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Caveolin-1–Mediated Negative Signaling Plays a Critical Role in the Induction of Regulatory Dendritic Cells by DNA and Protein Coimmunization

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Induction of Ag-specific regulatory T cells (iTregs) by vaccination is a promising strategy for treating autoimmune diseases. We previously demonstrated that DNA and protein co vaccination converted naive T cells to Ag-specific iTregs by inducing CD11c+ CD40lowIL-10+ regulatory dendritic cells (DCregs). However, it is unclear how coimmunization induces the DCregs. In this paper, we report that the event is initiated by coentry of sequence-matched DNA and protein immunogens into the same DC via caveolae-mediated endocytosis, which leads to inhibition of phosphorylation of caveolin-1 (Cav-1), the main component of caveolae, and upregulation of Tollip. This triggers downstream signaling that upregulates suppressor of cytokine signaling 1 and downregulates NF-κB and STAT-1. Silencing either Cav-1 or Tollip blocks the negative signaling, leading to upregulated expression of CD40, downregulated production of IL-10, and loss of iTreg-inducing function. We further show that DCregs can be induced in culture from primary DCs and JAWS II DC lines by feeding them sequence-matched DNA and protein immunogens. The in vitro-generated DCregs are effective in ameliorating autoimmune and inflammatory diseases in several mouse models. Our study thus suggests that DNA and protein coimmunization induces DCregs through Cav-1– and Tollip-mediated negative signaling. It also describes a novel method for generating therapeutic DCregs in vitro. The Journal of Immunology, 2012, 189: 2852–2859.

C onventional dendritic cells (DCs) are specialized APC that can be broadly classified into the CD11c+CD8α+ and CD11c+CD8α− subtypes, both of which have a remarkable functional plasticity in the induction of immunity or tolerance, depending on their maturation status (1). A large body of evidence shows that immature DCs can promote tolerance by converting naive T cells into the CD4 Foxp3+ regulatory T cells (Tregs) (2, 3). In addition, DCs alternatively activated with cholera toxin and LPS (4, 5), or modified with TGF-β and GM-CSF (6, 7), have also been shown to induce Tregs. However, these types of tolerogenic DCs are mostly generated in vitro, and there are numerous technical and regulatory obstacles to their use in human immunotherapy.

We recently demonstrated that coimmunization with sequence-matched DNA and protein immunogens could induce regulatory DCs (DCregs) of a CD11c+CD80−CD86−CD40lowIL-10+ phenotype in vivo. These DCregs could convert naive T cells into Ag-specific regulatory T cells (iTregs) of a CD4+CD25+Foxp3+IL-10+ phenotype in vitro and in vivo (8), which in turn mediated Ag-specific tolerance (8–12). Given the role of these DCregs in initiating the tolerogenic process, we wish to understand how they are generated during coimmunization.

To that end, we hypothesize that two signals, one from a DNA construct and the other from a sequence-matched protein, can act in a concerted manner to activate regulatory signals that convert normal DCs into DCregs (8). In the present report, we show that DCs differentiate into DCregs after taking up both DNA and protein immunogens. The change of cell property requires sequence match between the two immunogens and is triggered by caveolae-mediated coendocytosis of the two immunogens. We further show that DNA and protein cotreatment inhibited the activation of caveolin-1 (Cav-1) and increased the level of Tollip to initiate negative signaling, leading to downregulation of STAT-1α and NF-κB and expression of the DCreg phenotype. Our results thus support the DNA and protein two-signal hypothesis for DCreg induction.

Materials and Methods

Mice and reagents

Female BALB/c and C57BL/6 mice (8–10 wk of age) were from the Animal Institute of Chinese Medical Academy (Beijing, China). All animals received pathogen-free water and food.

Flexset IL-10 and fluorescently labeled anti-mouse mAbs including anti–IL-10-PE, anti–Foxp3-allophycocyanin, anti–IL-10-allophycocyanin, anti–CD40-allophycocyanin, anti–CD11c-allophycocyanin, anti–CD11c-FITC, anti–CD40-PE, and isotype controls were purchased from BD Biosciences (San Diego, CA). Alexa Fluor 546 (AF)-labeled goat anti-rabbit IgG was purchased from Invitrogen (Carlsbad, CA). CSF-1 was obtained from Molecular Probes (Eugene, OR). Abs against IL-1R–associated kinase-1
For in vitro function of DCregs, CD4+ T cells were purified using the CD4+Tollip double-knockdown DCs were fed with 10

\[ \text{effectants were selected on puromycin, from which stable Cav-1 and Tollip recombinant protein were prepared and described in our previous report (13).} \]

\[ \text{The OVA peptide (peptide 323–339, named as OVA323) or FITC-labeled OVA323 were synthesized by GL Biochem (Shanghai, China). All plasmids were purified to remove endotoxin with the EndoFree Plasmid Maxi kit (Qiagen, Tokyo, Japan) and used as the DNA vaccines by dissolving in PBS at 2 mg/ml. Recombinant proteins and peptides were dissolved in PBS at 2 mg/ml and sterilized by filtration.} \]

