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Tmem176B and Tmem176A are associated with the immature state of dendritic cells

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ABSTRACT

DCs play a central role in the development of innate and adaptive immunity but also in the induction and maintenance of immune tolerance. Identification of factors that govern DC activation, their maturation state, and their capacity to induce proinflammatory or tolerogeneic responses therefore represents a crucial aim of research. We previously identified a new molecule, Tmem176B (which we named TORID initially), as highly expressed in a model of allograft tolerance in the rat. We showed that its overexpression in rat DCs blocked their maturation, suggesting a role for this molecule in the maturation process. To characterize the function of Tmem176B further, we used a split-ubiquitin yeast, two-hybrid system to identify interacting partners and found that Tmem176B associated with itself but also with Tmem176A, a membrane protein similar to Tmem176B. Interestingly, these two molecules showed similar mRNA expression patterns among various murine tissues and immune cells and were both down-regulated following DC maturation. In addition, we showed that in using RNAi, these molecules are both involved in the maintenance of the immature state of the DCs. Taken together, these data suggest that Tmem176B and Tmem176A associate to form multimers and restrain DC maturation. Therefore, these two molecules may represent valid targets to regulate DC function. *J. Leukoc. Biol.* 88: 507–515; 2010.

Introduction

DCs represent professional APCs able to respond to danger signals and to induce efficient, naïve T cell activation. The immunostimulatory function of DCs can vary depending on their subtypes, their activation and maturation state, and the microenvironment within which they are located [1]. Immature DCs, on exposure to microbial products or proinflammatory cytokines, up-regulate costimulatory molecules, secrete cytokines, and elicit strong T cell priming. In contrast, in the absence of inflammatory signals or in the presence of immunomodulatory agents, such as IL-10 or TGF-β, DCs remain in an immature or resting state, characterized by low expression of costimulatory molecules and inflammatory cytokines. This "immature" state contributes to the silencing of antigen-specific T cells or the differentiation of regulatory T cells [2]. The comprehension of the mechanisms involved in the functional differentiation of immunostimulatory or tolerogeneic DCs represents a crucial aim of research to develop strategies to manipulate an immune response toward immunity or tolerance [3]. We previously identified an "unknown" molecule, Tmem176B (which we called TORID initially), as overexpressed by myeloid cells infiltrating a tolerated heart allograft in the rat [4]. We demonstrated that Tmem176B expression was strictly associated with the immature state of the DCs and that its overexpression by viral transduction decreases their maturation status and renders the cells resistant to the maturation induced by LPS [4]. These results suggested a role for Tmem176B in the immature state of the DCs. The human hortholog of Tmem176B (previously named LR8) was found initially to be expressed in a lung fibroblast subpopulation expressing the receptor for the collagen domain of the C1q molecule, and its mouse hortolog (previously named Clast1) was identified as up-regulated in B cells after stimulation by CD40 ligand [5]. Tmem176B KO mice have been generated and described to develop ataxia sporadically, as a result of a defect in cerebellar granules [6]. However, the function of Tmem176B remains unknown, and no study about immune cells was reported.

Abbreviations: AU=arbitrary unit(s), BMDC=bone marrow-derived dendritic cell, cDC=conventional dendritic cell, Cub=C-terminal half of ubiquitin, DC=dendritic cell, HPRT=hypoxanthine-guanine phosphoribosyltransferase, KO=knockout, MS4A=membrane-spanning four-domains subfamily, NCBI=National Center for Biotechnology Information, NubG=N-terminal half of ubiquitin, pDC=plasmacytoid dendritic cell, poly I:C=polyinosinic:polycytidylic acid, qRT-PCR=quantitative RT-PCR, RNAi=RNA interference, TLR4-L=TLR4-ligand

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TABLE 1. Oligonucleotides Used in This Study

5′ to 3′ Oligonucleotide sequences		
Mice HPRT	Forward	CGTGATTAGCGATGATGAACC
	Reverse	ATCCAGCAGGTCAGCAAAGA
Mice Tmem176B	Forward	CATCAGCATCCACATCCACC
	Reverse	ACTCCAGCTAGAATTGCCACAG
Mice Tmem176A	Forward	TCCTTCACAAGAAACGGGGT
	Reverse	TCTTCGGGAACTGGAGTGGT

In this study, we investigated possible interacting partners of Tmem176B and show that Tmem176B can interact with itself and with Tmem176A, a very similar protein. Therefore, we analyzed expression and regulation of these two molecules in mice and their role in DC maturation. Indeed, the molecular mechanisms by which modulation of DC maturation can occur are important to decipher to offer new targets to modulate an immune response.

