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Dab2, a negative regulator of DC immunogenicity, is an attractive molecular target for DC-based immunotherapy

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Abbreviations: BAT, blocking the TGF-β-activated translation element; BM, bone marrow; CFSE, 5, 6-carboxyfluorescein succinimidyl ester; CTL, cytotoxic T lymphocyte; *Dab2*^{KD}, *Dab2*-knockdown; *Dab2*, disabled-2 adaptor protein; DCs, dendritic cells; Foxp3, forkhead box P3; GM-CSF, granulocyte-macrophage colony stimulating factor; hMoDC, human monocyte-derived dendritic cell; hnRNP E1, heterogeneous nuclear ribonucleoprotein E1; imDC, immature DC; mDC, mature DC; OT-1 and OT-2 mice, $OVA_{257–264}$ and $OVA_{323–339}$-peptide-specific T cell receptor transgenic mice; OVA, ovalbumin; PI3K, phosphoinositide-3 kinase; STAT5, transducer and activator of transcription 5; TGF-β, transforming growth factor-β; Treg, regulatory T; WT, wild type.

Dab2 is an adapter protein involved in receptor-mediated signaling, endocytosis, cell adhesion, hematopoietic cell differentiation, and angiogenesis. It plays a pivotal role in controlling cellular homeostasis. In the immune system, the *Dab2* is a Foxp3 target gene and is required for regulatory T (Treg) cell function. *Dab2* expression and its biological function in dendritic cells (DCs) have not been described. In this study, we found that *Dab2* was significantly induced during the development of mouse bone marrow (BM)-derived DCs (BMDCs) and human monocyte-derived DCs (MoDCs). Even in a steady state, *Dab2* was expressed in mouse splenic DCs (spDCs). STAT5 activation, Foxp3 expression, and hnRNPE1 activation mediated by PI3K/Akt signaling were required for *Dab2* expression during GM-CSF-derived BMDC development regardless of TGF-β signaling. *Dab2*-silencing was accompanied by enhanced IL-12 and IL-6 expression, and an improved capacity of DC for antigen uptake, migration and T cell stimulation, which generated strong CTL in vaccinated mice. Vaccination with *Dab2*-silenced DCs inhibited tumor growth more effectively than did vaccination with wild type DCs. *Dab2*-overexpression abrogated the efficacy of the DC vaccine in DC-based tumor immunotherapy. These data strongly suggest that *Dab2* might be an intrinsic negative regulator of the immunogenicity of DCs, thus might be an attractive molecular target to improve DC vaccine efficacy.

Introduction

DCs are professional antigen presenting cells (APCs) that play a crucial role in activating T cell-mediated, antigen-specific adaptive immune responses.[1] DCs have heterogeneous origins, morphologies, phenotypes, and immunological functions.[2,3] Granulocyte-macrophage colony stimulating factor (GM-CSF) with or without interleukin (IL)-4 lead mouse BM cells and human monocytes to differentiate into DCs in *vitro*.[4] However, the molecular mechanism underlying DC differentiation and maintenance from BM cells or monocytes is not well understood in normal or disease conditions. Environmental and genetic factors are expected to be critical for DC differentiation and immunogenicity, but the detailed molecular mechanisms remain widely unknown.

A microarray analysis and qRT-PCR evaluation of mRNAs extracted from DCs differentiated from BM stem cells with GM-CSF revealed that the disabled-2 (*Dab2*) adaptor protein is significantly induced during DC development. Most studies of *Dab2* expression and functions have been performed with transforming growth factor-β (TGF-β) signaling in Treg cells, tumor cells, or nonlymphoid epithelial cells. However, we focused on the GM-CSF-mediated *Dab2* expression during DC development and its role in DC differentiation and immunogenicity.

Dab2 is a tumor suppressor/endocytic adaptor protein that is involved in receptor-mediated endocytosis/trafficking and TGF-β signaling.[5-7] The *Dab2* gene encodes two isoforms: p96 and p67.[8] p96 is predominantly expressed in adults, while p67 is mainly present during embryogenesis.[6] *Dab2* inhibits cell growth

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and proliferation in many cell types[9,10] and is significantly downregulated in various tumors[9,11,12] *Dab2*-deficient breast cancer cells had impaired ability to deplete TGF-β receptors through endocytosis, leading to TGF-β accumulation in the tumor microenvironment and immune tolerance.[13] Expression of *Dab2*, a forkhead box P3 (Foxp3) target gene, is restricted to CD4+ Treg cells in peripheral lymphocytes, and Treg cells lacking *Dab2* are functionally impaired.[14] Foxp3 expression and Treg function require TGF-β signaling.[15] *Dab2* is also broadly expressed in many nonlymphoid cells and organs[16-18] and is critical for embryogenesis and transformations, such as epithelial to mesenchymal transitions (EMT) induced by TGF-β/Smad signaling.[19,20] Heterogeneous nuclear ribonucleoprotein E1 (hnRNP E1) inhibits *Dab2* translation under normal conditions by blocking the TGF-β-activated translation (BAT) element in the 3′-untranslated region of *Dab2* mRNA transcripts.[21] TGF-β activation leads to hnRNP E1 phosphorylation by protein kinase B (PKB)-β/Akt2, inducing its release from the BAT element and translation of *Dab2* mRNAs.[21,22] *Dab2* contains an amino-terminal phosphotyrosine binding (PTB) domain, which interacts with transmembrane region of TGF-β receptors.[19] *Dab2* negatively regulates TGF-β-induced activation of c-Jun N-terminal kinase (JNK) without influencing the Smad pathway, suggesting that *Dab2* controls TGF-β signaling by balancing the Smad and JNK pathways.[23] Additionally, *Dab2* contains a C-terminal proline-rich domain (PRD) that interacts with SH3 domains in proteins such as Grb2.[24] *Dab2* competes with SOS for binding to Grb2, which modulates the growth factor receptor/Ras signaling pathways.[10]

GM-CSF is a critical regulator of DC development through intracellular signaling pathways, including the Janus kinase (JAK)/signal transducer and activator of transcription 5 (STAT5), mitogen-activated protein kinase (MAPK), phosphoinositide-3 kinase (PI3K), and canonical NF-kB pathways.[2] STAT5 signaling is essential for GM-CSF-dependent DC development.[25,26] STAT5 signaling is also needed for Foxp3 expression in Treg cells[27,28] and activates the PI3K/Akt and Ras/MAPK pathways.[29] Active protein kinase B (PKB)/Akt interacts with *Dab2* to facilitate *Dab2*-mediated albumin endocytosis and control the albumin overload-induced proximal tubule injury.[30]

Despite the important functions of *Dab2* in Treg and other nonlymphoid cell types, *Dab2* has not been addressed in association with GM-CSF signaling, DC development, or DC immunogenicity. In the present study, we found that *Dab2* was significantly expressed during GM-CSF-mediated BMDC development. *Dab2* was also found to be expressed in steady-state mouse spDCs and human MoDCs as well. GM-CSF-mediated *Dab2* expression required STAT5 signaling, Foxp3 expression, and PI3K/AKT-mediated hnRNP activation as shown by *Dab2* expression during the TGF-β-mediated EMT transition. However, *Dab2* expression during GM-CSF-derived BMDC development was found to have no relationship to TGF-β signaling. *Dab2*-silencing up-regulated the expression of surface immune-related molecules, antigen uptake capacity, DC migration and Th1 cytokine secretion. *Dab2*-silencing enhanced the DC vaccine efficacy in tumor immunotherapy. Finally, *Dab2* overexpression abrogated the efficacy of a DC vaccine. Our findings suggest that *Dab2* in DCs plays an important role as an intrinsic negative regulator in controlling DC immunogenicity. *Dab2* might be an attractive molecular target to improve DC vaccine efficacy.

Results

Dab2 was significantly induced during DC development

Dab2 expression was significantly induced at a later stage of DC development from mouse BM cells at both the mRNA (**Fig. 1A**) and protein (**Fig. 1B**) levels. Intracellular *Dab2* expression increased in parallel with CD11c expression during BMDC differentiation (**Fig. 1C**). *Dab2* was also highly expressed in primary major histocompatability complex class (MHC) II^{high} $CD11c^{+}$ spDCs isolated from normal mice as was shown in BMDCs (**Fig. 1D**). *Dab2* was also found to be expressed in human MoDCs (**Fig. 1E**). These results suggest that *Dab2* may play an important role in DC differentiation, immunogenicity, or both *in vivo*.

Dab2 expression in DCs requires STAT5 signaling, Foxp3, and hnRNP E1 activation

Most studies on *Dab2* have reported its expression in association with TGF-β or TGF-β related signaling.[13-16,19-23] None of the papers addressed *Dab2* expression with GM-CSF signaling. We examined whether TGF-β signaling pathways were involved in the *Dab2* expression in DCs induced by GM-CSF signaling. STAT5 signaling, which is essential for GM-CSF-mediated DC differentiation,[26] has not been examined in relation to *Dab2* expression. Treating DC precursor cells with STAT5 inhibitor downregulated GM-CSF-induced *Dab2* expression (**Fig. 2A**), suggesting that GM-CSF-mediated STAT5 activation is required for *Dab2* expression in DCs. Silencing Foxp3 in DC precursor cells also downregulated *Dab2* expression in GM-CSF-derived DCs (**Fig. 2B**). hnRNP E1 is involved in controlling *Dab2* translation by blocking the BAT element of *Dab2* mRNA. Once hnRNP E1 is phosphorylated by the PI3K/PKB/Akt pathway under TGF-β signaling, hnRNP E1 is released from the BAT element and *Dab2* is expressed.[21,22] The hnRNP E1 was significantly induced during GM-CSF-derived DC development at both the mRNA (left) and protein (right) levels and was activated in parallel with *Dab2* expression (**Fig. 2C**). Silencing hnRNPE1 decreased *Dab2* expression in DCs (**Fig. 2D**). A PI3K inhibitor blocked the GM-CSF-mediated activation of Akt/PKB, which was accompanied by impaired hnRNPE1 phosphorylation, resulting in significantly downregulated *Dab2* expression in a short time (**Fig. 2E**). TGF-β was undetectable in the culture supernatants of imDCs and mDCs (**Fig. 2F**). *Dab2* expression in BMDCs was not affected by TGF-β and/or by TGF-β receptor inhibitor (**Fig. 1G**). These data imply that GM-CSF-mediated *Dab2* expression during DC development from BM precursor cells is not associated with TGF-β signaling.

