**Disabled-2 (DAB2) Overexpression Inhibits Monocyte-Derived Dendritic Cells’ Function in Vogt-Koyanagi-Harada Disease**

Shenglan Yi,1 Rui Chang,1 Jianping Hu,1 Yiguo Qiu,1 Qingfeng Wang,1 Qingfeng Cao,1 Gangxiang Yuan,1 Guannan Su,1 Chunjiang Zhou,1 Yao Wang,1 Aize Kijlstra,2 and Peizeng Yang1

1The First Affiliated Hospital of Chongqing Medical University, Chongqing Key Laboratory of Ophthalmology and Chongqing Eye Institute, Chongqing, People’s Republic of China
2University Eye Clinic Maastricht, Maastricht, The Netherlands

**PURPOSE.** Recent studies reported that the tumor suppressor disabled-2 (DAB2) is a negative regulator of immune function. In this study, we investigated the role of DAB2 in monocyte-derived dendritic cells (DCs) from Vogt-Koyanagi-Harada disease (VKH) patients.

**METHODS.** The mRNA and protein levels of DAB2 were quantified by quantitative real-time PCR and Western blot. The Sequenom MassARRAY system was used to detect the promoter methylation level. An adenovirus carrying the DAB2 gene was transduced into immature DCs, isolated, and induced from active VKH patients. The surface markers of DCs, the frequency of T helper (Th) type 1 (Th1) and Th17 cells in CD4^+^ T cells, which were cocultured with DCs, were tested by flow cytometry. ELISA was used to analyze the inflammatory cytokines produced by DC and CD4^+^ T cell cocultures.

**RESULTS.** The mRNA and protein expression levels of DAB2 in DCs obtained from active VKH patients were decreased, while the DAB2 promoter methylation level was marginally increased when compared with inactive VKH patients and normal controls. The expression of CD80 on DCs was significantly downregulated by DAB2 overexpression. The DC-related inflammatory factors IL-6 and TNF-α were also decreased. The frequency of Th1 and Th17 cells and their related cytokines were reduced significantly after coculture with DAB2 overexpressing DCs. DAB2 overexpression did not affect autophagy in DCs from VKH patients.

**CONCLUSIONS.** These results suggest that the decreased expression of DAB2 in DCs plays a role in the pathogenesis of VKH disease. DAB2 overexpression inhibits DC function, but this is not mediated via autophagy.

**Keywords:** Vogt-Koyanagi-Harada disease, disabled-2, dendritic cells, promoter methylation, overexpression
transiently increased during the acute phase of multiple sclerosis, and it has been shown to exacerbate the severity of experimental autoimmune encephalomyelitis. Whether DAB2 affects the function of DCs in an autoimmune disease such as VKH is not yet known and was, therefore, the subject of the study reported here.

DNA methylation, one of the epigenetic controlling mechanisms, alters the expression of a gene but does not change the DNA sequence. Promoter hypermethylation of DAB2 has been shown to be involved in the metastasis of a variety of tumors. A number of recent studies have shown that DNA methylation was also involved in the pathogenesis of various autoimmune diseases. Several studies from our lab showed that DNA hypermethylation of interferon regulatory factor 8 (IRF8), IL-4, and TGF-β is involved in the development of VKH disease. However, whether aberrant DNA methylation of DAB2 is affected in VKH disease has not been reported.

In this study, we investigated the role of the expression and promoter methylation level of DAB2 in DCs in patients with VKH disease. We also overexpressed DAB2 in DCs from active patients to investigate its effect on DC maturation and function.

**MATERIALS AND METHODS**

**Subjects**

Blood samples from patients and controls were collected at the First Affiliated Hospital of Chongqing Medical University during December 2015 to January 2018. Forty-eight active VKH patients (17 females, 31 males; age, 38.21 ± 11.89 years), 24 inactive VKH patients (8 females, 16 males; age, 38.33 ± 11.26 years), and 41 normal controls (13 females, 28 males; age, 36.80 ± 12.06 years) without any inflammation were enrolled for this study. The diagnosis of VKH was made according to the criteria of the international nomenclature committee. In these 48 active VKH patients, 7 were in the acute stage and showed diffuse chorioiditis and serious retinal detachment, and the other 41 active VKH patients were in the recurrent stage and showed granulomatous anterior uveitis and sunset glow fundus. When blood samples were collected, all active VKH patients had not been on systemic immunosuppressive therapy for at least one week or only used a very low dose of oral prednisone (<20 mg/d). The inactive patients had quiet eyes and were still on a treatment regimen with prednisone (20 mg/d). Informed consent was obtained from all participants before sampling. All procedures adhered to the tenets of the Declaration of Helsinki.

