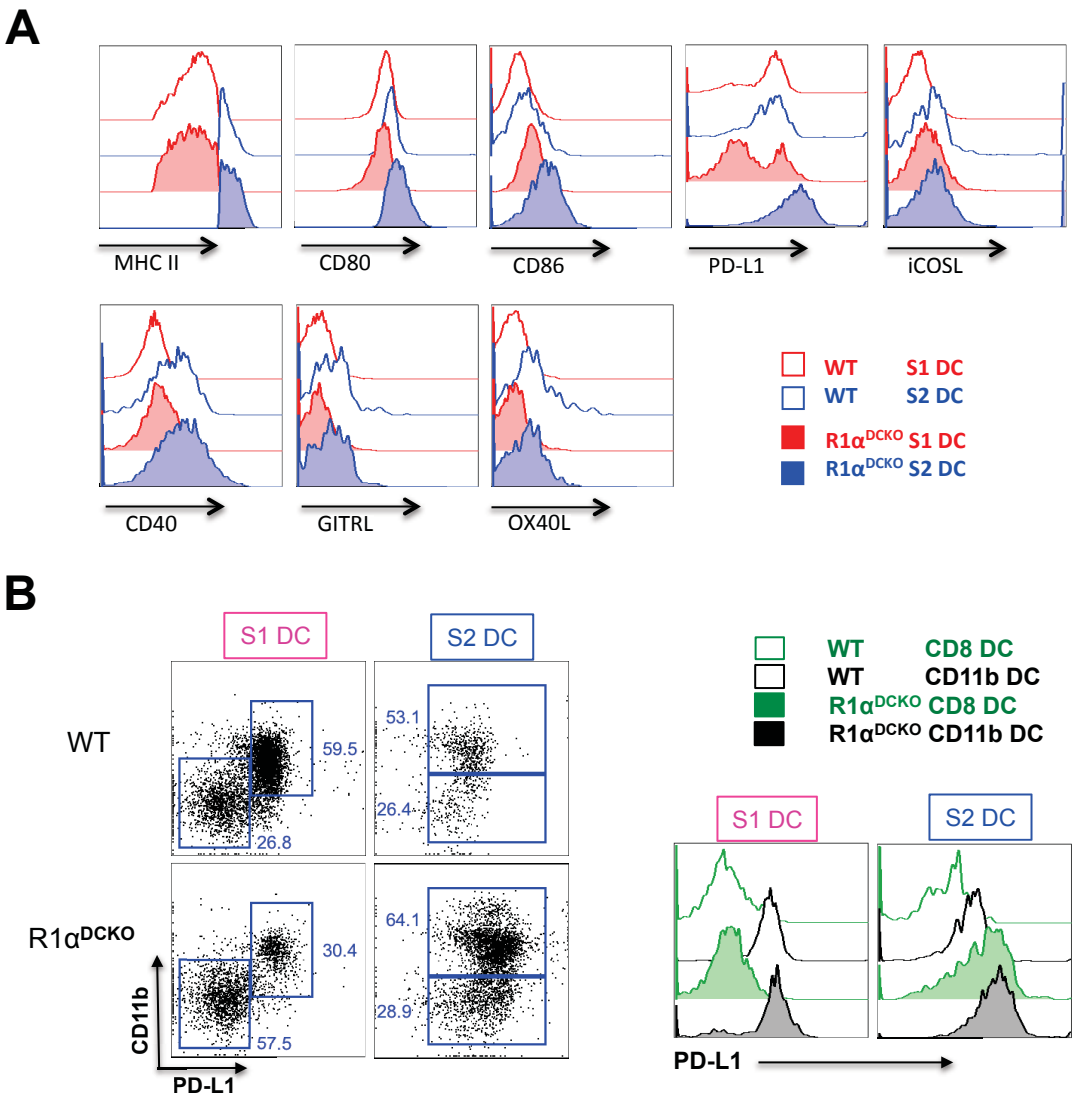

Figure 3–8: **PKAR1α deficient DCs display an activated phenotype of various co-stimulatory molecules including PD-L1.** (A) DCs from WT and DC-PKAR1α ko mice were analyzed for the expression of MHC II, CD80, CD86, PD-L1, iCOSL, CD40, GITRL, OX40L by flow cytometry. S1 DC and S2 DC were shown in red and blue respectively. (B) PD-L1 expression in S1 and S2 DCs from WT and DC-PKAR1α ko mice. Data are representative of 2-3 independent experiments.