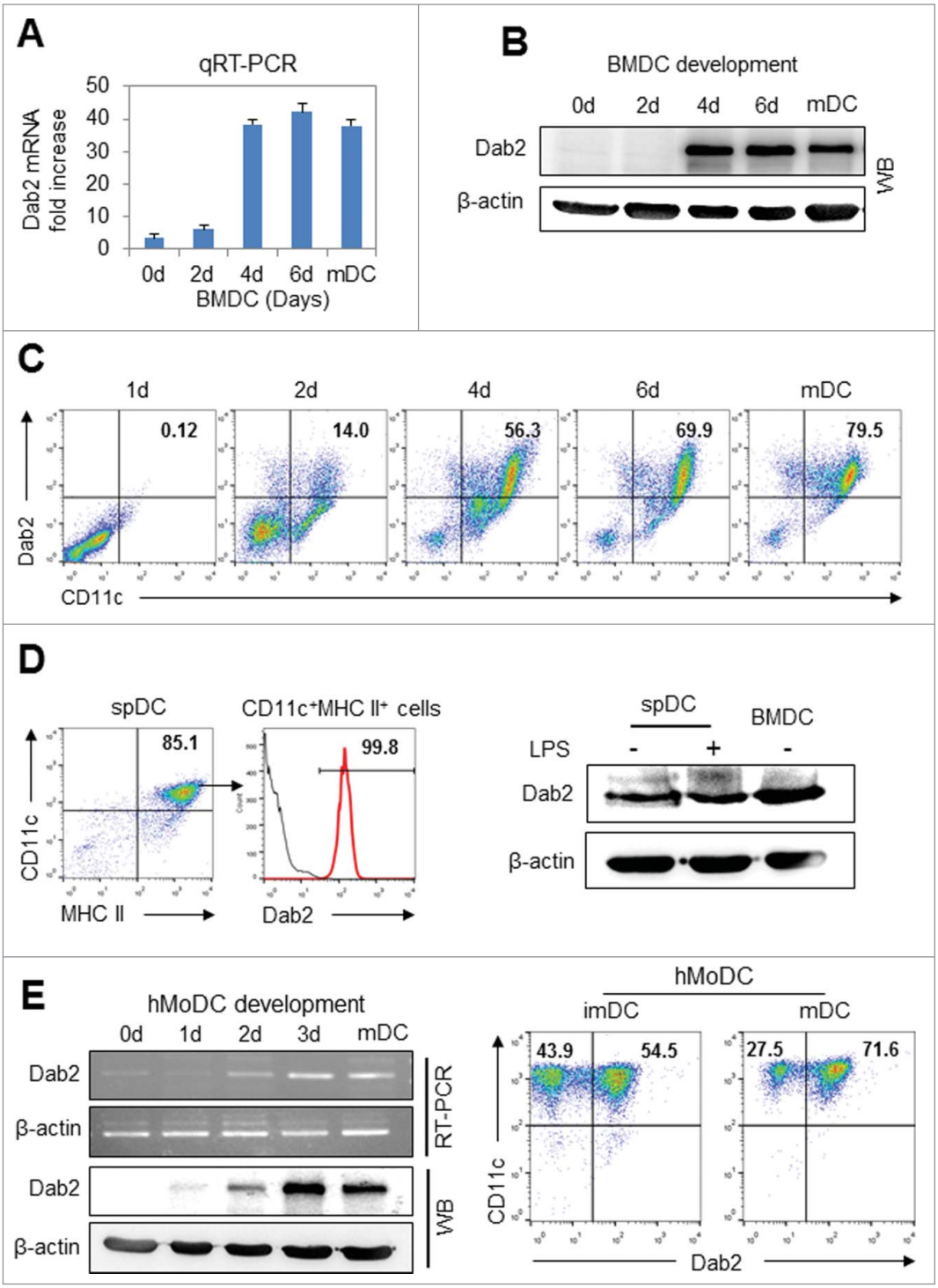

Figure 1. *Dab2* expression in BMDC, splenic DC and human monocyte-derived DC (**A and B**) *Dab2* expression was assessed during BM (C57 BL/6) cell-derived DC (BMDC) development. Cells developing into BMDCs were harvested on days 0, 2, 4, and 6, and *Dab2* mRNA and protein levels were assessed by quantitative real-time (qRT)-PCR with Fast SYBR® Green Master Mix kit (Life Technologies) and by Western blot with rabbit anti-mouse *Dab2* polyclonal antibody (Protein Tech), respectively. qRT-PCR data are shown as mean ± SD of nine samples pooled from three independent experiments. (**C**) Cells were harvested on days 0, 2, 4, and 6 during DC development, and assessed by flow cytometry after staining with FITC-labeled CD11c and PE-labeled intracellular *Dab2* antibodies. (**D**) Splenic DCs were isolated from mouse (C57BL/6) spleen using $CD11c^+$ isolation kit (Miltenyi Biotech) and treated with or without LPS (100 ng/mL) for 24 h. Intracellular *Dab2* expression in splenic DCs was assessed by FACS (left) and Western blot assay (right). (**E**) *Dab2* mRNA and protein expression were assessed by real-time (RT)-PCR and by Western blot with rabbit anti-mouse *Dab2* polyclonal antibody (Protein Tech), respectively, during human monocyte-derived DC (hMoDC) development (left) as described in Materials and Methods . Intracellular *Dab2* expression in MoDCs was also assessed by FACS (right).

Dab2 controls the expression of MHC and co-stimulatory molecules during DC development

Next, we investigated the surface phenotypes of BMDCs in the absence of *Dab2*. *Dab2*-specific siRNA (si-*Dab2*) was used to block *Dab2* expression by over 80% at both the mRNA (qRT-PCR) and protein levels in Western blot and flow cytometry (**Fig. 3A**). In *Dab2*-knockdown ($Dab2^{KD}$) DCs, the expression of MHC I and MHC II molecules and co-stimulatory molecules CD40 and CD80 increased in both imDCs and mDCs (**Fig. 3B**). These phenotypic changes by *Dab2*-silencing in both imDCs and mDCs were statistically significant (**Fig. 3C**). These data imply that *Dab2* expression during BMDC differentiation is likely involved in the negative regulation of DC immunogenicity.

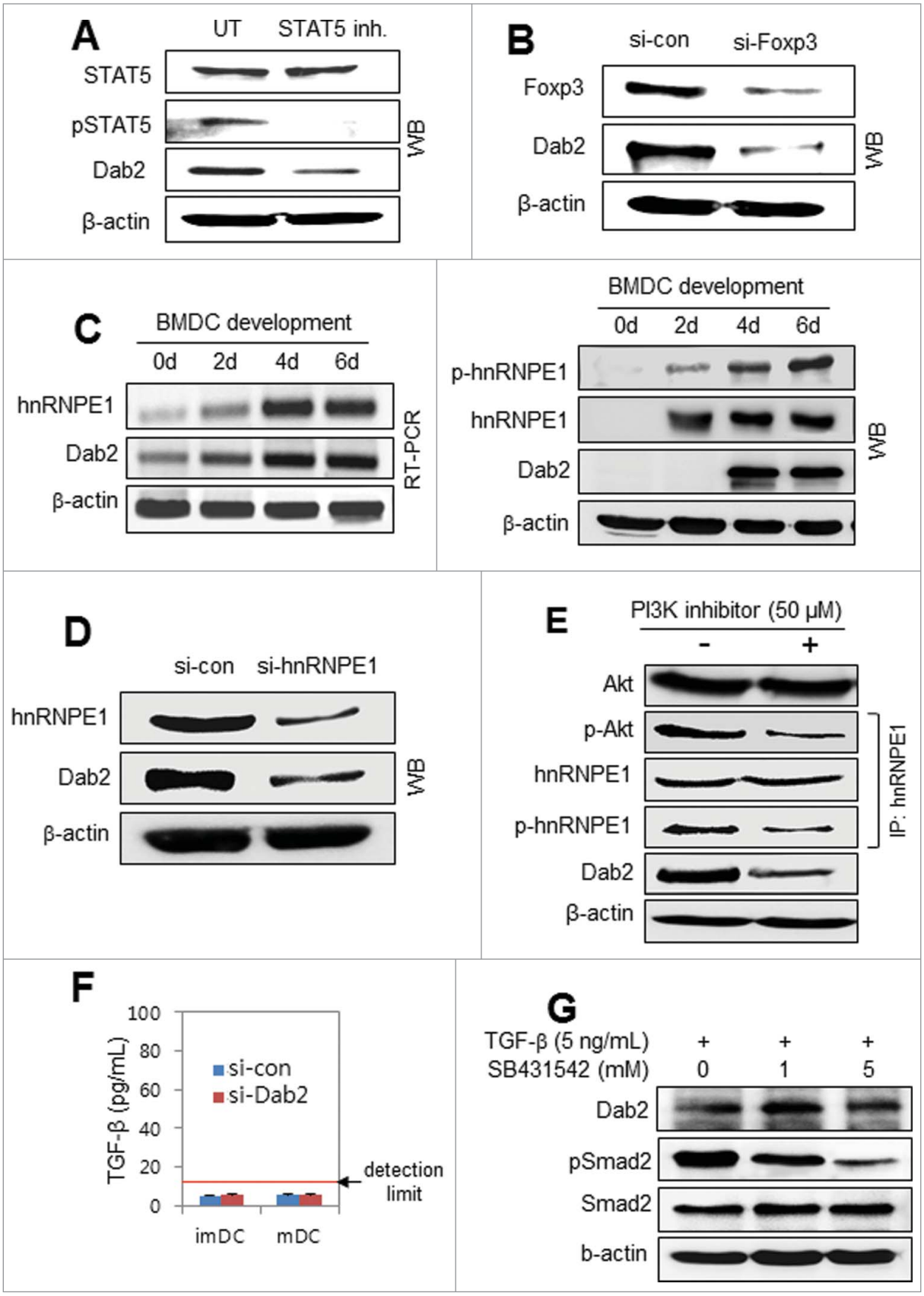

Figure 2. For figure legend, see next page.