**Cell Isolation and Culture**

CD4+ T cells and CD14+ monocytes were separated by human CD4 and CD14 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) from peripheral blood mononuclear cells (PBMCs) of normal controls and VKH patients and then cultured with RPMI 1640 medium (10% fetal bovine serum, 100 U/ml penicillin/streptomycin). IL-4 (50 ng/ml; AcroBiosystems, Newark, NJ, USA) and granulocyte-macrophage colony-stimulating factor (100 ng/ml; AcroBiosystems) were added for 7 days to drive CD4+ T cells into monocyte-derived immature DCs (iDCs). Lipopolysaccharide (LPS; 100 ng/ml; Sigma-Aldrich, St. Louis, MO, USA) and granulocyte-macrophage colony-stimulating factor (100 ng/ml; AcroBiosystems) were added for 7 days to drive CD4+ T cells into monocyte-derived immature DCs (iDCs). Lipopolysaccharide (LPS; 100 ng/ml; Sigma-Aldrich, St. Louis, MO, USA) was added to these iDCs during the last 24 hours of culture to obtain the mDCs. CD4+ T cells were cocultured with DAB2-overexpressing mDCs at a CD4+ T cell:iDC ratio of 5:1 for 5 days. Preparation of these DAB2-overexpressing DCs is shown below.

**RNA Preparation and Real-Time PCR**

Total RNA was separated from DCs by the TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and then reverse transcribed into complementary DNA by the PrimeScript RT kit (Takara Biotechnology, Dalian, China). The mRNA expression was measured by SYBR Premix (Bio-Rad Laboratories, Hercules, CA, USA) with the ABI Prism 7500 system (Applied Biosystems, Foster City, CA, USA). The sequences of DAB2 and β-actin PCR primer pairs were as follows: DAB2 forward 5′-CTAGAAAA CAAGTGCCAACCAATGGGT-3′, DAB2 reverse 5′-GCCT TTGAACCTTGCTAAGAGA-3′, and β-actin forward 5′-GAGAGG CATGC-3′, β-actin reverse 5′-CTGGGGTG TTGAAGGTCTC-3′.

**Western Blot Analysis**

Matured DCs were washed twice with phosphate-buffered saline and lysed with Lysis Buffer (Beyotime Biotechnology, Haimen, China) on ice for 30 minutes. SDS-PAGE was used to separate the proteins in the lysates, and proteins were then transferred to polyvinylidene difluoride sheets. Sheets were incubated with primary antibodies (Abcam, Cambridge, MA, USA) overnight at 4°C, followed by an incubation with secondary antibodies for 1 hour. The Pierce ECL Western Blotting Substrate (Advanta, Menlo Park, CA, USA) and gel densitometry (Bio-Rad Laboratories) were used to develop and then quantitate the protein bands.

**Transduction**

The plasmid vector pHBAD-EFl1-MCS-3flag-CMV-GFP was used for carrying the target gene DAB2 (NM 001343) in the adenoviruses. DAB2-containing and blank plasmid vector adenoviruses (Hanbio, Shanghai, China) were transduced into DCs at 50, 100, 200, 300, 400, or 500 multiplicity of infection (MOI) on the third and fourth day. The medium was changed after an incubation at 37°C in 5% CO2 for 6 hours. On the sixth day, LPS was added to stimulate imDCs into mDCs. The optimal MOI (200) was chosen for subsequent experiments, according to the results of fluorescence microscopy and flow cytometry (Supplementary Fig. S1). The mDCs and supernatants after transduction were collected for flow cytometry, Western blot analysis, and ELISA.