3.4.2 PKAR1α ko DCs produce high basal level of IL-2, but not IL-10 or TGF-β

In addition to the phenotypic changes in the expression of co-stimulatory molecules, DCs may also regulate Treg homeostasis indirectly by secreting functional cytokines. For example, IL-2 is known to be essential for Treg survival and proliferation, and both IL-10 and TGFβ have been shown to promote the conversion of conventional CD4+ T cells to iTregs. To determine whether PKAR1α deficient DCs regulate Treg homeostasis through these cytokines, we first measured IL-2, IL-10 and TGFβ production of different cDC subsets in vitro. We found that the mutant DCs, both S1 and S2 DCs, were able to produce certain amount of IL-2 even without any stimulation, while for unstimulated WT DCs, their IL-2 production was not detectable (Figure 3-9.A). This result indicates that PKAR1α ko mutation makes DCs acquire the capability of producing IL-2 in steady state. Specifically, at the cell concentration of 1×10^6/ml, PKAR1α ko S1 DCs produced 10 pg/ml IL-2, and PKAR1α ko S2 DCs produced 30 pg/ml IL-2 (Figure 3-9.B). Although the mutant DCs produced extremely higher amount of IL-10 than their WT counterparts when stimulated with LPS or CpG, the basal level (without stimulation) of IL-10 production in mutant DCs was not significantly changed (Figure 3-9.C), and also, TGFβ secretion was not enhanced in the mutant DCs as compared to WT DCs (Figure 3-9.D). These findings again support our previous conclusion that the Treg expansion in PKAR1α ko mice is not caused by enhanced conversion, which can be mediated by IL-10 or TGFβ.

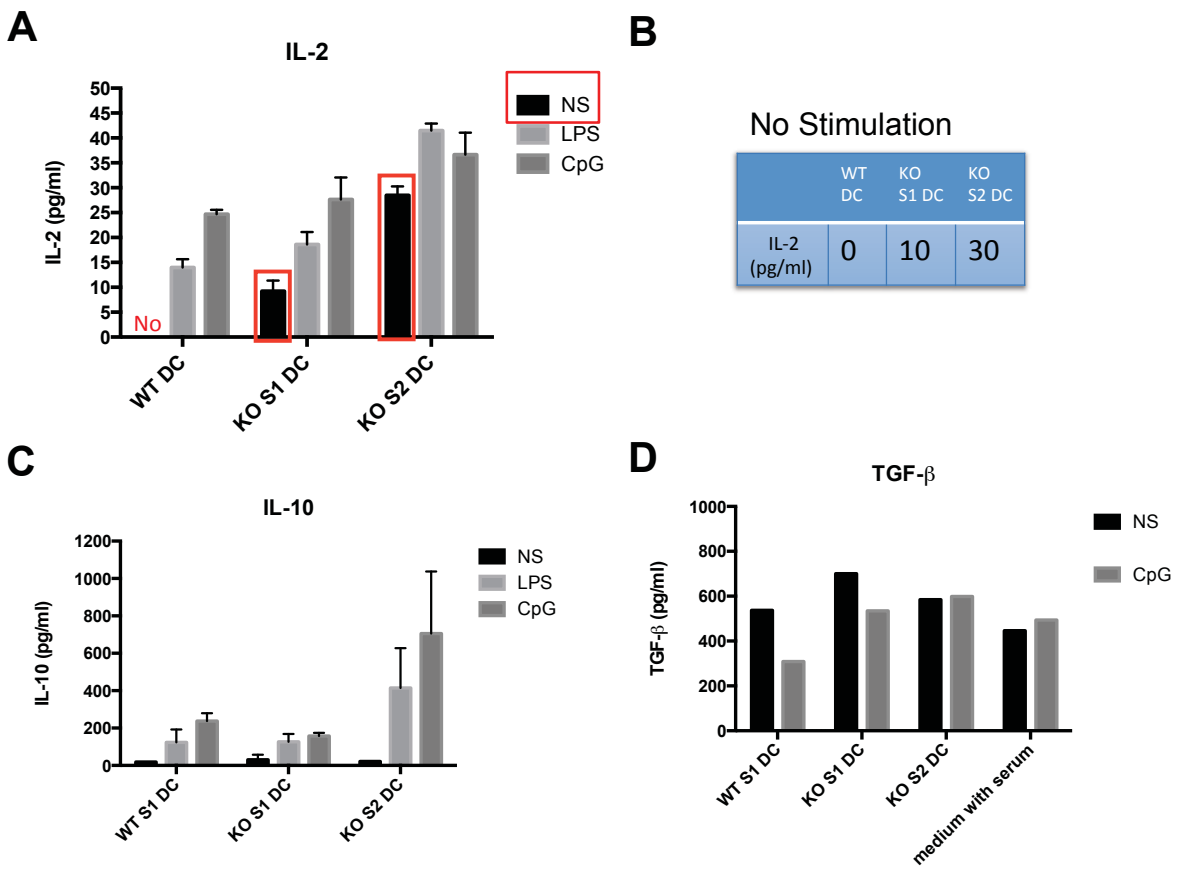

Figure 3–9: **PKAR1α deficient DCs produce high basal level of IL-2, but not IL-10 or TGF-β.** (A, C and D) IL-2 (A), IL-10 (C) and TGFβ (D) production of purified DCs from WT and DC-PKAR1α ko mice were measured after 22-26 hours' culture at a concentration of 1×10^6/ml without (NS) or with 1μg/ml LPS or 1μM CpG. (B) The amounts of IL-2 produced by 1×10^6/ml purified DCs from WT and DC-PKAR1α ko mice without stimulation. Results are representative of 2-3 independent experiments. Error bars are SEM.