**Culture and stimulation of JAWS II cells and purified CD11c+ cells**

The JAWS II mouse DC line was purchased from the American Type Culture Collection (Manassas, VA) and maintained in complete growth medium containing MEMα with ribonucleosides, deoxyribonucleosides, 4 mM glutamine, and 1 mM sodium pyruvate (Invitrogen) and supplemented with 20% FBS (American Type Culture Collection) and 5 ng/ml murine recombinant GM-CSF (R&D Systems, Minneapolis, MN). The cells were incubated at 37°C with 5% CO2 and treated with different Ags (10 μg/ml) such as pVAX, pOVA, OVA, pOVA323, and OVA323 for 24 h. For stimulation of purified CD11c+ cells, CD11c+ cells were isolated from single-cell suspensions from spleen using CD11c MicroBeads, according to the manufacturer’s protocol (Miltenyi Biotech, Auburn, CA). For treatment with inhibitors, JAWS II cells were pretreated with filipin (10 μg/ml) or MDC (50 μM) for 30 min at 37°C or with amiloride (5 mM) for 10 min at 37°C, washed with medium, and then stimulated with Ags.

**Silencing Cav-1 and Tollip in JAWS II and treatment by DNA and protein**

The JAWS II cell line was transfected with Cav-1 and Tollip-specific small interfering RNA (siRNA) carried by the pFIV U6/H1 puro vector (System Biosciences, Mountain View, CA), according to the vendor’s instructions. The sequences for the siRNA targeting Cav-1 were sense, 5'-AAAGAAGGGAAGATGCTGTCGAC-3', and antisense, 5'-AAAATGTGACGACGCTTCTTT-3'. The sequences for the siRNA targeting Tollip were sense, 5'-AAAGTGCGAGCGAGGCTTACT-3', and antisense, 5'-AAAAATCGACTCTGCGTCCAC-3'. Oligonucleotides were annealed, phosphorylated, and then inserted into pFIV-H1/U6 puro. Transfecteds were selected on puromycin, from which stable Cav-1 and Tollip knockdown clones were collected.

Wild-type (WT), Cav-1 knockdown, Tollip knockdown, and Cav-1 and Tollip double-knockdown DCs were fed with 10 μg/ml pOVA323 and OVA323 or pvAX and OVA323 (added directly into the medium) for 24 h. For in vitro function of DCregs, CD4+ T cells were purified using the CD4+ T Cell Isolation Kit II, according to the manufacturer’s protocol (Miltenyi Biotech), from the spleen of mice that had been immunized with OVA in IFA and labeled with CFSE. CFSE-labeled CD4+ T cells were cocultured with the DCs for 5 d, and then, T cell proliferation and expression of Foxp3 and IL-10 were detected by flow cytometry. Mean fluorescence intensity (MFI) values were determined for each sample and normalized to 100% for each antigen.

For detection of NF-kB, cytoplasmic and nuclear proteins were extracted separately as described previously (14). Nuclear and cytoplasmic extract was analyzed by immunoblotting. The ECL (GE Healthcare Europe, Uppsala, Sweden) method was used for protein detection.

**Induction of inflammatory bronchitis and autoimmune ovarian disease in mice**

Inflammatory bronchitis was induced in BALB/c mice as described previously (12, 15). Autoimmune ovarian disease (AOD) was induced in C57BL/6 mice as described previously (11). For treatment of inflammatory bronchitis and AOD, 5 × 10^6 CD11c+ DCs or JAWS II cells in PBS were i.v. transferred into the mouse for 3 d consecutively.

**Histology analysis**

Lung or ovarian tissue was fixed in 4% paraformaldehyde or Bouin’s solution and embedded in paraffin blocks. Sections were cut and stained with H&E. Histopathology of the lung or ovaries was evaluated under a light microscope.