**Flow Cytometry**

DAB2-overexpressing and control DCs were collected separately and incubated with anti-human CD40 (peridinin-chlorophyll-cyanin5.5; BioLegend, San Diego, CA, USA), CD80 (allophycocyanin; BioLegend), CD83 (phycoerythrin-cyanin7; BioLegend), CD86 (allophycocyanin; BioLegend), and HLA-antigen D related (HLA-DR; phycoerythrin-cyanin5; BioLegend) at 4°C for 30 minutes. The CD4+ cells that were cocultured with mature DAB2-overexpressing and control DCs were incubated with ionomycin (1 μg/ml; Sigma-Aldrich Corp., St. Louis, MO, USA) and phorbol 12-myristate 13-acetate (100 ng/ml; Sigma-Aldrich Corp.) for 1 hour at 37°C. Then, brefeldin A (10 μg/ml; Sigma-Aldrich Corp.) was added for another 4 hours. CD4+ cells were incubated with anti-human IL-17A (phycoerythrin; eBioscience, San Diego, CA, USA) and anti-human IFN-γ (phycoerythrin-cyanin7, eBioscience). Fluorescence-activated cell sorting (BD Biosciences, Franklin Lakes, CA, USA) was used to detect the median fluorescence intensity changes of the DC markers and the frequency of CD4+ IL-17A+ and CD4+ IFN-γ+ cells. The results were analyzed by FlowJo software (Tree star, Ashland, OR, USA).
Enzyme-Linked Immunosorbent Assay

The protein levels of IL-1β, IL-6, TNF-α, IL-17, and IFN-γ in culture supernatants were measured by human Duoset ELISA development kits (R&D Systems, Minneapolis, MN, USA). The IL-12p70 high sensitivity ELISA kit (eBioscience) was used to detect the concentration of IL-12p70.

Statistical Analysis

The results were analyzed by SPSS v.17.0 statistical software and GraphPad Prism v.5.0 software. Data are shown as mean ± standard deviation (SD). The normal distribution of the data was tested by the Shapiro-Wilk normality test, and the homogeneity of variance was tested by the F-test. The statistical significance between two groups was analyzed by independent-sample t-test, Mann-Whitney U test, Wilcoxon rank sum test, or paired samples t-test. Data were corrected for multiple comparisons with the Bonferroni correction method. A (corrected) P value less than 0.05 was considered significant.

RESULTS

Decreased mRNA and Protein Expression of DAB2 in DCs from Active VKH Patients

Previous studies suggested that DAB2 plays a role during oncogenesis and regulates immunogenicity of DCs as well as macrophage polarization.16,21 The role of DAB2 in autoimmune diseases is not yet clear however. To investigate the role of DAB2 in VKH, we first measured the mRNA and protein levels of DAB2 in monocyte-derived DCs from normal controls and active VKH patients. The results showed that mRNA expression of DAB2 in DCs was significantly decreased in active VKH patients as compared with normal controls (Fig. 1A). Protein expression in DCs obtained from active patients was also decreased (Figs. 1B, 1C). In a subsequent experiment, we investigated the expression levels of DAB2 in DCs from patients in whom the disease was controlled after having received treatment. The mRNA and protein expression were increased in inactive VKH patients when compared with active patients (Figs. 1D, 1E, 1F).

Effect of Promoter Hypermethylation on the Expression of DAB2 in Active VKH Patients

To study the reason for the decreased DAB2 expression in DCs, we measured the promoter methylation level of DAB2 in these cells. We analyzed the first exon of the DAB2 gene at the CpG island locations between −300 and +200 from the transcription initiation site. The methylation levels of CpG-3.4.5.6, CpG-7.8.9, CpG-18, and CpG-20 in active VKH patients were higher than in normal controls. After Bonferroni correction, only the increased CpG-18 methylation status remained significant (Table; Fig. 2A). To further analyze the relationship between the methylation level with the expression of DAB2 in DCs, we also investigated a group of inactive VKH patients. This experiment showed that only the methylation status of the CpG-3.4.5.6 (Fig. 2B) unit was decreased compared with the active patients following Bonferroni correction for multiple comparisons.

Effect of DAB2 Overexpression on Immune Surface Markers of DCs Obtained from Active VKH Patients

To further study the role of DAB2 in VKH disease, DAB2 was overexpressed in DCs obtained from active patients by using adenovirus transduction. Flow cytometry was used to examine CD40, CD80, CD83, CD86, and HLA-DR surface markers. The expression of CD40, CD83, and CD86 was inhibited by DAB2 overexpression, but the expression of CD80 and HLA-DR was not affected (Fig. 3). After multiple comparison correction, only CD86 still showed a statistically significant difference.
TABLE. Promoter Methylation Levels of the DAB2 Gene in Active VKH Patients and Normal Controls