3.4.3 PKAR1α ko DCs support Treg survival in an IL-2 dependent manner

To determine whether the IL-2 produced by PKAR1α deficient DCs is sufficient to support Treg survival, we co-cultured Treg cells with different DC subsets and then examined Treg apoptosis by Annexin V. Culture of Treg cells with mutant S2 DCs significantly reduced the apoptotic rate of Treg cells (Figure 3-10.A), and this benefit

in Treg survival was abolished by the presence of anti-IL-2 antibody that can block the function of IL-2 (Figure 3-10.C), suggesting IL-2 is also necessary for the survive of Treg cells. Moreover, when cultured with different concentrations of IL-2, the culture of Treg cells with the presence of 30 pg/ml IL-2 improved Treg survival (Figure 3-10.B). These results indicate that enhanced IL-2 production is at least one of the reasons for the altered functionality of PKAR1α deficient DCs to support Treg survival/proliferation.

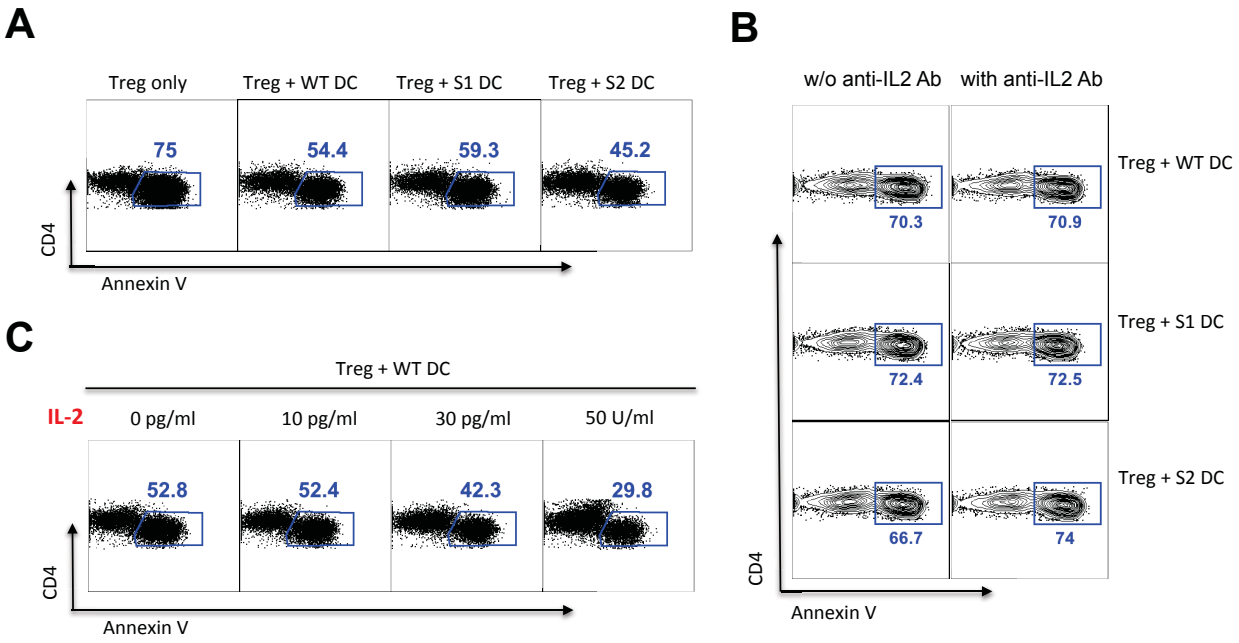

Figure 3–10: **PKAR1α ko DCs support Treg survival in an IL-2 dependent manner.** (A and B) Treg cells (CD4+CD25+CD44-) were sorted out from WT C57BL/6 mice, and co-cultured with purified DCs from WT and DC-PKAR1α ko mice at a ratio of 2:1 for 16-22 hours, in the presence (A) or absence (B) of anti-IL2 antibody. Annexin V positive cells were analyzed by flow cytometry. (C) As in (A and B), sorted Treg cells were co-cultured with WT DCs with different concentrations of IL-2 as indicated. Results are representative of two independent experiments.

3.5 DC-PKAR1α ko mutation does not protect NOD mice from Type 1 Diabetes

Treg cells can suppress various immune responses including autoimmune responses, therefore the enhanced Treg homeostasis in DC-PKAR1α ko mice suggests that the mutant mice might be resistant to autoimmune diseases such as Type 1 Diabetes (T1D). To test this possibility, we backcrossed DC-PKAR1α ko/C57BL/6 mice to T1D-prone NOD mice for fifteen generations, and by SNP analysis we also confirmed that 99.9% of the resulted DC-PKAR1α ko/NOD mice' genome were derived from NOD mice.