**FACS analysis**

Dcs or T cells were stained with the appropriate PE-, FITC-, or allophycocyanin-conjugated mAbs in PBS for 30 min at 4°C, according to previous studies (8, 11). The cells were collected by flow cytometry (FACScalibur) and analyzed with FlowJo.

**Statistics**

Nonparametric test was used for data analysis, except data in Fig. 3B, which one-way ANOVA was used. Differences were considered statistically significant if p < 0.05.

**Results**

Low expression of CD40 is a marker for coimmunization-induced DCregs

We previously demonstrated that CD11c+CD40lowIL-10high DCregs were induced in vivo after coadministration of sequence-matched DNA and protein immunogens (8). To test whether the low CD40 expression is a reliable phenotype of coimmunization-induced DCregs, a cukaryotic expression construct encoding the full-length hen OVA (pOVA) was constructed and used in combination with the protein (OVA). We injected pOVA and OVA i.m. into one group of mice (pOVA + OVA). As a control for gene specificity, a DNA construct containing the noncoding strand of OVA (pOVArev) and OVA was coimmunized into another group of mice (pOVArev + OVA). On day 2, we isolated DCs from both groups, together with a group of noninjected mice (naive), and compared their expression of CD40 by FACS. Expression of CD40 in the pOVA + OVA group was higher than that in the naive group but lower than that in the pOVArev + OVA group (Fig. 1A), confirming the low expression of CD40 (CD40<sup>low</sup>) phenotype. We also tested an additional combination of DNA and protein immunogens, composed of a DNA construct coding for the murine ZP3 and the ZP3 protein, and observed a similar result (Fig. 1A). These results suggest that the low CD40 expression is a consistent phenotype induced by coadministration of sequence-matched DNA and protein immunogens.
We next repeated the experiment in culture with primary DCs and the DC line JAWS II. We added pOVA and OVA, or pVAX and OVA (control), directly to freshly isolated CD11c+ cells and JAWS II cells for 24 h. The result showed that, in both cell types, CD40 expression was lower following the pOV A + OVA treatment than following the control treatment (Fig. 1B), suggesting that the CD40low phenotype can also be induced in vitro in cultured primary DCs and DC lines.

Our previous studies showed that DCregs induced in vivo by coinmunization could convert naive T cells into Tregs in vivo and in vitro (8). To determine whether the in vitro-induced CD40low DCs could do the same, we tested the activity of the CD40low JAWS II cells by coculturing them with CFSE-labeled syngeneic CD4+ T cells from OVA-sensitized mice. The expressions of Foxp3 and IL-10 within the CFSE+ cells were analyzed after 5 d coculture. The result showed that the CD40low JAWS II cells caused expansion of Foxp3+ and IL-10+ T cells (Fig. 1C), confirming that the CD40low DCs generated in vitro were in fact DCregs.

Because the appearance of the CD40low phenotype required matching sequence between DNA and protein, we speculated that it might require uptake of both DNA and protein by the same DC. To test this hypothesis, we labeled pOVA323 (a DNA construct encoding the OVA323–339 dominant epitope) and pVAX (the empty vector) with Cy5 and FITC labeled-OVA323 (the OVA323–339 peptide) was obtained from GL Biochem. As depicted in Fig. 1D, low expression of CD40 was observed only in individual DCs taking up both Cy5-pOVA323 and FITC-OVA323, as observed by confocal microscopy. Taken together, these results suggest that CD40low is a reliable marker for DCregs generated by coinmunization because the display of this maker requires coupled uptake of sequence-matched DNA and protein immunogens.

DCs cotake up DNA and protein immunogens via clathrin- and caveolae-mediated endocytosis

DCs take up exogenous Ags via various mechanisms including clathrin-mediated endocytosis, caveolae-mediated endocytosis, and macropinocytosis (18). To define which pathway(s) was involved in the cotype of DNA and protein immunogens, JAWS II cells were pretreated with MDC, a specific inhibitor of clathrin formation (19, 20), or filipin, an inhibitor of caveolae trafficking (21), before being fed pOVA323 + OVA323. Using CD40low as the marker, we found that both MDC and filipin could prevent the CD40low phenotype. This suggests that the CD40low phenotype is primarily the result of caveolae-mediated endocytosis and/or clathrin-mediated endocytosis (Fig. 2A, 2B). Another inhibitor, amiloride, which blocks macropinocytosis (22), had no effect on CD40 expression (data not shown).