<table>
<thead>
<tr>
<th>CpG Unit</th>
<th>VKH</th>
<th>Normal Controls</th>
<th>P Value (P &lt; 0.05)</th>
<th>Pc Value (P &lt; 0.005)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CpG-1.2</td>
<td>0.0243 ± 0.0150</td>
<td>0.0250 ± 0.0134</td>
<td>0.6473</td>
<td>0.6473</td>
</tr>
<tr>
<td>CpG-3.4.5.6</td>
<td>0.1593 ± 0.0954</td>
<td>0.1343 ± 0.0122</td>
<td>0.0149*</td>
<td>0.0149</td>
</tr>
<tr>
<td>CpG-7.8.9</td>
<td>0.0529 ± 0.0120</td>
<td>0.0456 ± 0.0101</td>
<td>0.0360*</td>
<td>0.0360</td>
</tr>
<tr>
<td>CpG-11.12</td>
<td>0.0307 ± 0.0121</td>
<td>0.0300 ± 0.0218</td>
<td>0.7266</td>
<td>0.7266</td>
</tr>
<tr>
<td>CpG-13.14</td>
<td>0.0486 ± 0.0146</td>
<td>0.0550 ± 0.0250</td>
<td>0.5591</td>
<td>0.5591</td>
</tr>
<tr>
<td>CpG-15.16.17</td>
<td>0.0357 ± 0.0165</td>
<td>0.0356 ± 0.0191</td>
<td>0.7530</td>
<td>0.7530</td>
</tr>
<tr>
<td>CpG-18</td>
<td>0.1886 ± 0.0300</td>
<td>0.1550 ± 0.0271</td>
<td>0.0046**</td>
<td>0.0046</td>
</tr>
<tr>
<td>CpG-20</td>
<td>0.0843 ± 0.0477</td>
<td>0.0650 ± 0.0401</td>
<td>0.0293*</td>
<td>0.0293</td>
</tr>
<tr>
<td>CpG-21</td>
<td>0.1179 ± 0.0451</td>
<td>0.1244 ± 0.0433</td>
<td>0.7618</td>
<td>0.7618</td>
</tr>
<tr>
<td>CpG-22.23</td>
<td>0.0686 ± 0.0235</td>
<td>0.0671 ± 0.0295</td>
<td>0.6921</td>
<td>0.6921</td>
</tr>
</tbody>
</table>

The unpaired t-test and Mann-Whitney U test were used to compare the difference between active VKH patients and normal controls (significance level at 5% *P < 0.05, **P < 0.01; significance level at 0.5% *Pc < 0.005 by Bonferroni correction).

Effect of DAB2 Overexpression in DCs from Active VKH Patients on the Production of IL-6, TNF-α, and IL-12p70

To investigate the effects of inflammatory cytokine secretion after DAB2 overexpression in DCs, the production of DC-related inflammatory factors, such as IL-1β, IL-6, TNF-α, and IL-12p70, were measured by ELISA. The data showed that IL-6, TNF-α, and IL-12p70 were decreased in DAB2 overexpressing cells as compared with the controls. However, the concentration of IL-1β was not affected. After multiple comparison correction, the IL-6, TNF-α concentration still showed a statistically significant difference (Fig. 4A, 4B) and the IL-12p70 and IL-1β did not (Figs. 4C, 4D).

DAB2 Overexpression in DCs from Active VKH Patients Negatively Regulates Th1 and Th17 Differentiation

DCs have a strong antigen-presenting function and can stimulate Th cell differentiation. To investigate whether overexpression of DAB2 in DCs can regulate the differentiation of Th1/Th17 cell subsets, we cocultured DAB2 overexpressing DCs or control DCs with CD4+ T cells. The results showed that the frequency of IL-17A- and IFN-γ-positive CD4+ T cells were decreased when cocultured with DAB2 overexpressing DCs (Figs. 5A, 5B, 5C). The production of IL-17 and IFN-γ in coculture supernatants was also tested and both showed a lower concentration in the presence of DAB2 overexpressing DCs (Fig. 5D).

Autophagy Protein LC3 and P62 Expression Were Not Affected by the Upregulation of DAB2 Expression

Autophagy can directly affect the maturation of DCs, and DAB2 has been mentioned as an inhibitor of autophagy. To investigate the role of DAB2 in the autophagy of DCs, we used Western blots to measure the autophagy proteins LC3 and P62 in DCs overexpressing DAB2. The data did not show an effect of DAB2 on the expression of both autophagy proteins tested (Fig. 6A, 6B, 6C).