Dab2 inhibits the antigen-uptake and migration capacities of BMDCs

Immature DCs (imDCs) take up antigens through receptor-mediated endocytosis and receptor-independent macropinocytosis.[31] *Dab2* is well established as an endocytic adaptor protein in endoderm cells.[6] To examine the role of *Dab2* in antigen uptake by imDCs, we explored the antigen uptake capacity of $Dab2^{KD}$ DCs. Unexpectedly, *Dab2*-sliencing significantly increased antigen uptake by imDCs (**Fig. 4A**), suggesting that *Dab2* in DCs, unlike in other cell types, may be a negative regulator for antigen uptake by imDCs. It was reported that, differing from *Dab2*, TGF-β-induced protein (βIg-h3) selectively upregulated in imDCs stimulates antigen uptake by DCs through endocytosis.[32] Detailed molecular mechanisms underlying *Dab2*-mediated inhibition of antigen uptake of imDCs remain to be established. Next, we examined the migration of $Dab2^{KD}$ DCs. We analyzed CCR7 expression on $Dab2^{KD}$ imDCs and mDCs. CCR7 expression was significantly enhanced by *Dab2*-silencing in mDCs while the expression was not affected by *Dab2*-silencing in imDCs (**Fig. 4B**). mDC migration toward CCL19 in a transwell plate increased by about 25% after silencing *Dab2* as compared with WT mDCs (**Fig. 4C**). The number of DCs that migrated from the injection site to the regional lymph node in mice also significantly increased by *Dab2*-silencing (**Fig. 4D**). We next analyzed cytokine secretion from WT and $Dab2^{KD}$ DCs. IL-12 and IL-6 secretions were significantly enhanced by silencing *Dab2* in mDCs while IL-10 secretion was significantly reduced by silencing *Dab2* as compared with control DCs (**Fig. 4E**). Other pro-inflammatory cytokines, such as TNF-α and IL-1β, were not affected by *Dab2*-silencing. These data suggest that $Dab2^{KD}$ DCs would be more effective than WT mDCs in activating antigen-specific T cell responses.

***Dab2* expression inhibits T cell stimulation by DCs**

Next, we assessed the ability of $Dab2^{KD}$ DCs to stimulate T-cell proliferation by using ovalbumin (OVA)-specific T cells obtained from C57BL/6-originated OVA-specific T cell receptor transgenic OT-1 and OT-2 mice. $Dab2^{KD}$ BMDCs were pulsed with OVA peptide ($OVA_{257–264}$ and $OVA_{323–339}$) and co-cultured with 5,6-carboxyfluorescein succinimidyl ester (CFSE)-labeled naïve OT-1 and OT-2 T cells, respectively. $Dab2^{KD}$ DCs were much more effective in stimulating OT-1 and OT-2 T cell proliferations as compared with WT DCs (**Fig. 5A**). The proliferation profiles of OT-1 and OT-2 T cells in DC/T cell co-cultures at three different ratios (**Fig. 5B**) revealed that the T (both $CD4^+$ and $CD8^+$) cell stimulation capacity of DCs was significantly improved by *Dab2*-silencing. To determine the major subset(s) of $CD4^+$ T cells that respond to *Dab2*-silencing of DCs, Th1, Th2, and Th17 cytokines were assessed in the supernatants of $Dab2^{KD}$ DCs/OT-2 T cell co-cultures. Among the cytokines in the supernatants of $Dab2^{KD}$ DC/T cell co-cultures, the IL-17A (Th17 cytokine) level was significantly enhanced by *Dab2*-silencing while the γ-interferon (IFNγ) level (Th1 cytokine) was much less enhanced and the IL-4 (Th2 cytokine) level did not change (**Fig. 5C**). In good agreement with the cytokine analysis, intracellular cytokine staining also showed that the Th17 population significantly increased in DC/T cell co-culture experiments after *Dab2*-silencing (**Fig. 5D**). *Dab2*-silencing slightly reduced the population of $CD4^+CD25^+Foxp3^+$ Treg cells in DC/OT-2 T cell co-cultures, but the reduction was not statistically significant (**Fig. 5E**). These results suggest that *Dab2* expression in DCs regulates the DC-mediated Th1 and Th17 immunity without affecting Th2 and Treg responses.

***Dab2*-silencing enhanced the DC vaccine efficacy in tumor immunotherapy**

To elucidate the effects of *Dab2*-silencing on DC-mediated effector T cell proliferation *in vivo*, OT-1 T cell-transfused mice were injected with $OVA_{257–264}$-pulsed $Dab2^{KD}$ or WT mDCs. Thereafter, the OVA-specific $CD8^+$ T cells in the spleen were assessed by tetramer assay and intracellular IFNγ staining. Both populations of tetramer-positive (**Fig. 6A**) and IFNγ-expressing (**Fig. 6B**) $CD8^+$ effector T cells were significantly increased in mice injected with $Dab2^{KD}$ DCs as compared with WT DCs. We examined CTL activity in mice vaccinated with $Dab2^{KD}$ or WT DCs both pulsed with $OVA_{257–264}$ peptide. The IFNγ levels in culture supernatants of T cells isolated from the spleen and the draining lymph node (DLN) of mice vaccinated with $Dab2^{KD}$ DCs were much higher than those assessed in mice vaccinated with WT DCs (**Fig. 6C**). In addition, the CTL activity in the DLNs of mice vaccinated with $Dab2^{KD}$ DCs was significantly enhanced as compared with those in mice vaccinated with WT DCs (**Fig. 6D**). These data indicate that *Dab2* expression in DCs plays an important role in controlling DC-mediated Th1 immunity and CTL activity. To assess *Dab2* function in DC-based tumor immunotherapy, C57BL/6 tumor-bearing mice were

Figure 2 (See previous page). *Dab2* expression in DCs requires STAT5 signaling, Foxp3 expression, and hnRNPE1 activation but has no association with TGF-β signaling. (**A**) Mouse (C57BL/6) BMDCs were treated with 100 μM STAT5 inhibitor for 4 h before harvest. Harvested cells were subjected to Western blot analysis of STAT5 and *Dab2* expression with STAT5, phospho-STAT5, and *Dab2* antibodies. (**B**) DC precursor cells on day 4 during BMDC development were transfected with control and Foxp3 siRNAs and then harvested after 48 h. *Dab2* expression was assessed by Western blot with *Dab2* and Foxp3 antibodies. (**C**) mRNA (left) and protein (right) expressions of hnRNPE1 and *Dab2*, together with hnRNPE1 phosphorylation, were assessed during BMDC ←development by RT-PCR and Western blot analysis. (**D**) BMDC precursor cells on day 4 were transfected with si-con and si-hnRNPE1 and harvested after 48 h. *Dab2* and hnRNPE1 expression was assessed by Western blot with *Dab2* and hnRNPE1 antibodies. (**E**) BMDCs were treated with 50 μM PI3K inhibitor (Calbiochem) for 20 min before harvest and subjected to Western blot analysis of Akt and *Dab2*. hnRNPE1 was immunoprecipitated from BMDC lysates with anti-hnRNPE1 antibody, followed by immunoblot analysis with phospho-hnRNPE1 (phospho-serine) and phospho-Akt antibodies. (**F**) BMDC precursor cells on day 4 were transfected with *Dab2*-specific siRNAs (si-*Dab2*) or control siRNA (si-con). After 48 h, the level of TGF-β in culture supernatant was examined using TGF-β ELISA kit (BioLegend). (**G**) BMDCs were treated or untreated with 5 ng/mL TGF-β for 24 h in the presence or absence of SB431524 (TGFβRI). *Dab2* and Smad2 expressions, and phospho-Smad2 were assessed by Western blot.