Coimmunization downregulates NF-κB and STAT-1α by activating negative signaling pathways

The transcription factor NF-κB regulates the expression of CD40 (23, 24), and IRAK-1 regulates the activation of NF-κB. Inter-
Interestingly, Cav-1, a component of caveolae, was previously shown to form a complex with Tollip to suppress IRAK-1’s kinase activity under the steady-state condition (25, 26). We found that phosphorylation of Cav-1 Tyr14 was strongly inhibited in spleen DCs isolated from mice that had been coimmunized with pOVA + OVA, as compared with those isolated from mice immunized with pOVA or OVA or coimmunized with pVAX + OVA (Fig. 3A). Lack of phosphorylated Cav-1 was also seen in JAWS II cells fed pOVA + OVA in culture (Supplemental Fig. 1A).

Following that lead, we investigated the expression of Tollip and the activation of IRAK-1 in spleen DCs in response to pOVA + OVA coimmunization. We observed that the transcription of Tollip, and TGF-β and IL-10 as well, was upregulated in coimmunized mice, whereas the transcription of CD40 and TNF-α was downregulated (Fig. 3B). Similar results were also observed in JAWS II cells fed pOVA + OVA in culture (Supplemental Fig. 1B). The expressions of PD-L1, PD-L2, OX40L, and B7-H3 were also tested by either RT-PCR or FACS, and no significant differences were observed among DNA, protein, and DNA + protein treatment (data not shown). Phosphorylation of IRAK-1 was also significantly inhibited in coimmunized mice (Fig. 3A), which agrees well with inhibited Cav-1 phosphorylation and increased expres-
sion of Tollip because nonphosphorylated Cav-1 and Tollip formed a complex with IRAK-1 to inhibit the phosphorylation of IRAK-1.

Because SOCS negatively regulates the activation of IRAKs and the JAK–STAT pathway (27, 28), we analyzed the level of the SOCS1 protein. SOCS1 was significantly increased in response to pOVA + OVA coimmunization (Fig. 3A). Taken together, these results indicate that coimmunization alters phosphorylation of Cav-1 and expression of Tollip and SOCS1 to activate negative signaling.

Next, we analyzed the activation of the transcription factors NF-κB and STAT-1α. The phosphorylation of NF-κB p65Ser536 and STAT-1αTyr701 was strongly inhibited in pOVA + OVA coimmunized mice (Fig. 3C). The translocation of NF-κB and STAT-1α was also inhibited because the concentration of NF-κB p65 and STAT-1α in nuclear was decreased in the coimmunized group (Fig. 3D), suggesting downregulated activation of NF-κB and STAT-1α after coimmunization.

Taken together, these results demonstrate that coimmunization activates negative pathways mediated by Cav-1 and Tollip, leading to downregulation of the activity of NF-κB and STAT-1α and reduced expression of CD40.

Silencing Cav-1 and Tollip prevents the induction of DCregs

To address the role of Cav-1 and Tollip in the induction of DCregs, we used RNA interference to silence the expression of Cav-1 and Tollip. The efficiency of RNA interference reached ~80% for both genes in JAWS II cells (Supplemental Fig. 2A). Silencing of both Cav-1 and Tollip completely prevented JAWS II cells from differentiating into DCregs when fed pOVA323 + OVA323, as judged by increased CD40 expression and decreased IL-10 production following silencing, whereas silencing of either Cav-1 or Tollip alone was partially effective (Fig. 4A). Furthermore, translocation of NF-κB was increased following Cav-1 silencing (Supplemental Fig. 2B).

The function of Cav-1– and/or Tollip-deficient DCs was detected in the induction of iTreg using the DCs–T cell coculture system. WT or Cav-1– and/or Tollip-deficient DCs were fed with pOVA323 + OVA323 or pVAX + OVA323 for 24 h, and then were cocultured with CFSE-CD4+ T cells from mice sensitized for OVA. Functionally, Cav-1– and/or Tollip-deficient and pOVA323 + OVA323-treated JAWS II cells were unable to inhibit the proliferation of responder T cells in a coculture assay, or induce iTreg conversion or IL-10 expression (Fig. 4B). These data show that both Cav-1 and Tollip play a critical role in the induction of the DCreg phenotype and function following coimmunization.