DISCUSSION

This study shows that the DAB2 expression in DCs from patients with active VKH was significantly decreased when compared with healthy controls. We hypothesized that a low expression of DAB2 in DCs may contribute to the pathogenesis of VKH and found that the methylation level of DAB2 was only marginally increased in active patients. Investigation of VKH patients in whom the inflammation was controlled showed that the mRNA and protein expression were increased and that only one site in the promoter region was demethylated. Whether hypermethylation plays a significant role in the low expression of DAB2 is therefore not yet clear, and other unknown factors may also be involved. The relationship between DAB2 methylation and expression levels have been reported in several tumors, but this is the first report investigating the role of this protein in a clinical autoimmune disease.

The role of DAB2 has been studied earlier in experimental models of autoimmune disease. Our data in humans are, however, in disagreement with studies where it was shown that DAB2 is upregulated in macrophages/microglia in the inflamed spinal cord of mice and rats undergoing murine experimental autoimmune encephalomyelitis, a model of multiple sclerosis. These authors also showed that DAB2 expression is positively correlated with disease severity and that DAB2-deficient mice have a milder form of the disease than control animals. The discrepancy may be due to different types of cells investigated, macrophages versus DCs, or a species effect.

To investigate the potential roles of DAB2 in DCs from active VKH patients, we overexpressed DAB2 to investigate its effect on DC function. The results showed that the expression...
of DC costimulatory molecules, such as CD40, CD83, and CD86, were reduced. These molecules can activate the TCR signaling pathway and T-cell differentiation. We also tested the effect of DAB2 overexpression on the release of inflammatory cytokines, such as IL-6, IL-1β, IL-12p70, and TNF-α. In agreement with an earlier study, IL-6 and IL-12p70 protein were decreased, although no effect was observed on the release of the IL-1β protein. However, the expression of TNF-α was also decreased in our study, which was different from data reported in mouse bone marrow–derived DCs. This discrepancy may be due to the different effects of DAB2 on mouse bone marrow–derived DCs compared with its effect on human monocyte–derived DCs.

The activation of Th1 and Th17 lymphocyte subsets plays an important role in several autoimmune diseases, including VKH. According to a previous study from our lab, inhibiting the hyperactivity of Th1 and Th17 responses effectively controls ocular inflammation in patients with active VKH. As mentioned above, DC activity directly affects pathogenic Th1 and Th17 cell differentiation, as identified by the secretion of IL-17 and the IFN-γ protein. Having defined the possible role of DAB2 as a suppressor of DC activity, we also investigated the possible influence of DAB2 overexpression in DCs on the differentiation of Th1 and Th17 cells. Our data show that when naïve CD4+ T cells were cocultured with DAB2 overexpressing DCs, the Th1 and Th17 response was significantly decreased. Supernatants from CD4+ T cells cocultured with DAB2 overexpressing DCs showed low levels of IL-17A and IFN-γ. These results collectively support the powerful inhibitory effect of DAB2 upregulation on the activity of DCs obtained from active VKH patients.

NF-κB and mitogen-activated protein kinase are two important pathways related to inflammation, and DAB2 has been reported to have an effect on these pathways. Moreover, an increased DAB2 expression regulates the maturation of DC cells by inhibiting the Src/P38K/Akt-NF-κB pathway. Src, P38K, and Akt are key genes affecting autophagy, and autophagy has been implicated in the antigen presentation ability and cytokine response of DCs. Earlier reports showed that DAB2 was found to be an autophagy inhibitor by blocking the interaction between vacuolar protein sorting 34 and beclin-1. To find out whether DAB2 affects the function of DCs through autophagy, we measured the autophagy proteins LC3 and P62 after overexpression of DAB2 in DCs but did not find a difference compared with controls. These findings suggest that the mode of action of DAB2 overexpression in patients with active VKH downregulated the expression of CD40, CD83, and CD86 in DCs. After being corrected with multiple comparison correction, only CD86 still had significance. (A) Overlapping histograms from a representative experiment of DAB2-overexpression or controls in active VKH patients. (B) The difference in mean fluorescence intensity (MFI) between DC surface markers in DAB2-overexpressing cells compared with cells transduced with control adenovirus. The data were analyzed with the paired samples t-test (CD40, CD83, HLA-DR) and Wilcoxon rank sum test (CD80, CD86) (n = 10, *P < 0.05, **P < 0.01, *P < 0.01 by multiple comparison correction).