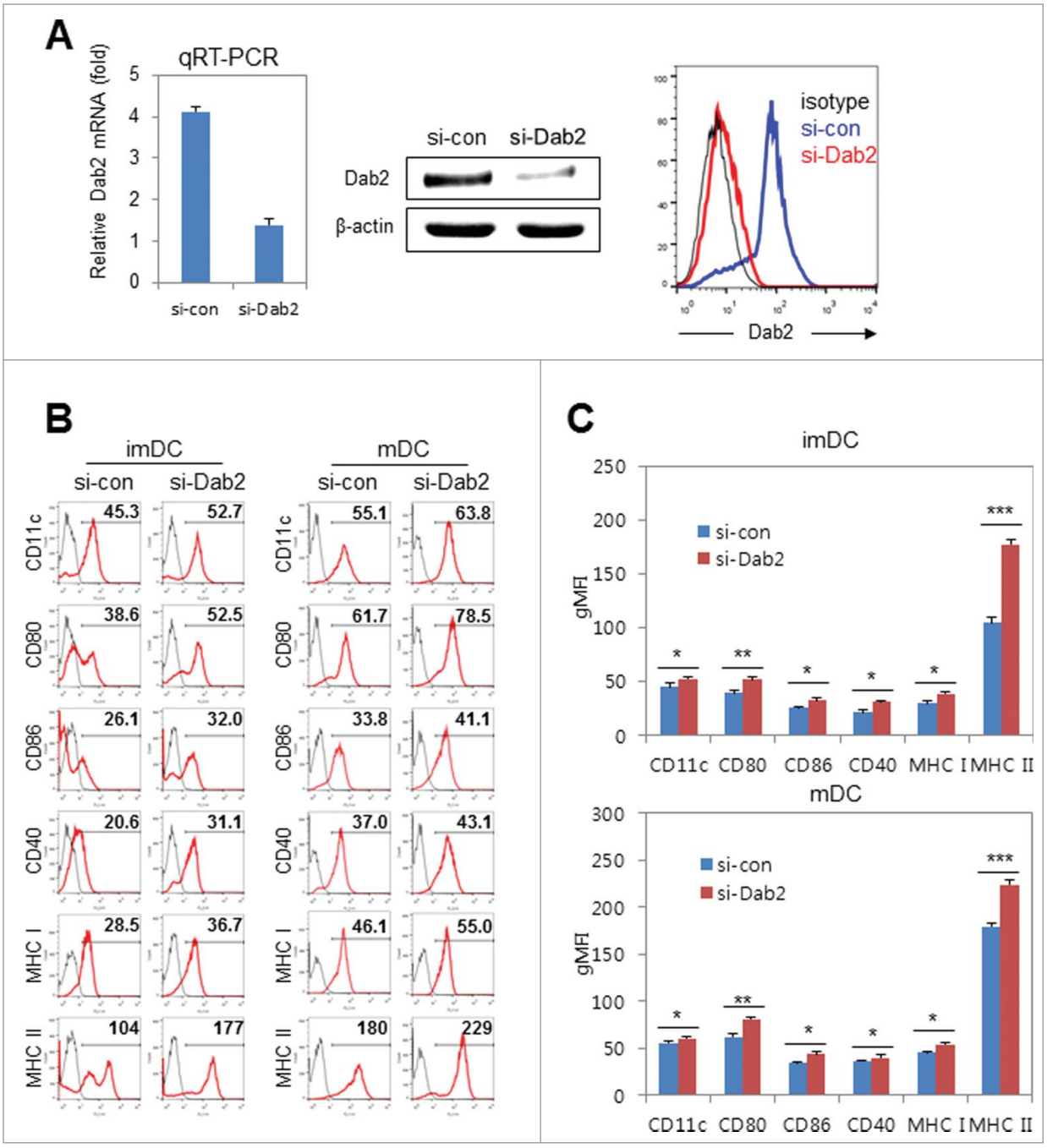

Figure 3. Effects of *Dab2*-silencing on BMDC surface phenotypes. (**A**) BMDC precursor cells on day 4 were transfected with si-*Dab2* or si-con and harvested on day 6 as WT or $Dab2^{KD}$ BMDCs. *Dab2* silencing was measured by qRT-PCR (left), Western blot (middle) and flow cytometry (right). qRT-PCR data are shown as mean ± SD of $n = 9$ samples pooled from three independent experiments. (**B**) Representative flow cytometry data showing surface phenotypes of $Dab2^{KD}$ (si-*Dab2*) and WT (si-con) immature (imDC) and mature (mDC) BMDCs. (**C**) Geometric mean fluoescence intensities (gMFI) of each BMDC surface molecule shown in flow cytometry (**B**) are presented as mean ± SD of nine samples pooled from three individual experiments. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared with control WT DCs, Student's *t*-test.

vaccinated twice with OVA peptide ($OVA_{257–264}$ and $OVA_{323–339}$)-pulsed $Dab2^{KD}$ or WT DCs vaccine. The $Dab2^{KD}$ DC vaccine inhibited E.G7 tumor growth more significantly than did WT DCs. EL4 tumors did not change, even when vaccinated with $Dab2^{KD}$ DCs (**Fig. 6E**). These results suggest that *Dab2*-silencing made the DC vaccine more effective for tumor immunotherapy by strengthening specific antitumor immunity.

DC immunogenicity was significantly impaired by overexpression of *Dab2*

We next examined the effect of *Dab2* over-expression during DC development on the DC phenotype and immunogenicity *in vitro* and *in vivo*. DC precursor cells were transfected with a plasmid expressing *Dab2* (pEF-Myc/His-p96), and *Dab2* overexpression in DCs was examined by Western blot and flow cytometry (**Fig. 7A**). *Dab2* over-expression downregulated the expression of costimulatory molecules CD80, CD86, and MHC I and MHC II on the surface of DCs (**Fig. 7B**). IL-12 secretion from mDCs was also significantly reduced by *Dab2* overexpression (**Fig. 7C**). *Dab2* overexpression obliterated the efficacy of the DC vaccine for tumor immunotherapy (**Fig. 7D**). These results strongly support our conclusion that *Dab2* is an intrinsic negative regulator of DC immunogenicity probably to maintain immune homeostasis *in vivo*.

Discussion

Adapter proteins play an important role in signal transduction. These proteins contain a variety of protein-binding modules that link binding partners together to create large signaling complexes. The adaptors have no intrinsic enzymatic activity, but they mediate specific protein-protein interactions to drive the formation of a protein complex. The Dab was originally identified in *Drosophila* as the product of a gene with a key role in a neural development.[33] *Dab2*

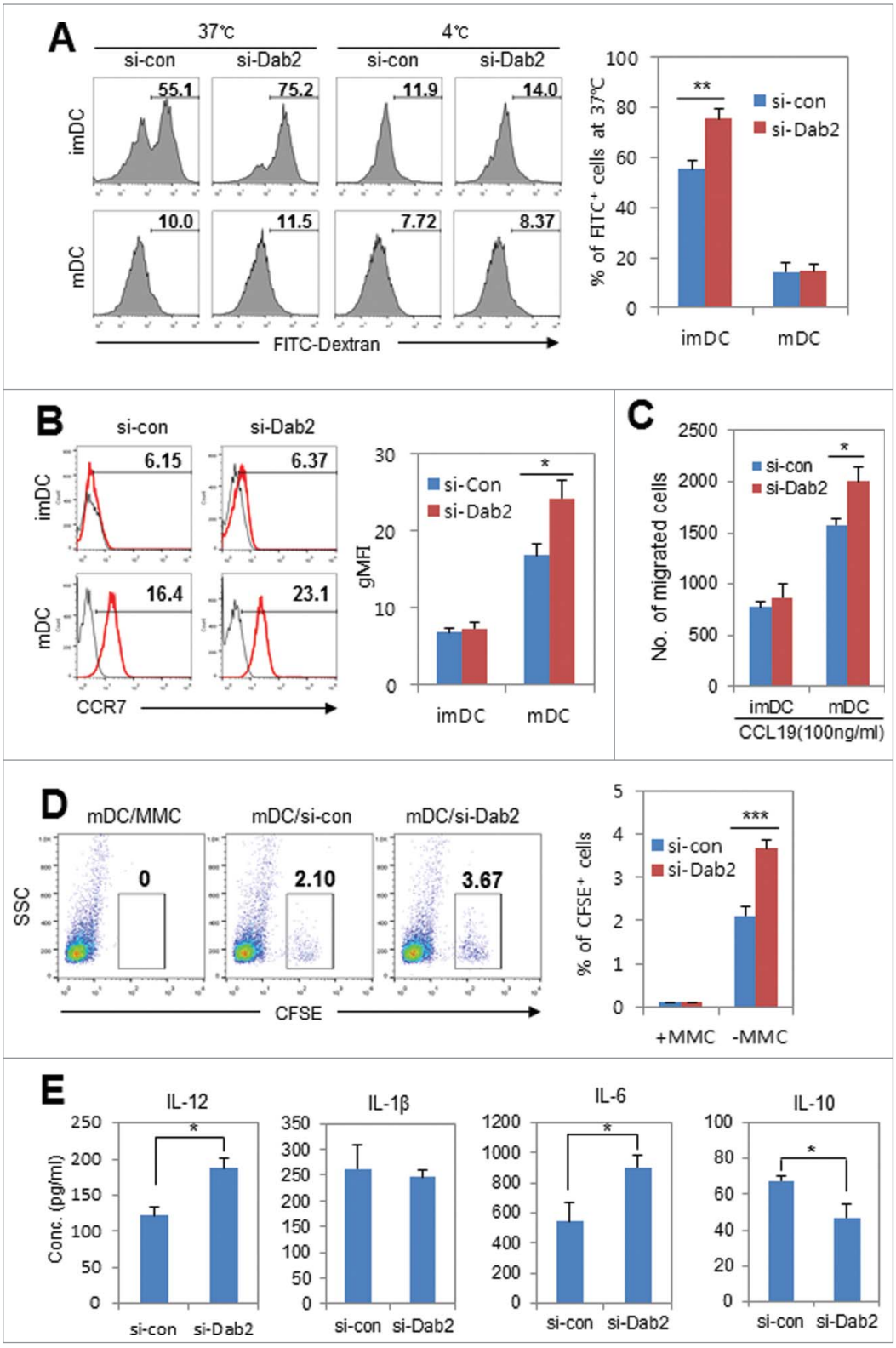

Figure 4. For figure legend, see next page.

(also called p96) is an ortholog of Dab.[9] *Dab2* was identified as a cytosolic adaptor that regulates endocytosis and growth factor signaling.[34] It has also been implicated in several receptor-mediated signaling and cell adhesion functions,[35] hematopoietic cell differentiation,[36] and angiogenesis.[37] *Dab2* is broadly expressed in various tissues and organs[16-18] and is a Foxp3 target gene required for Treg cells function.[14] *Dab2* also acts as a tumor suppressor in various tissues like ovary,[11] prostate,[38] and nasal carcinomas.[39] The function of *Dab2* in DC differentiation and immunogenicity has not been reported.