Cav-1– and/or Tollip-deficient DCs are not tolerogenic in vivo

To determine whether the Cav-1– and/or Tollip-deficient DCs had also lost their ability to promote tolerance in vivo, we i.v. transferred them into syngeneic mice after feeding them pOVA323 + OVA323. The recipient mice were then challenged by immunization with OVA in IFA. Although transfer of WT JAWS II cells (control) inhibited the induction of DTH and OVA-reactive T cells by guest on October 30, 2017 http://www.jimmunol.org/ Downloaded from
and increased the expression of Foxp3 and production of IL-10 in CD4+CD25+ T cells [CD25+ iTreg (8, 11)], the silenced JAWS II failed to the same (Fig. 5). This result confirms that the silenced JAWS II cells are not tolerogenic.

**Covimmunization-induced DCregs ameliorate inflammatory bronchitis and autoimmune ovarian disease in mice**

To assess the potential of covimmunization-induced DCregs as a therapeutic for inflammatory and autoimmune disease, we fed cultured primary DCs pOVA + OVA and used the resulting DCregs to treat BALB/c mice with OVA-induced inflammatory bronchitis (Fig. 6A). Adoptive transfer of the primary DCregs significantly decreased the level of IgE in recipient mice (Fig. 6B). Histological analysis of lung sections from the mice revealed a nearly normal lung morphology that was free of cell infiltration (Fig. 6C). As expected, the anti-inflammatory effect of the transferred DCs was absent if they were pretreated with filipin. To investigate whether the suppression is Ag specific, pcD-mZP3 + mZP3-specific DCregs were used to treat OVA-induced inflammatory bronchitis. Although the frequency of IL-10+ and Foxp3+ CD4+ T cells was increased by pcD-mZP3 + DCregs treatment (Supplemental Fig. 3A), the level of IgE was not decreased by such treatment (Fig. 6D). Histological analysis showed cell infiltration similar to the nontreated model (data not shown).

To determine whether a similar therapeutic effect could be reproduced with a DC line, we fed cultured JAWS II cells pcD-

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**FIGURE 5.** Cav-1- and/or Tollip-deficient DCs are not tolerogenic in vivo. Cav-1- and/or Tollip-deficient JAWS II cells were adoptively transferred into syngeneic mice (day 0). The mice were then immunized with OVA in IFA on days 0 and 7. On day 14, DTH response was tested. On day 15, T cell proliferation, expression of Foxp3 in T cells, and IL-10 levels in supernatant were determined. Shown is one of three independent experiments with similar results. *p = 0.049 (footpad thickness), p = 0.05 (count of CD25+ Foxp3+) or p = 0.027 (IL-10); **p = 0.0055; all between WT DCs fed pOVA323 + OVA323 and WT DCs fed pVAX + OVA323.

**FIGURE 6.** Covimmunization-induced DCregs ameliorate inflammatory bronchitis. (A) Experimental design: BALB/c mice were injected with 0.1 ml 1 mg/ml OVA/alum complexes in PBS on days 0 and 7 by i.p. and subsequently challenged with 100 μg OVA intratracheally on days 14, 16, and 18 to establish the “model.” Control mice were given PBS intratracheally on days 14, 16, and 18 and designated as the “shame” control. On day 21, 5 × 10⁵ CD11c+ cells from syngeneic donor mice were transferred into model mice once daily for 3 consecutive days by i.v. (n = 3/group). Prior to the transfer, the donor CD11c+ cells purified from spleen of naive mice were pretreated with or without filipin and subsequently fed pOVA + OVA or pVAX + OVA for 24 h. On day 14 after the final transfer, serum samples were taken to analyze the level of IgE or cytokines. Sections of lung tissues were made to evaluate disease severity. (B) The level of Ag-specific IgE was analyzed by ELISA following adoptive transfer of indicated DCs. *p < 0.033 between the pOVA + OVA DC group and all other groups except the sham group. (C) Lung sections were examined by H&E staining and recorded under a light microscope at ×100 and ×200 magnification. (D) The level of Ag-specific IgE was analyzed by ELISA following adoptive transfer of indicated DCs. *p < 0.05 between the pOVA + OVA-induced DC group and all other groups, except the sham control.
mZP3, a DNA construct encoding the mouse ZP3 protein, and the mZP3 protein (pcD-mZP3 + mZP3). The resulting DCregs were adaptively transferred into C57BL/6 mice with mZP3-induced AOD (Fig. 7A) (29). Subsequently, we observed reduced production of IFN-γ, IL-5, and TNF-α (Fig. 7B) and reduced the severity of AOD (Fig. 7C) in the recipient mice. Histological analysis of ovarian sections from the treated mice revealed a nearly normal histological structure without notable cell infiltration (Supplemental Fig. 3B). FACS analysis of the spleen further showed increased frequency of IL-10+ and Foxp3+CD4+ T cells (Fig. 7D). pOVA + OVA-induced OVA-specific DCregs were also used to treat mZP3-induced AOD. The results showed that pOVA + OVA–DCregs had no therapeutic effects on AOD (data not shown). Taken together, these results suggest that DCregs generated in culture by feeding primary DCs or DC lines sequence-matched DNA and protein immunogens are potentially useful for adoptive immunotherapy via an Ag-specific manner.