In the resulted DC-PKAR1α ko/NOD mice, we found a similar increase of Foxp3+CD4+ Treg cells as in DC-PKAR1α ko/C57BL/6 mice (Figure 3-11.A and B), indicating that PKAR1α deficient DCs also regulate Treg homeostasis in NOD mice. However, the Treg expansion in NOD mice failed to improve the overall survival rate of T1D in NOD mice (Figure 3-11.C), suggesting that DC-PKAR1α ko mutation does not protect NOD mice from Type 1 Diabetes.

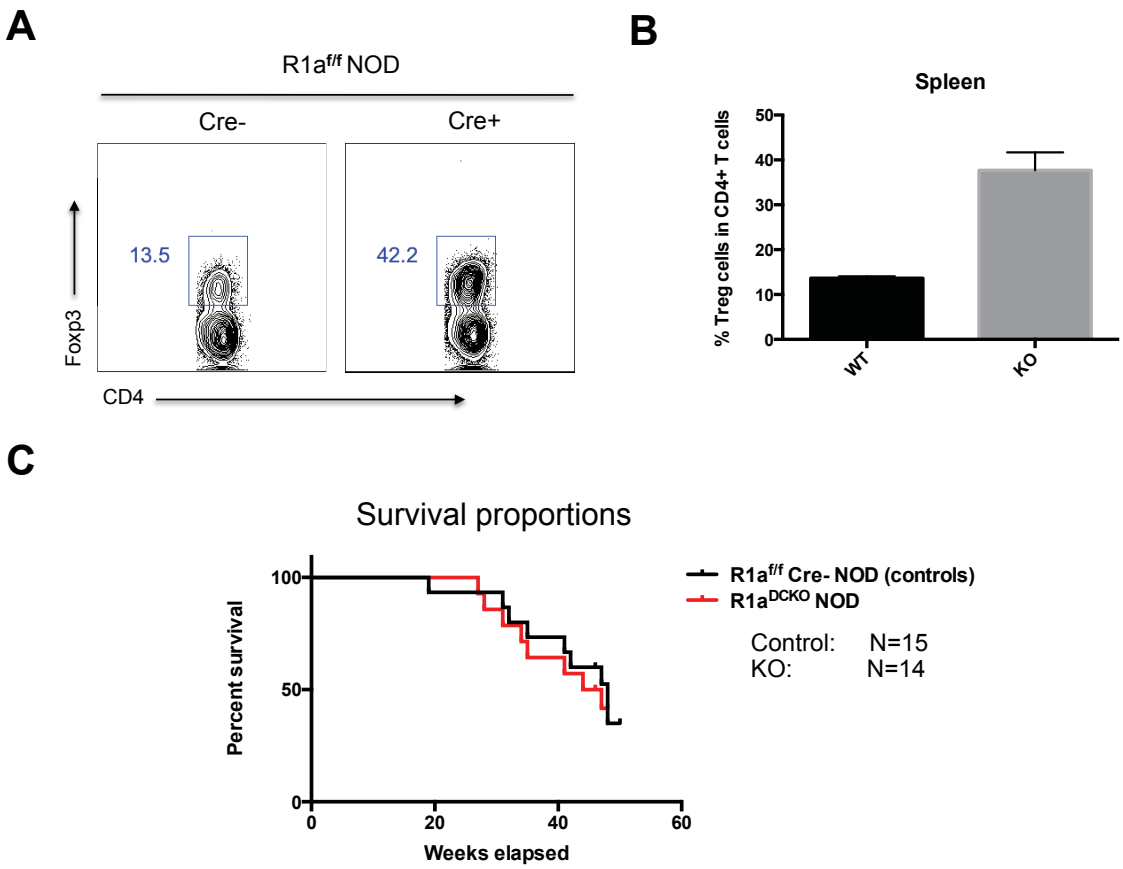

Figure 3–11: **DC specific PKAR1α ko mutation does not protect NOD mice from T1D.** (A and B) Flow cytometric analysis (A) and statistical analysis (B) to show the percentage of splenic Foxp3+CD4+ Treg cells in total CD4+ T cells from DC-PKAR1α ko/NOD mice and their Cre- littermate controls. Data are representative of at least two independent experiments. (C) Survival curves of DC-PKAR1α ko/NOD mice (n=14) and their Cre- littermate controls (n=15).

3.6 PKA signaling pathway in DCs is a potential target for tumor immunotherapy

Tumor is another class of diseases that involve Treg cells, and it has been widely noticed that tumors often have a proportionally increased number of Treg cells, which protect them from immune attack [74, 75]. However, the mechanism used by tumor to maintain the homeostasis of tumor regionally expanded Treg cells remains unclear. Since our results demonstrate that PKA activated DCs have the ability to boost Treg expansion, and it is also known that DCs are found to be existed in tumor microenvironment [92], we next sought to test whether it is possible that PKA activated DCs play a role in the regulation of tumor regional Treg homeostasis.

3.6.1 Tumor infiltrating DCs phenocopy PKA activated DCs

We first examined whether tumor infiltrating DCs acquired the phenotypes of PKA activated DCs. Due to the extremely low frequency of DCs in tumor infiltrating leukocytes, we used Flt3L-secreting B16 melanoma cell line as our tumor model for inoculation. Flt3 ligand is known as a growth factor of DCs, therefore using B16-Flt3L melanoma cells allows us to expand tumor infiltrating DCs in vivo. Similar to PKA activated DCs, including both PKAR1α ko DCs and PKACα-on DCs, tumor infiltrating DCs also possessed the new subset 2 DCs, which were barely observed in WT DCs (Figure 3-12).