In this manuscript, we showed *Dab2* expression in DCs and its role in DC immunogenicity. Dab2 expression increased during BMDCs development and was also present in steady-state spDCs and human MoDCs as well (**Fig. 1**). STAT5 activation, Foxp3 expression, and PI3K/Akt signaling-mediated hnRNPE1 activation, all of which are needed for *Dab2* expression in a TGF-β-mediated EMT transition,[19,20] were also required for *Dab2* expression in DCs during GM-CSF-mediated BMDC development (**Fig. 2**). Treating BMDCs with PI3K inhibitor for even 20 min before harvest was enough to down-regulate *Dab2* expression in DCs (**Fig. 2E**), suggesting that *Dab2* expression is rapidly controlled by intracellular PI3K/Akt signaling in DCs. hnRNP E1 is involved in controlling *Dab2* translation by blocking the BAT element of Dab2 mRNA. Once hnRNP E1 is phosphorylated via the PI3K/PKB/Akt pathway under TGF-β signaling, hnRNPE1 is released from the BAT element and *Dab2* is expression is significantly induced.[21,22] However, TGF-β in culture supernatants of BMDCs was undetectable (**Fig. 2F**), and the treatment of BMDCs with TGF-β or TGF-β receptor inhibitor did not affect the expression of *Dab2* in BMDCs (**Fig. 2G**), suggesting that *Dab2* expression in BMDCs was not associated with TGF-β signaling. Taken together, our data strongly suggest that *Dab2* expression during GM-CSF-mediated BMDC development is contingent on the PI3K/Akt-dependent phospho-hnRNP E1-mediated translational control, rather than general transcriptional control, regardless of TGF-β signaling pathway. Silencing *Dab2* in DCs enhanced the expression of surface immunogenic molecules (**Fig. 3**), DC migration and antigen uptake, and expression of IL-12 and other proinflammatory cytokines as compared with WT DCs (**Fig. 4**). These data indicated that $Dab2^{KD}$ DCs might have higher immunogenic potential than do WT DCs, probably because *Dab2* acts as an intrinsic negative regulator. In particular, we found that $Dab2^{KD}$ DCs secreted more IL-12 and IL-6 cytokines than did WT DCs (**Fig. 4E**). IL-12 inhibits STAT5-mediated activation of the Foxp3 promoter.[40] IL-6 is needed with TGF-β for Th17 cell differentiation.[41] *Dab2* expression requires TGF-β-Smad signaling,[19] and IFNγ inhibits *Dab2* expression in macrophages.[42] These data support our findings that $Dab2^{KD}$ DCs have more immunogenic potential than WT DCs.

Next, we demonstrated that *Dab2*-silencing potentiated DC immunogenicity, thus stimulating both antigen-specific $CD4^+$ T cells and $CD8^+$ T cells significantly (**Fig. 5**). An analysis of cytokine secretion and intracellular staining of OT-2 T/DC co-cultures revealed that *Dab2*-silencing in DCs significantly increased the population of Th17 subsets (**Figs. 5C and D**). Treg cells lacking *Dab2* have been reported to be functionally impaired.[14] However, in T/DC co-culture experiments, DCs lacking *Dab2* did not affect the $CD25^+Foxp3^+$ Treg population (**Fig. 5E**), indicating that the increase in Th17 cells caused by *Dab2*-silencing in DCs was not due to downregulation of Treg cells. A $Dab2^{KD}$ DC vaccine was more effective at inducing antigens-specific cytotoxic T lymphocytes (CTL) and inhibiting tumor growth than a WT DC vaccine (**Fig. 6**). Ectopic overexpression of *Dab2* in DCs abrogated the DC vaccine efficacy in tumor immunotherapy (**Fig. 7**). Taken together, our findings indicate that *Dab2*, which is induced during DC development, is an intrinsic negative regulator of DC differentiation and immunogenicity. Because *Dab2* is a phosphoprotein,[43] other molecules might interact with *Dab2* adaptor protein to activate signaling pathways.

DCs play a pivotal role in activating T cell responses against tumors. The DC-based tumor immunotherapy is an attractive approach to treat cancer because of its safety and feasibility. However, DC-based tumor immunotherapies have not been as effective as expected because of the limited immunogenicity and immunosuppressive conditions in tumor microenvironments. Modifying genes of negative regulators in DCs improves the efficacy of DC vaccines in DC-based tumor

Figure 4 (See previous page). Effects of *Dab2*-silencing on antigen uptake, migration, and cytokine secretion of BMDCs. (**A**) WT or $Dab2^{KD}$ imDCs and LPS-stimulated (100 ng/mL for 24 h) mDCs (2×10^5 cells) were equilibrated with 1 mg/mL FITC-conjugated dextran for 1 h at 37°C or 4°C, respectively. Cells were washed and analyzed by flow cytometry. Representative flow cytometry of $FITC^+$ cell populations is shown (left) with the mean ± SD of 9 samples pooled from three independent experiments (right). $^{**}p < 0.01$, $^{*}p < 0.05$ compared with control imDCs and mDCs. Student's *t*-test. (**B**) WT or $Dab2^{KD}$ imDCs and LPS-stimulated mDCs were stained with PE-conjugated anti-CCR7 mAb followed by FACS analysis. Representative flow cytometry analysis of the $CCR7^+$ cell populations (%) in imDCs and mDCs (left) and accumulated statistical data (right) are shown with the mean ± SD of 9 samples pooled from three independent experiments. $^{**}p < 0.01$, Student's *t*-test. (**C**) The migration of WT and $Dab2^{KD}$ imDCs and mDCs was assessed by an *in vitro* chemotaxis assay as described in the materials and methods. The data are shown as mean ± SD of nine samples pooled from three independent experiments. $^{*}p < 0.05$, Student's *t*-test. (**D**) *In vivo* migration assay with $Dab2^{KD}$ BMDC was performed as described in materials and methods. CFSE-labeled $Dab2^{KD}$ or WT mature DCs (1×10^6 cells) were inoculated s.c in the right flank region of C57BL/6 mice. After 24 h, CFSE-labeled DCs in the inguinal lymph nodes were examined by flow cytometry. Mitomycine C (MMC)-treated (50 μg/mL for 30 min) CFSE-labeled BMDCs were used as a negative control. Representative FACS data (left) and statistical data from three independent experiments are shown as mean ± SD (right). $^{***}p < 0.001$. (**E**) Cytokine production was assessed by ELISA of WT and $Dab2^{KD}$ BMDC culture supernatants after stimulating with LPS (100 ng/mL) for 24 h. The data are shown as mean ± SD of nine samples pooled from three independent experiments. $^{*}p < 0.05$ compared with control mDCs, Student's *t*-test.

immunotherapy. In the present study we found that *Dab2*, which is expressed during DC development, seems to play an important role in controlling DC immunogenicity, probably to prevent immune exaggeration and maintain homeostasis after immune reactions. Therefore, molecular targeting of *Dab2* would be an attractive approach for the development of more effective DC vaccines in tumor immunotherapy. The detailed molecular mechanism underlying *Dab2*-mediated negative regulation of DC immunogenicity remains to be elucidated by further studies.

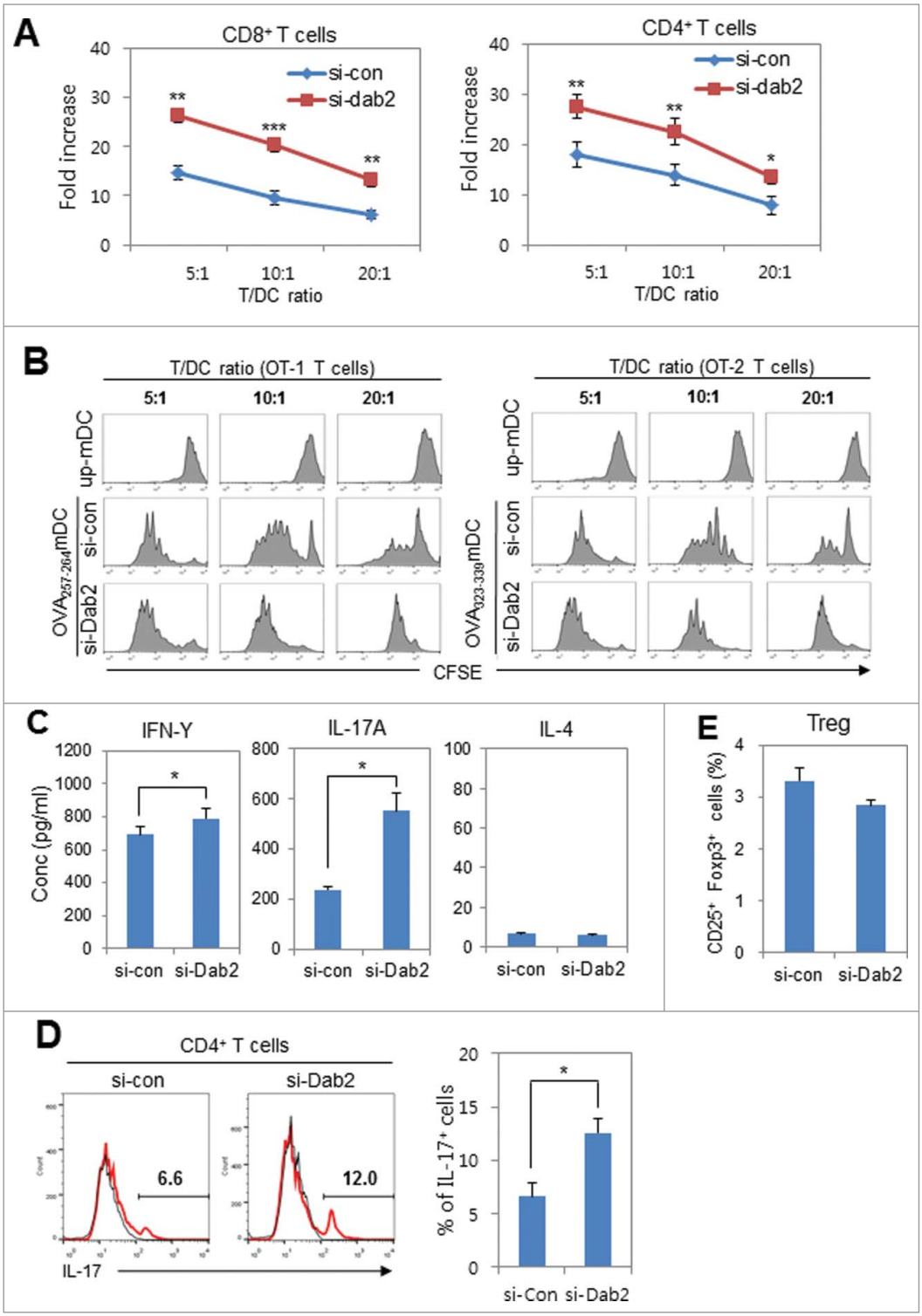

Figure 5. For figure legend, see next page.