Discussion

We have shown that DNA and protein coinmunization induces DCregs by allowing coinulation of the DNA and protein immunogens by the same DC via caveolae-mediated endocytosis. This event downregulates the phosphorylation of Cav-1 and upregulates Tollip, which in turn initiates downstream signaling that upregulates SOCS1 and downregulates NF-κB and STAT-1α. The downregulation of NF-κB explains the CD40low and IL-10+ phenotype of the coimmunization-induced DCregs. We have also shown that DCregs may be generated in vitro in both primary DCs and DC lines by feeding them DNA and protein immunogen for as short as 24 h. The in vitro-generated DCregs are effective as a therapeutic for mouse models of inflammatory and autoimmune diseases, presumably by inducing Ag-specific CD25+ iTreg (8, 11).

Coinmunization-induced DCregs show CD11c+CD80+CD86+CD40lowIL-10+ phenotype and CD40low may be due to the active downregulation or lack of the activation signal. Our results showed that DNA and protein coinulation in the presence of LPS results in a CD40high phenotype (data not shown), suggesting that the second possibility (lack of activation signal) is more likely. The DCregs are different with normal mature DCs that are CD11c+CD80+CD86+CD40high and either IL-10+ (tolerogenic) or IL-12+ (immunogenic).

Cav-1 is the key protein to form caveolae. It also regulates signal transduction through compartmentalization of numerous signaling molecules (30, 31). Cav-1, Tollip, and IRAK-1 form a complex to suppress the IRAK-1’s kinase activity during the resting condition. Cav-1 dissociates from the complex once phosphorylated, which leads to phosphorylation of IRAK-1 in the cytosol and activation of the downstream signaling cascade, including translocation of NF-κB (25). Interestingly, coinulation of DNA and protein by caveolae-mediated endocytosis downregulates phosphorylation of Cav-1, thereby preventing the activation of NF-κB. Cav-1–silencing DCs cotreated by DNA and protein could induce T cell proliferation. However, Ohnuma et al. (32) have demonstrated that Cav-1 is necessary for T cell proliferation via CD26 ligation. In Ohnuma’s experiment, monocytes were preincubated with rsCD26 and then incubated with T cells to detect the T cell proliferation. Cav-1–silenced monocytes cannot be activated by rsCD26 to result in the inhibition of T cell proliferation. In our experiment, DCs were treated with DNA and protein and then incubated with T cells. Cav-1–silenced DCs could take up Ags through caveolae-independent endocytosis to activate DCs to result in T cell proliferation. We speculate that the way the APCs were stimulated might explain the discrepancy.

Tollip plays an important role in inhibition of innate responses and maintenance of the resting state (33, 34). Coinulation of DNA and protein immunogens upregulates Tollip, and silencing of Tollip or Cav-1 partially blocks the differentiation of DCs into DCregs, suggesting that Tollip may act independently of Cav-1 in downregulating NF-κB. Further investigation will be needed to delineate the respective role of Cav-1 and Tollip.

In conclusion, we previously hypothesized that a DNA immunogen and a sequence-matched protein immunogen could convert normal DCs into DCregs. The data presented in this paper support
this hypothesis by demonstrating 1) that coupptake of the two immunogens by the same DC is required for acquisition of the DCreg phenotype and function and 2) that the coupptake event triggers Cav-1 and Tollip coregulatory signaling that upregulates SOCS1 and downregulates NF-kB and STAT-1x. A question that remains to be answered is why coupptake of sequence-mismatched DNA (pVAX) or nontranslated sequence-matched DNA (pOVAR) and protein immunogens fails to trigger the same signaling cascade. We supposed that DNA receptors or RNA polymerase in caveolea might be involved in distinguishing the signals from DNA. Because of the pOVAR (contained the OVA sequence but cannot be translated into OVA protein) + OVA cannot induce DCregs, RNA polymerase may play critical role in distinguishing DNA signals. Future investigation will be needed to explore the possibility of additional molecules and molecular processes involved in recognizing the match.

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Disclosures
The authors have no financial conflicts of interest.

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