MATERIALS AND METHODS

Animals

Six- to 8-week-old C57Bl/6 and Balb/c mice were obtained from the Centre d'Elevage Janvier (Le Genest-Saint-Isle, France) and maintained in an animal facility under standard conditions, according to our institutional guidelines.

Reagents and flow cytometry

Cells were grown in RPMI 1640 (Gibco, Grand Island, NY, USA), supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine (Gibco), and 50 μm 2-ME (Sigma-Aldrich, St. Louis, MO, USA). Purified, unlabeled FITC-, PE-, PerCp-Cy5.5-, Pe-Cy7-, or allophycocyanin-labeled antibodies were purchased from BD Biosciences (San Diego, CA, USA) and include anti-CD45R (B220), anti-CD3, anti-CD11c, anti-class II MHC (I-A^b), anti-CD11b (Mac1), anti-Gr1, anti-DX5, anti-CD25, anti-CD4, anti-CD8, anti-CD80, anti-CD86, anti-CD40, and anti-class I MHC. Cells were sorted on a FACSAria (BD Biosciences) or analyzed on a FACS LSRII (BD Biosciences) using FlowJo software (Tree Star, Ashland, OR, USA).

Generation of BMDCs

BMDCs were generated using the method described by Lutz et al. [7]. Briefly, C57Bl/6 marrow was sieved through a 100-μm nylon mesh in complete medium, red cells lysed using ammonium chloride, and cells plated at 2×10^6/10 cm bacteriological petri dishes in complete medium, supplemented with 10 ng/ml murine rGM-CSF, supplied as a culture supernatant. Medium (10 mL) was added on Day 3, and half of the medium was replaced on Day 6. Finally, BMDCs were harvested on Day 8 by gentle pipetting. For maturation, 0.1 μg/ml LPS (Sigma-Aldrich) or 12.5 μg/mL poly I:C (Sigma-Aldrich) was added on Day 8, and cells were harvested after 10 or 24 h of stimulation.

Cell separation

Lymphoid cells

Cell suspensions from spleens were subjected to ammonium chloride to lyse erythrocytes and then labeled using the appropriate antibodies. $CD4^+$ T cells ($CD3^+$ $CD4^+$ $CD8^-$), $CD8^+$ T cells ($CD3^+$ $CD4^-$ $CD8^+$), NK cells ($CD3^-$ $DX5^+$), and B cells ($CD3^-$ $CD45R^+$) were then purified from total spleen by flow cytometry.

Myeloid cells

Cell suspensions from spleens were subjected to ammonium chloride to lyse erythrocytes, and then, T and B cell depletion was achieved by using anti-CD3 and anti-CD19 antibodies and anti-rat IgG-coated magnetic beads (Dynal, Oslo, Norway), according to the manufacturer's recommendations. cDC (CD11c Hi), pDC (CD11c Lo $CD45R^+$), granulocytes ($Gr1^+$ $CD11b^+$ $CD45R^-$), and monocytes/macrophages ($CD11b^+$ $Gr1^-$ $CD45R^-$) were then purified by flow cytometry. The purity of the sorted cells was >98%.

Split-ubiquitin yeast two-hybrid system

A regulatory T cell yeast split-ubiquitin library, derived from cDNA of peptide and TGF-β-stimulated A1.$RAG^{-/-}$ T cells, was a gift of Prof. Herman Waldmann (Oxford University, UK). The split-ubiquitin yeast two-hybrid system has been performed as described previously [8]. Briefly, the library "DBYT.IL2/NubG-x" was directionally cloned into the library vector pPR3-N (Dualsystems Biotech, Zurich). The bait construct was made by amplification of the coding sequence of Tmem176B by RT-PCR from RNA derived from mouse splenocytes using the primers in **Table 1**. This was cloned into the bait vector pBT3-STE (Dualsystems Biotech). Bait and library constructs were transformed into the yeast strain NMY51 [MATa his3δ200 trp1-901 leu2-3,112 ade2 LYS2::(lexAop)4-HIS3 ura3::(lexAop)8-LacZ ade2::(lexAop)8-ADE2 GAL4] using standard procedures. The pro-