Materials and Methods

Mice, cell lines, and reagents

C57BL/6, C57BL/6-background OT-1 ($OVA_{257–264}$-specific $CD8^+$ T cell receptor transgenic) and OT-2 ($OVA_{323–339}$-specific $CD4^+$ T cell receptor) mice (6 ~8 week old) were used for the present study. OT-1/OT-2 mice were Rag-1/Rag-2 normal. All mice were maintained in the animal care facility at Sungkyunkwan University according to the University Animal Care and Use guidelines. EL4 (C57BL/6 mouse-derived thymoma) and E.G7 (OVA-expressing EL4) cells were obtained from the American Type Culture Collection (ATCC). CFSE (5,6-carboxyflouroscein succinimidyl ester) (BioLegend^R), Dextran (40 KDa)-FITC (Sigma–Aldrich), STAT5 inhibitor and PI3K inhibitor (Calbiochem), TGF-β receptor inhibitor (TGFβRI) (SB431524, Sigma–Aldrich) and Protein A/G PLUS-Agarose (Santa Cruz Biotech) were used. A PE-conjugated monoclonal antibody to CCR7 was purchased from BioLegend, rabbit anti-mouse *Dab2* polyclonal antibody from Protein Tech, Foxp3 monoclonal antibody from eBioscience, and 24-well transwell chambers (8-μM pore size) from Corning Costar (Cambridge, MA). Anti-phospho-tyrosine-STAT5,

anti-phospho-Smad2, anti-pan-STAT5, anti-pan-Akt and anti-phospho-serine-Akt antibodies were obtained from Cell Signaling Inc. Anti-β-actin , HRP-conjugated anti-rabbit and anti-mouse IgGs were purchased from Sigma, anti-Smad2 and anti-phospho-serine antibodies from Abcam, murine GM-CSF from Creagene Inc., recombinant human TGF-β1 from R&D system and lipopolysaccharide (LPS, from *Escherichia coli* O111:B4) from Sigma–Aldrich. Synthetic ovalbumin peptides [$OVA_{257–264}$ (SIINFEKL] and $OVA_{323–339}$ (ISQAVHAAHAEINEAGR)] were provided by Peptron (Daejon, Korea). FITC- or PE-conjugated anti-CD4, CD8, CD25, CD11c, CD40, CD80, CD86, MHCII, MHCI, and Foxp3 antibodies were purchased from BD Pharmingen and BioLegend. Cytokine ELISA kits for murine IL-6, IL-12p70, IL-10, IL-17A, IL-1β, TNF-α, TGF-β and γIFN were purchased from BioLegend and the kit for IL-13 was from Abcam.

Bone marrow-derived DCs (BMDC), splenic DCs (spDC) and human monocyte-derived DCs (hMoDC)

BMDCs were generated from mouse BM progenitor cells as described previously.[25,26,44] In brief, BM cells collected from the femurs and tibiae of 6-week-old female C57BL/6 mice were washed and cultured in complete RPMI 1640 media [RPMI 1640 (Gibco^{R}) supplemented with 10% FBS and 10 ng/mL mGM-CSF (Creagene Inc.)]. After 2 d, non-adherent cells were washed and re-fed with 2 mL of fresh complete medium containing mGM-CSF. On day 4, 1 mL of fresh medium containing mGM-CSF was added to the culture. On day 6, non-adherent cells were collected as imDCs. imDCs were matured by further culturing in the presence of 100 ng/mL LPS for 24 h. spDCs were isolated from the spleen of 6–8 week old C57BL/6 mice using CD11c microbeads according to manufacturer's instruction (Miltenyi Biotech). Human MoDCs were generated as described[45,46] with minor modifications. Peripheral blood mononuclear cells (PBMC) were isolated from the blood of healthy donors by Ficoll gradient centrifugation. Monocytes were isolated by plastic adherence for 1 h and subsequently cultured in the presence of 30 ng/mL rhGM-CSF and 20 ng/mL rhIL-4 (Creagene Inc.) in RPMI 1640 medium supplemented with 1% human AB serum (Lonza). On day 3 of culture, nonadherent cells (used as immature MoDCs) were collected and matured for 18 h with 100 ng/mL lipopolysaccharide (LPS) (Sigma–Aldrich).

***Dab2* silencing with small interfering RNA (siRNA)**

Dab2-specific siRNAs were designed by BLOCK-IT RNAi Designer (Invitrogen) to achieve full specificity without any off-target effects, such as inducing type-1 interferon. Two siRNAs specific to *Dab2* 5′-cctgttgtctacagtcctt-3′ (si-*Dab2*-1) and 5′-ccacctcttgttccctcaa-3′(si-*Dab2*-2) and a control siRNA 5′-ccttgtatcgacctgtctt-3′(si-con) were synthesized by Invitrogen. DC precursor cells on day 4 or day 5 of mouse BM cell culture were transfected with the 2 *Dab2* siRNAs (si-*Dab2*) or control siRNA (si-con) using Lipofectamine RNAiMAX (Life Technologies) as reported previously.[25,26] After 48 h, cells were washed and used as a *Dab2*-silenced (*Dab2*^{KD}) immature BMDCs (imDCs) for subsequent experiments.

Flow cytometry analysis

Direct immunofluorescence staining was performed to analyze the DC surface phenotypes as described previously.[26] DCs were stained with appropriate antibodies at 4°C for 20 min. After washing, cells were then analyzed by FACS Caliber (BD) using CellQuest or FlowJo software. For intracellular *Dab2* staining, cells prestained with FITC-labeled hamster anti-mouse CD11c antibody were fixed and permeabilized with the BD Cytofix/Cytoperm^{TM} kit (BD Bioscience Pharmingen). Cells were then stained with PE-labeled rabbit anti-mouse *Dab2* antibody (Bioss^{R}) or PE-labeled rabbit isotype control antibody in BD perm/wash buffer for 1 h. In the case of intracellular staining for IL-17 or IFNγ in T cells, cells prestained with FITC-labeled anti-mouse CD4 or CD8 antibody were fixed, permeabilized, and then stained with PerCP-Cy5.5-labeled anti-IL-17 or anti-IFNγ antibodies together with PerCP-Cy5.5-labeled mouse isotype control antibody (BioLegend). After washing with BD perm/wash buffer, cells were analyzed by flow cytometry.

T-cell proliferation assay

A T cell proliferation assay was performed with WT and *Dab2*^{KD} BMDCs as described previously.[25] T cells were purified from the spleen or DLN of OT-1 and OT-2 mice using a mouse $CD4^{+}$ and $CD8^{+}$ T-cell Isolation Kit II (Miltenyi Biotech) and labeled with CFSE (1 μM for 10 min) as described previously.[26]

Figure 5 (See previous page). The effects of *Dab2*-silencing on the ability of BMDCs to stimulate T cell proliferation. (**A**) WT and *Dab2*^{KD} mDCs (from C57BL/6 mice) that were pulsed with $OVA_{257–264}$ or $OVA_{323–329}$ peptides were co-cultured with CFSE-labeled OT-1 or OT-2 T cells, respectively, for 4 d at three different ratios. CFSE-positive proliferated (CFSE-diluted) T cells were gated, calculated, and represented by fold increases as described in materials and methods. Data are shown as mean ± SD of six samples pooled from three independent experiments. *$p < 0.05$ and **$p < 0.01$, Student *t*-test. (**B**) OVA peptide ($OVA_{257–264}$ or $OVA_{323–329}$)-pulsed WT and *Dab2*^{KD} mDCs were co-cultured with CFSE-labeled OT-1 or OT-2 T cells, respectively, for 4 d at different T:DC cell ratios. CFSE-labeled proliferating T cells were gated and represented by histogram. (**C**) Th1 (IFNγ), Th17 (IL-17), and Th2 (IL-4) cytokines in the supernatant of DC/T cell co-cultures at a 1:10 ratio were assessed by ELISA at day 3. The data are shown as mean ± SD of six samples pooled from three independent experiments. *$p < 0.05$ compared with WT (si-con) DC, student *t*-test. (**D**) Treg cell populations were assessed by intracellular Foxp3 staining and CD25 surface staining from co-cultures of mDCs (C57BL/6) pulsed with $OVA_{323–339}$ peptide and OT-2 T cells at a ratio 1:10 for 5 d. $CD25^{+}$ $Foxp3^{+}$ Treg cells were assessed by flow cytometry and are shown as mean ± SD of six samples pooled from three independent experiments. (**E**) $OVA_{323–329}$ peptide-pulsed WT and *Dab2*^{KD} mDCs were co-cultured with OT-2 T cells for 3 d, and Th17 cells among the OT-2 cells were assessed by FACS after intracellular staining with anti-IL-17 antibody (left). Statistical data (right) are shown as mean ± SD of six samples pooled from three independent experiments. *$p < 0.05$, Student *t*-test.