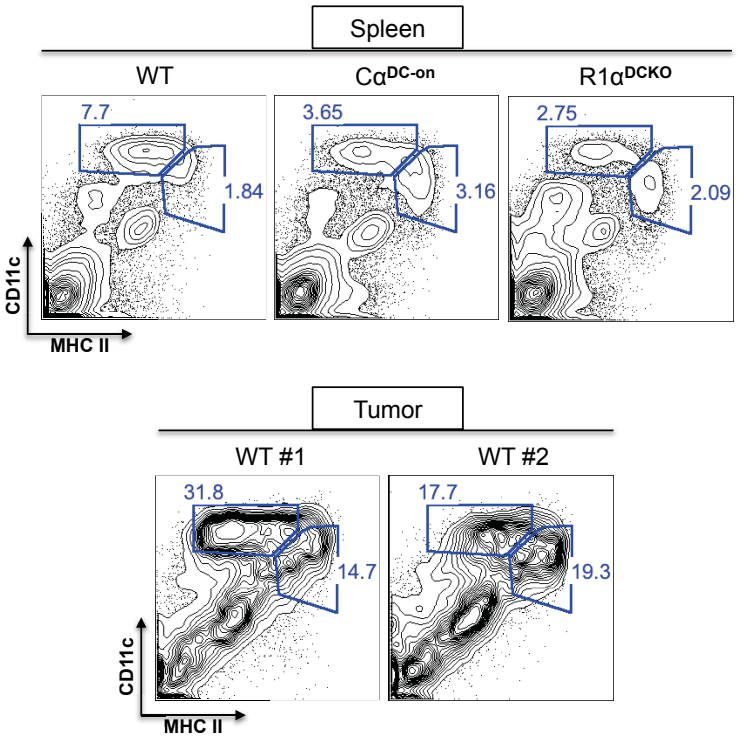

Figure 3–12: **Tumor infiltrating DCs possess MHC II^{high}CD11c^{int} subset 2 DCs.** Representative flow cytometric data of tumor infiltrating DCs (lower panel), as compared to splenic DCs from WT, DC-PKAR1α ko and DC-PKACα-on mice (upper panel). Tumor bearing mice were taken for analysis 12-14 days post injection of 5×10^6 Flt3L-secreting B16 melanoma cells. Cells shown above were gated on CD3-B220-NK1.1-TER119- cells. The experiment was repeated twice with similar results.

Further analysis of expressions of various co-stimulatory molecules together with MHC II revealed that S2 DCs, including tumor infiltrating S2 DCs, PKAR1α ko S2 DCs and PKACα-on S2 DCs, shared a similar expression pattern, which is distinct from that of conventional WT S1 DCs (Figure 3-13). Taken all together, these data indicate that tumor infiltrating DCs phenocopy PKA activated DCs, and given the fact that tumor is enriched for adenosine, which is a potent stimulator of A2a-PKA signaling, these data also suggest that tumor infiltrating DCs are probably PKA activated DCs.