**Figure 3.** DAB2 overexpression in patients with active VKH downregulated the expression of CD40, CD83, and CD86 in DCs. CD80 and HLA-DR expression were not affected. After being corrected with multiple comparison correction, only CD86 still had significance. (A) Overlapping histograms from a representative experiment of DAB2-overexpression or controls in active VKH patients. (B) The difference in mean fluorescence intensity (MFI) between DC surface markers in DAB2-overexpressing cells compared with cells transduced with control adenovirus. The data were analyzed with the paired samples t-test (CD40, CD83, HLA-DR) and Wilcoxon rank sum test (CD80, CD86) (n = 10, *P < 0.05, **P < 0.01, *P < 0.01 by multiple comparison correction).

**Figure 4.** DAB2 over-expressing DCs from active patients reduced the expression of inflammatory factor IL-6, TNF-α, and IL-12p70, except for IL-1β. After correction for multiple comparisons, the IL-6, TNF-α still remained significantly different. The difference in expression of (A) IL-6, (B) TNF-α, (C) IL-1β, and (D) IL-12p70 in DCs from active patients between DAB2 overexpressing and adenovirus control DCs. The paired samples t-test (D) and Wilcoxon rank sum test (A–C) were used in this figure (n = 12, *P < 0.05, ***P < 0.001, *P < 0.0125).
DAB2 might be different in DCs as compared with other cell types used in previous studies.36 It is essential to point out that our study has a number of limitations. First of all, it is often difficult to obtain sufficient numbers of active VKH patients that are not yet undergoing systemic immunosuppressive treatment, which might affect the outcome of the experiments. VKH has multiple disease phases, each having a different type of disease activity, and it is not clear whether the immune mechanisms driving the acute uveitic, chronic/convalescent, and recurrent phases are necessarily the same. For this study, we used patients with active uveitis irrespective of the stage of the disease. Further longitudinal studies in a cohort of well-defined patients are needed to examine the effects of immunosuppressive therapy and control of the inflammation on the expression and methylation level of DAB2. We only tested a limited number of parameters concerning the effect of DAB2 on DC function and found a weak correlation between the methylation status with DAB2 expression. Further experiments investigating the role of specific methylation sites on gene expression or using specific methylation inhibitors are needed to address this issue. Although the effects of DAB2-silencing in mouse DCs have been studied thoroughly, the role of DAB2 inhibition on human DCs is still largely unknown and also deserves further investigation.

In summary, our data showed a decreased level of DAB2 in DCs obtained from patients with active VKH. Overexpression of DAB2 in DCs not only inhibited inflammatory cytokine production and immune surface marker expression on DCs but also controlled Th1 and Th17 cell differentiation. Further studies are needed to examine whether pharmacologic control of DAB2 expression may ameliorate autoimmune diseases, such as VKH.

**FIGURE 5.** The frequency of CD4⁺IL-17⁺ and CD4⁺IFN-γ⁺ cells were significantly lower in T cells cocultured with DAB2-overexpressing DCs than adenovirus controls (DCs from active VKH patients). IL-17 and IFN-γ secreted in DAB2 overexpressing cocultures were also decreased significantly. A representative experiment of (A) CD4⁺IL-17⁺ (B) and CD4⁺IFN-γ⁺ T cells cocultured with DAB2-overexpressing or adenovirus control DCs. (C) The difference in frequencies of CD4⁺IL-17⁺ and CD4⁺IFN-γ⁺ T cells were evaluated by flow cytometry (n = 8, *P < 0.05, **P < 0.01). (D) The protein concentration of IL-17 and IFN-γ in supernatants were decreased after coculture with DAB2-overexpressing DCs. The paired samples t-test and Wilcoxon rank sum test were used for statistical analysis (n = 11, *P < 0.05).

**FIGURE 6.** The autophagy of DCs was not significantly different in DAB2 overexpressing DCs when compared with controls. (B) The autophagy proteins LC3 and P62 expression were normalized to GAPDH. The density ratio of (A) LC3B-II/LC3B-I and (C) P62 in DCs with DAB2 overexpression or controls. The results were analyzed with the paired samples t-test (n = 6, ns > 0.05).
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