Dab2^{KD} or WT immature BMDCs (imDC) were matured by culturing with LPS (100 ng/mL) for 24 h, and then pulsed with 1 μg/mL OVA peptide ($OVA_{257–264}$ or $OVA_{323–339}$) for 1 h. Pulsed mDCs were washed with cold phosphate-buffered saline (PBS), and then co-cultured with CFSE-labeled T cells at different ratios for 3–4 d. Cells grown in co-cultures were harvested and analyzed by flow cytometry. CFSE-positive proliferating (CFSE diluted) T cells were gated and calculated by using the following formula (proliferation index; PI = 1000/geometric sum of gated CFSE). T cell proliferation capacity of antigen-pulsed DCs was represented by fold increase on the basis of the PI of the T cells co-cultured with unpulsed DCs.

Antigen-uptake assay

As described previously,[26] *Dab2*^{KD} or WT immature and mature BMDCs (2×10^5 cells) equilibrated at 37°C or 4°C for 45 min in FACS tubes, were pulsed with 1 mg/mL FITC-conjugated dextran for 1 h. The reaction was stopped with cold PBS buffer. The cells were washed and stained with PE-conjugated anti-CD11c and analyzed with a FACS Calibur flow cytometer.

Chemotaxis of DCs

Dendritic cell chemotaxis was measured by migration in 24-well transwell chambers (Corning Costar, Cambridge, MA, USA). *Dab2*^{KD} or WT immature and mature BMDC (1×10^5 cells in 0.1 mL) were washed with PBS and resuspended in serum-free RPMI 1640 medium, and then placed in the upper chamber of the transwell plate. The lower chamber of transwell plates contained CCL19 (300 ng/mL) diluted with 0.6 mL of serum free RPMI 1640. Plates were incubated at 37°C for 3 h to allow DC migration. Migrated cells were harvested from the lower chamber and analyzed by flow cytometry.

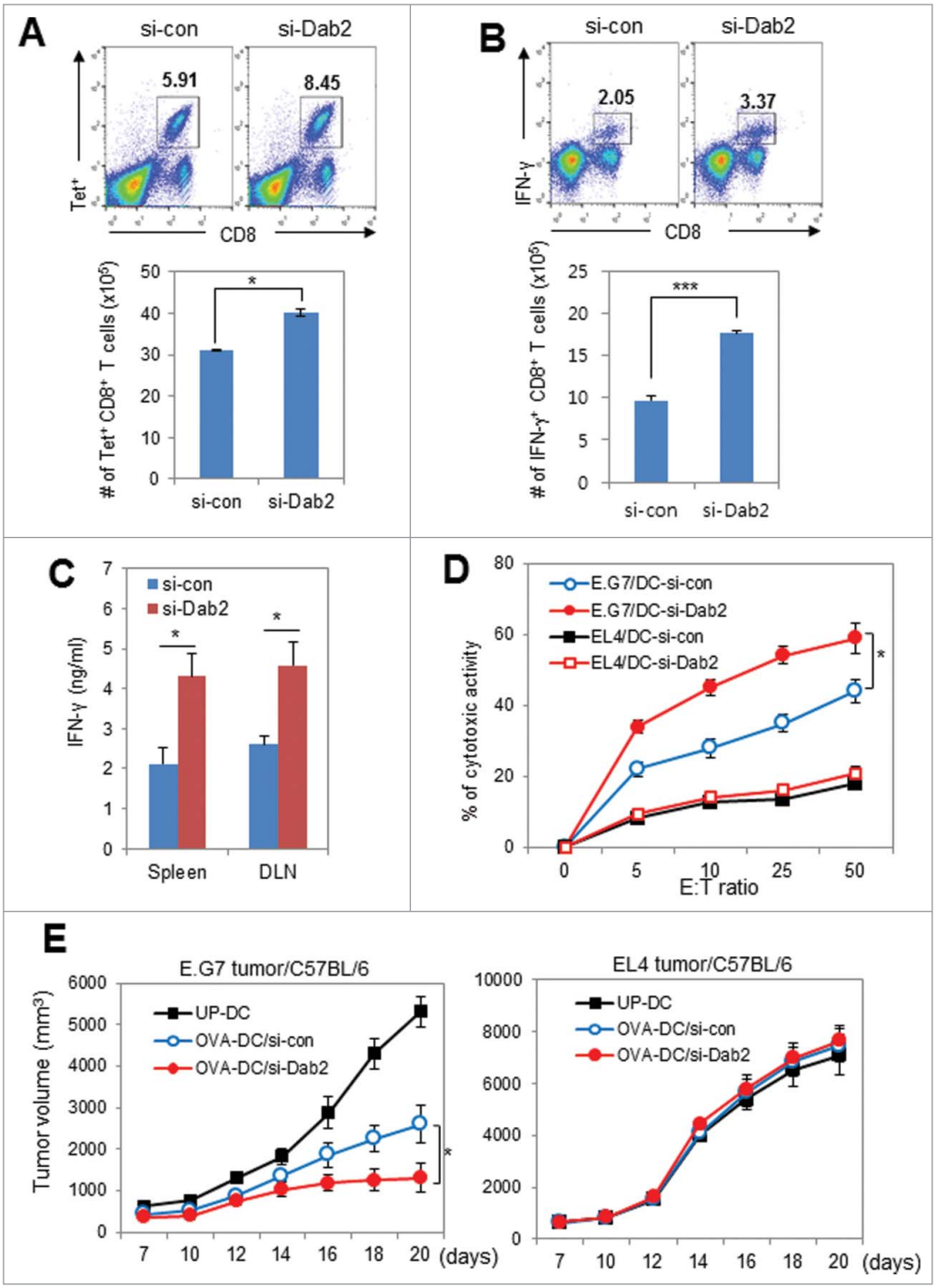

Figure 6. For figure legend, see next page.

In vivo migration assay of DCs

As described previously,[47] Dab2^{KD} or WT mature BMDCs were labeled with 5 μM CFSE (BioLegend) at 37°C for 10 min. A total of 1×10^6 CFSE-labeled DCs were inoculated subcutaneously (s.c.) in the right flank region of C57BL/6 mice.

Twenty-four hour later, inguinal DLNs were obtained from the mice, and the labeled DCs that had migrated from the injection site to the inguinal DLNs were examined by FACS. As a negative control, CFSE-labeled BMDCs that were pre-treated with 50 μg/mL mitomycine C (MMC) for 30 min were used.

Quantitative real time (qRT)-PCR

Total RNA was isolated and purified from BMDCs using the Ribospin^{TM} kit (GeneAll). cDNA was synthesized using Maxima Enzyme Mix (Thermo Scientific) and 5X reaction mix (Thermo Scientific). Quantitative PCR was performed using the Fast SYBR® Green Master Mix kit (Life Technologies) with the following primer sets: Dab2 (S)5′-tgctcgtgatgtgacagaca-3′, (AS)5′-agggtcattagggcctcact-3′; GAPDH (S)5′-aatgtgtccgtcgtggatct-3′, (AS)5′-tccaccaccctgttgctgta-3′; and β-actin (S) 5′-gtatgcctcggtcg-tacca-3′, (AS) 5′cttctgcatcctgtcagcaa-3′.

Western blot analysis

Western blot analysis was performed as described previously.[26,48] In brief, cells were washed in cold PBS, and lysed with lysis buffer containing 50 mM Tris-HCl (pH-7.4), 150 mM NaCl, 1 mM DTT, 30 mM NaF, 10 mM Na3VO4, 0.5% NP40, and a protease inhibitor cocktail (Roche). Cell lysates were subjected to 8–12% SDS-PAGE and transferred to a PVDF membrane (Millipore). Membranes were incubated overnight at 4°C with primary antibodies in 4% nonfat dry milk, and then further incubated for 1 h with HRP-conjugated secondary antibodies. Bound antibodies were detected by using chemiluminescent HRP substrate (Millipore, USA) and a Chemiluminescent Imaging System (Davinch Chemi^{TM}).

Immunoprecipitation

Immunoprecipitation (IP) was performed as described previously.[49] In brief, BMDCs were lysed with IP lysis buffer containing 25 mM HEPES (pH-7.4), 150 mM NaCl, 1 mM EDTA and 0.5% TritonX-100, incubated for 5 min on ice, and sonicated for 3–4 five-second pulses on ice using an ultrasonicator (Sonic Vibra-Cell VC 750). After centrifugation, the supernatants were incubated with primary antibody at 4°C for overnight, followed by incubation with Protein A/G PLUS-Agarose (Santa Cruz Biotech). Agarose bead pellets were washed, resuspended in a loading buffer, and subjected to SDS-PAGE. The resolved proteins were transferred to nitrocellulose membranes and stained with specific antibodies. Blotted protein was assessed by ECL.

ELISA

BMDC precursor cells on day 4 of DC culture were transfected with si-Dab2 or si-con. After 48 h, cells were stimulated with LPS (100 ng/mL) for 24 h. The BMDC (si-con/si-Dab2) culture supernatants were assessed using ELISA kits for IL-6, IL-12p70, TNF-α, IL-1β, TGF-β and IL-10. The amounts of IFNγ, IL-17A, and IL-4 cytokines were also assessed in the supernatants of DC/T cell co-cultures at day 3 with an ELISA reader (Molecular Device).

Tetramer assay

Tetramer assay was performed as described previously.[50] OVA-pulsed Dab2^{KD} or WT BMDCs (1×10^6) and OT-1 T cells (5×10^6) were transferred intravenously into naive C57BL/6 mice through a tail vein. After 7 d, the mice were boosted by intravenous (i.v) injection with 0.1 mM OVA I peptide (100 μL). Three day later, splenocytes from these mice were strained with PE-labeled H-2K^b/OVAI tetramer (kindly provided by Dr. Chang, Ewha Womans University, Seoul, Korea) and FITC-anti-CD8 antibodies, followed by flow cytometry analysis.