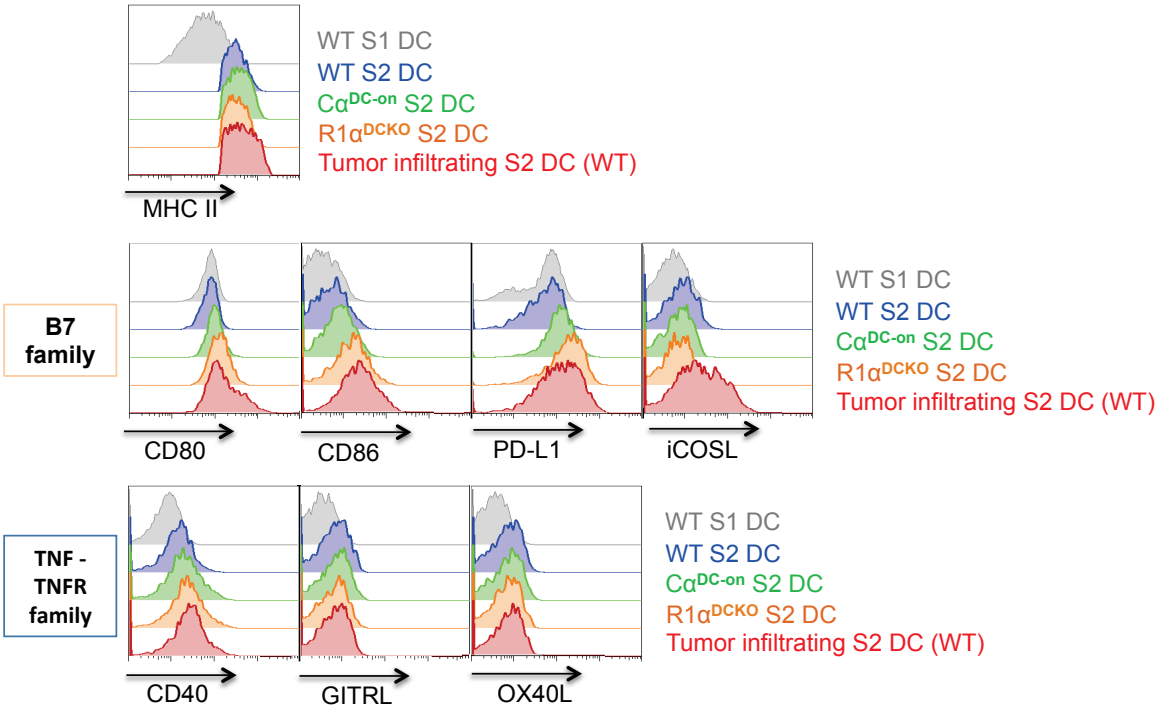

Figure 3–13: **Tumor infiltrating S2 DCs have a similar expression pattern of various co-stimulatory molecules as PKA activated S2 DCs.** Expression of MHC II and various co-stimulatory molecules by different groups of DCs as indicated. WT S1 DCs were gated on MHC $II^{int}CD11c^{high}$ cells and S2 DCs were MHC $II^{high}CD11c^{int}$. Data are representative of two independent experiments.

3.6.2 Tumor infiltrating nTreg cells are mostly proliferating Treg cells

The expansion of Treg cells in DC-PKAR1α ko mice is mostly through the proliferation of peripheral nTregs, to test whether it is the same for Treg expansion in tumor, we inoculated WT mice with B16-OVA melanoma cells, and analyzed tumor infiltrating Treg cells two weeks after inoculation by flow cytometry. We found that Foxp3+CD4+ Treg cells in tumor tissues were indeed enriched by approximately 3-4 times as compared to that in the spleen or lymph nodes of the same tumor bearing mice (Figure 3-14.A). Among the total tumor infiltrating Treg cells, approximately half of them were nTregs expressing Nrp1 (Figure 3-14.B and C).

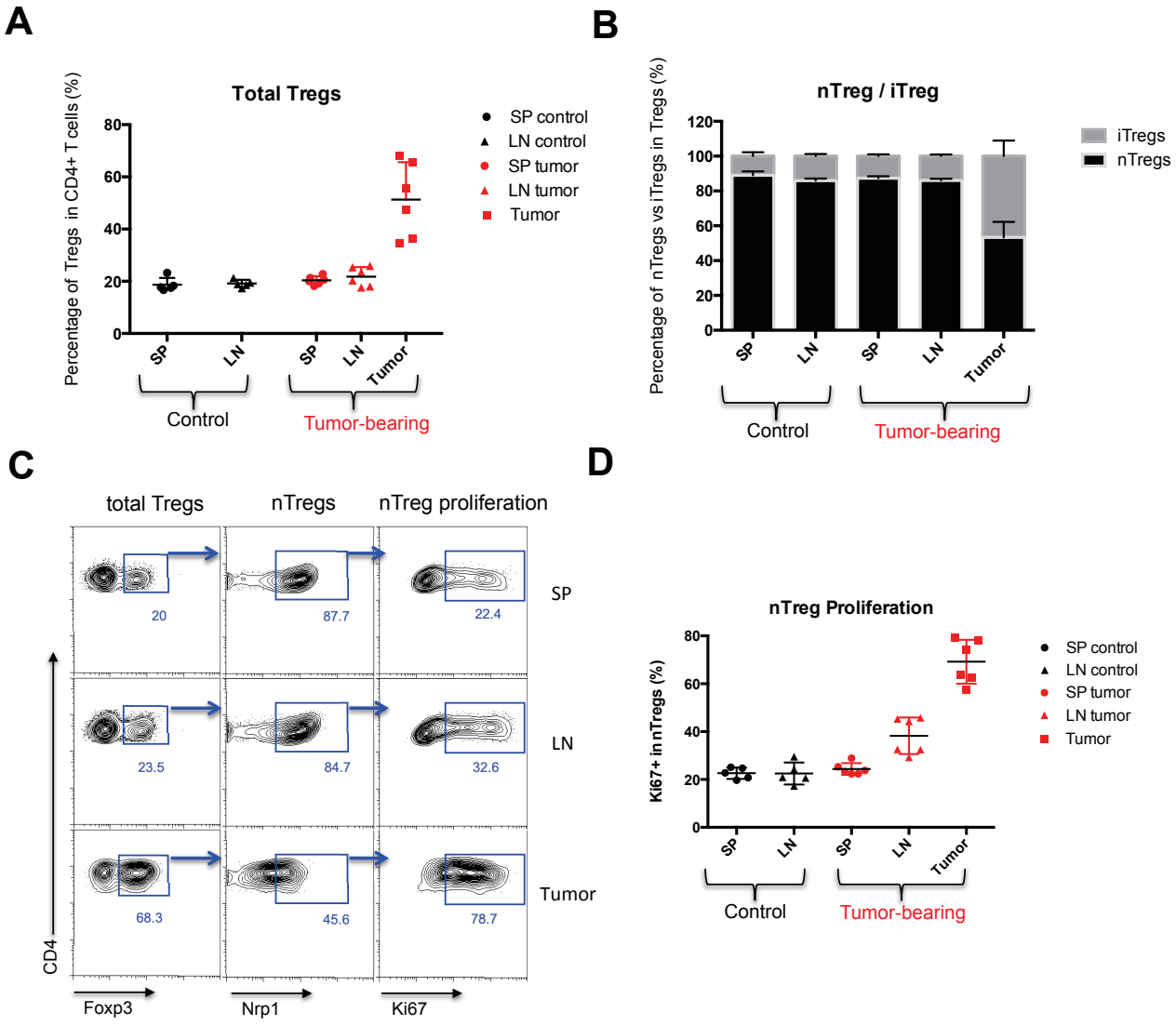

Figure 3–14: **Tumor infiltrating natural Treg cells are mostly proliferating Treg cells.** (A) Percentage of Treg cells in total CD4+ T cells from indicated tissues of non-tumor-bearing mice and tumor-bearing mice. (B) Composition of nTregs versus iTregs in spleen, lymph nodes and tumor from tumor bearing mice and controls. (C) Representative flow cytometric analysis of Treg cells in tissues from tumor bearing mice to show the expression profile of Nrp1 and Ki67. (D) nTreg proliferation represented by percent Ki67+ cells. Tissues from tumor bearing mice were shown in red. B16-OVA melanoma cells were used as the tumor model.

Moreover, compared to nTregs in spleen or lymph nodes, tumor infiltrating nTregs had a significantly increased proportion of Ki67+ cells, indicating that most of them are proliferating (Figure 3-14.C and D). Thus, these results demonstrate that in addition to iTreg generation, proliferation of nTregs also contributes to the Treg expansion in tumor microenvironment.

3.6.3 DC-PKAR1α ko mutation protects tumor growth

To test whether the increased Treg cells in DC-PKAR1α ko mice protect tumor growth, we inoculated both WT and DC-PKAR1α ko mice with 1×10^6 EG7 cells as tumor model, and monitored tumor growth every two days.

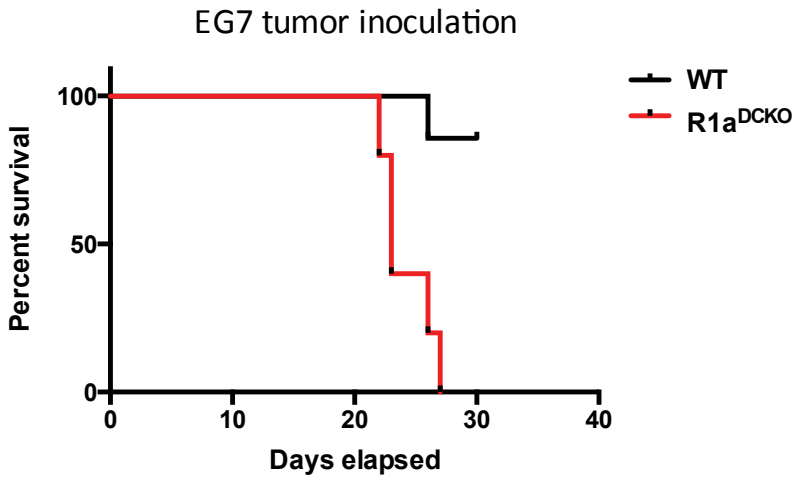

Figure 3–15: **DC-PKAR1α ko mutation protects tumor growth.** 1×10^6 EG7 tumor cells were subcutaneously injected into DC-PKAR1α ko mice (n=5) and their littermate controls (n=5). Tumors were measured every two days. Mice were euthanized when the tumor volume reached 2,000 mm^3, and the days after tumor challenging were recorded to draw the survival curve.

We found that all five DC-PKAR1α ko mice died because of tumor within four weeks (mice with tumor size larger than 2,000 mm^3 were euthanized), while only one out of five WT mice were dead during that time (Figure 3-15), indicating that tumors were protected

for their growth in DC-PKAR1α ko mice, possibly due to the expanded Treg cells that are caused by DC-PKAR1α ko mutation.

CHAPTER 4
Discussion

We achieved DC-specific PKA activation, either the deletion of PKA regulatory subunit R1α or the expression of activated PKA catalytic subunit Cα specifically in DCs, by using CD11c-Cre Tg mice. Although CD11c is considered as a specific marker for DCs, it has been reported that, in CD11c-Cre EYFP reporter mice (by crossing CD11c-Cre Tg mice to R26-EYFP reporter mice), Cre recombination could notably leak into 12% of NK cells and 5% of B cells [89]. To exclude the possibility that the enhanced Treg homeostasis in DC-PKAR1α ko mice is caused by PKA activated NK cells, we also analyzed NK cells, and found that NK cells were developed normally (data not shown), and there was no obvious sign of any abnormalities in NK cells. In addition, our previous result in the lab also indicated that PKA activation might be lethal for B cell development. Thus, we conclude that it is the PKA activated DCs that are responsible for the increase of Treg cells in our DC-PKAR1α ko mice.