CTL assay and ELISA for IFNγ

OT-1 mice were injected twice at a 1-week interval with WT (si-con) or Dab2^{KD} (si-Dab2) DCs pulsed with $OVA_{257–264}$ peptide. Fourteen day after injection, splenocytes and draining lymph nose (DLN) cells from vaccinated mice were cultured for 7 d in the presence of OT-1 peptide (10 μg/mL) in 6-well plates (2×10^6 cells/well) and co-cultured with CFSE-labeled target cells (EL4 and E.G7 tumor cells) at different ratios for 4 h. After PI staining, CTL activity of splenocytes and DLN cells were analyzed by flow cytometry as described.[26,51] IFNγ levels in the culture supernatants were assessed by ELISA.

Figure 6 (See previous page). *Dab2*^{KD} DC vaccine was more effective than WT DC vaccine in the induction of antitumor immunity in tumor immunotherapy. (**A**) CD8$^+$ T cells proliferation *in vivo* by WT or *Dab2*^{KD} BMDCs was assessed by tetramer assay as described in materials and methods. Naive C57BL/6 mice were transferred i.v with OT-1 T cells together with OVA-pulsed *Dab2*^{KD} or WT mDCs, and then boosted with $OVA_{257–264}$ peptide on day 7. Three day later, splenocytes from the mice were stained with PE-labeled H-2K^b/$OVA_{257–264}$ tetramer and FITC-anti-CD8 antibodies, and subsequently analyzed by flow cytometry. Shown is representative FACS data (upper) and the number of tetramer(Tet)$^+$CD8$^+$ T cells is represented as mean ± SD of six samples from two mice (lower). *$p < 0.05$, Student *t*-test. (**B**) The splenocytes prepared for tetramer assay in (**A**) were activated with phorbol myristate acetate (PMA)/ionomycin (40 ng/mL each, Sigma–Aldrich) for 4 h. Cells were then examined by flow cytometry after surface and intracellular staining with FITC-anti-CD8 antibody and PerCP/Cy-anti-IFNγ antibody, respectively. Shown is representative FACS data (upper), and the number of IFNγ+CD8+ T cells is represented as mean ± SD of six samples from two mice (lower). ***$p < 0.001$, Student *t*-test. (**C**) OT-1 mice were immunized twice at a 1-week interval with 1×10^6 OVA peptide ($OVA_{257–264}$)-pulsed *Dab2*^{KD} or WT mBMDCs (C57BL/6). Cells from the spleen and DLN of vaccinated mice were harvested and cultured for 5–7 d in the presence of $OVA_{257–264}$ peptide. The level of IFNγ in the culture supernatants on day 2 of culture was assessed by ELISA, and the data are shown as mean ± SD of six simples from two independent experiments. *$p < 0.05$, Student *t*-test. (**D**) CTL activity in the DLNs of vaccinated OT-1 mice was assessed by flow cytometry using CFSE-labeled E.G7 and EL4 as target cells. Quantitative CTL activities are shown as mean ± SD ($n = 3$). *$p < 0.05$, Student *t*-test. (**E**) C57BL/6 mice were inoculated s.c. with E.G7 and EL4 tumor cells (5×10^5) in the right flank and immunized twice on day 3 and day 10 with 1×10^6 *Dab2*^{KD} (si-*Dab2*) or WT (si-con) mDCs (from C57BL/6 BM cells) that were pulsed with OVA peptide. Tumor growth was monitored and represented as mean ± SD of four mice from each of two experiments (bottom). *$p < 0.05$, Student *t*-test.

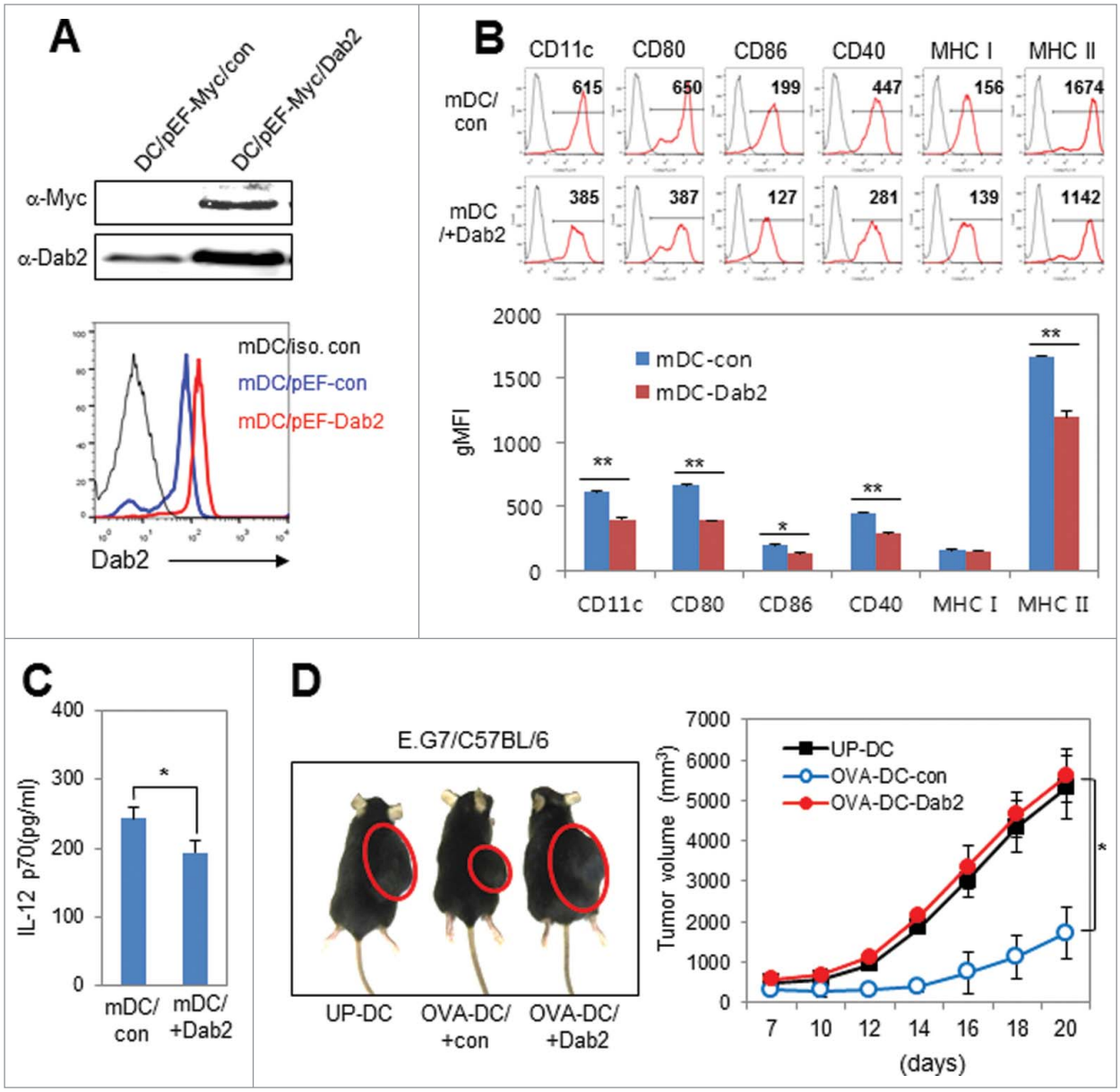

Figure 7. *Dab2*-overexpression impaired the efficacy of the DC vaccine for tumor immunotherapy. (**A**) *Dab2* expression plasmid (pEF-Myc/*Dab2*) and control vector (pEF-Myc/con) were transfected into DC precursor cells on day 4 during BMDC development. The transfected cells were harvested after 48 h, and matured with LPS (100 ng/mL) for 24 h. *Dab2* expression in the transformed mDCs was assessed by Western blot (upper) and FACS analysis (lower) using anti-Myc and anti-*Dab2* antibodies. (**B**) The surface phenotypes of *Dab2* transfected mDCs were assessed by flow cytometry, and the gMFI value of each DC surface marker from three independent experiments is shown as mean ± SD of nine samples. *$p < 0.05$, **$p < 0.01$ compared with control vector-transfected DCs, Student's *t*-test. (**C**) IL-12 levels were assessed by ELISA of the culture supernatant of *Dab2*-transfected DCs and are shown as mean ± SD of nine samples. *$p < 0.05$ compared with control vector transfected DCs, Student's *t*-test. (**D**) C57BL/6 mice were inoculated s.c. with E.G7 tumor cells (5×10^5) in the right flank, and then immunized on day 3 and day 10 with 1×10^6 *Dab2*-expressing mDCs (OVA-DC-*Dab2*) or control vector-transfected mDCs (OVA-DC-con), which were derived from C57BL/6 BM cells and pulsed with OVA peptides ($OVA_{257–264}$ and $OVA_{323–339}$). Representative images of E.G7 tumors are shown on day 20 after DC vaccination (left). Tumor growth was monitored every 2–3 d and presented as mean ± SD of four mice from each of two experiments. *$p < 0.05$, Student's *t*-test.

DC-based tumor immunotherapy

Tumor immunotherapy was performed on tumor-bearing C57BL/6 mice as described previously.[26] In brief, mice were injected with EL4 and E.G7 cells (5×10^5 cells /mouse) s.c. in the right flank. On day 3 and day 10 after tumor inoculation, the mice were vaccinated with WT (si-con), *Dab2*-silenced, or *Dab2* overexpressing DCs that had been pulsed with $OVA_{257–264}$ peptide. Tumor growth was monitored every 2–3 d using a slide caliper. Tumor masses were calculated with the formula $V = (A^2 \times B)/2$, where A is the short axis (width) and B is the long axis.

Statistical analysis

All experiments were performed at least three times with consistent results. Statistical data are presented as mean ± SD. Group comparisons were analyzed with student's *t*-test. A *p*-value of less than 0.05 ($p < 0.05$) was considered statistically significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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