Although we were able to show that after PKA activation, DCs could secrete certain amount of IL-2 to support Treg cells in vitro, we didn't detect an elevated level of IL-2 in the serum of mutant mice. Meanwhile, the amount of IL-2 produced by PKAR1α ko DCs was much less as compared to that produced by CD4+ T cells. Therefore, it's likely that Treg cells were expanded by PKA activated DCs in local microenvironment where DCs may interact with Treg cells and IL-2 can be accumulated. Indeed, it has been reported that DCs and Treg cells can form long-lasting cell clusters [93]. However, to confirm the role of IL-2 production by PKAR1α ko DCs in the regulation of Treg homeostasis, future experiments, such as IL-2 signaling block assay by either antibody injection or conditional knockout of IL-2 in DCs, should be performed.

In addition to IL-2 production, DCs may also use other mechanisms that are not yet fully explored to expand Treg cells. Among them, we noticed that PD-L1 expression pattern was significantly changed in DCs after PKAR1α deletion. PD-L1 is ligand for PD1, which plays an essential role in the induction and maintenance of T cell tolerance [94]. Our finding of altered expression of PD-L1 in PKAR1α deficient DCs is quite interesting, yet unfortunately very little research has been done on how PD-L1 in DCs can regulate Treg homeostasis. Thus, generation of DC specific PD-L1 knockout mice followed by Treg analysis would be a good start to figure out whether PD-L1 in DCs is involved in the regulation of Treg homeostasis.

Another interesting finding would be the newly occurred S2 DC population in DC-PKAR1α ko mice. The S2 DCs are indeed DCs as they are both MHC class II and CD11c positive, and we can easily see their dendrites when we cultured them after sorting. Although the MFI of MHC class II expression in S2 DCs is higher than that in WT DCs, it is unlikely that S2 DCs are migratory DCs, since we have also compared their MFIs and found migratory DCs in lymph nodes express even higher level of MHC class II. Moreover, we've also confirmed that PKAR1α is successfully deleted in both S1 DCs and S2 DCs by western blot, thus excluding the possibility that it might be the different deletion efficiency that splits DC population in DC-PKAR1α ko mice.

In physiological conditions, cell deaths continuously occur throughout life, which as we discussed earlier, not only release large quantities of auto-antigens, but also increase the extracellular level of adenosine. The increased adenosine may serve as stimulus to trigger the intracellular PKA signaling pathway of nearby cells. As the half life of adenosine in vivo is very short (few seconds), there seems to be limited chance under physiological conditions for DCs to be PKA activated through adenosine-adenosine receptors-PKA axis. However, under pathological conditions such as tissue damage, ischemia, inflammation or tumor, massive cell death can provide a long lasting increase of adenosine, therefore in such cases, it is highly possible that PKA signaling can be

activated in DCs that are recruited to those areas. Our DC-PKAR1α ko mutation artificially activates PKA in DCs, thus providing a strategy to study what happens to DCs in vivo under the above pathological conditions.

We expect and have also assumed that PKA signaling is activated in both PKAR1α ko DCs and PKACα-on DCs based on previous literature, nevertheless, we haven't demonstrated so far that this is the case. Therefore, one of the future experiments that can be done is to provide a solid and direct evidence showing that PKA from PKAR1α ko DCs or PKACα-on DCs is able to phosphorylate its substrates, and this can be done by measuring their PKA kinase activity.

Treg cells are known for their ability to suppress inflammatory responses including autoimmune responses. Surprisingly, our resulted DC-PKAR1α ko mice in NOD background were not benefited from the enhanced Treg homeostasis. The reason behind this is not clear. Our previous data have shown that the increased Treg cells in DC-PKAR1α ko mice are functional Treg cells that can suppress the proliferation of conventional CD4+ T cells. Thus, It is possible that the expanded Treg cells in periphery may fail to be recruited and migrate to pancreas, therefore not be able to suppress the autoimmune responses in pancreas, however, to confirm this, more experiments on DC-PKAR1α ko/NOD mice are required, for example, further analysis of the Treg cells in both pancreases from DC-PKAR1α ko/NOD mice and their Cre- littermate controls.

PKA is the downstream effector of adenosine-A2a receptor singnaling, and given that A2a receptor has been reported to protect tumor growth [86], it might be also interesting to know whether this protection works through PKA signaling, and more specifically, though which cell type. Our present data suggest that DCs may be involved in this protection by PKA signaling pathway, as direct evidence comes from the fact that DC-PKAR1α ko mutation protects EG-7 tumor growth. Future directions will be to generate mouse models such as DC-PKACα ko mice, and to further investigate the role of PKA

signaling in DCs in tumor microenvironment. These studies together may provide a new strategy for the purpose of clinical tumor immunotherapy.

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