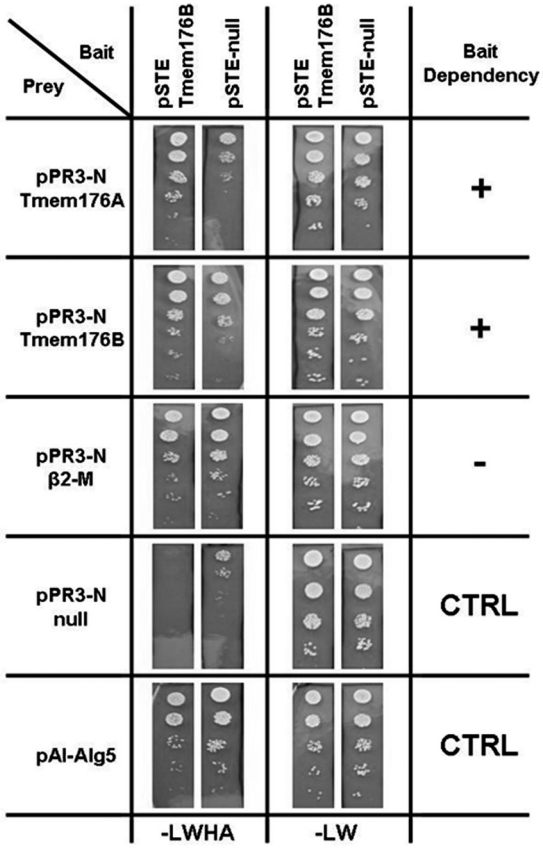

Figure 1. Interacting partners of Tmem176B. Three clones, Tmem176A, Tmem176B, and β2-microglobulin (β2-M), were selected from the screening of a split-ubiquitin yeast two-hybrid system and subjected to a bait-dependency test to confirm the interaction as described in Materials and Methods. Representative pictures illustrate the bait tests for Tmem176B, Tmem176A, β2-microglobulin, vector null, and pAl-Alg5. Data are representative of three independent experiments. CTRL, Control.

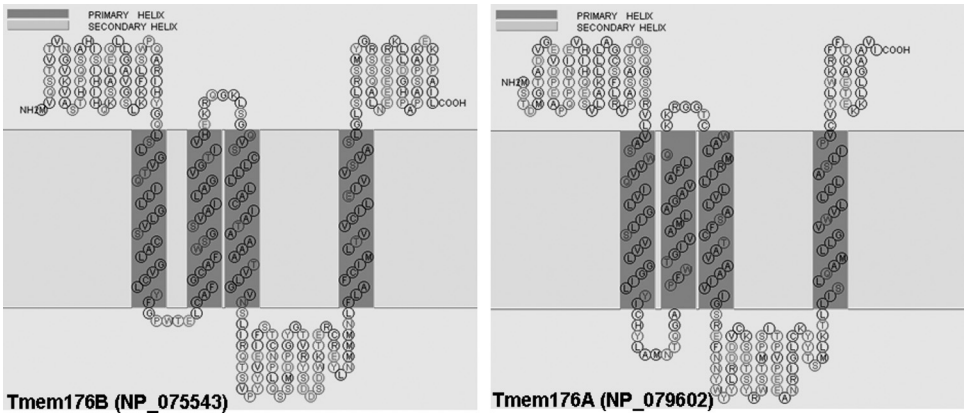

Figure 2. Comparison of Tmem176B and Tmem176A structures. Schematic representations of the amino acid sequence of Tmem176B and Tmem176A in mice using SOSUI representation (http://bp.nuap.nagoya-u.ac.jp/sosui/), showing N- and C-terminal orientation, the predicted transmembrane domains based on hydropathy profiles.

tein of interest, Tmem176B (the bait), was fused to the Cub and the artificial transcription factor LexA-VP16. Protein of the library (the prey) was fused to the mutated NubG. When bait and prey interact together, the NubG and Cub are forced into close proximity, resulting in the reconstitution of split-ubiquitin, which is recognized immediately by a ubiquitin-specific protease, which then cleaves the polypeptide chain between Cub and LexA-VP16. As a result, the artificial transcription factor is released from the membrane and translocated to the nucleus, where it binds to the LexA operators situated upstream of a reporter gene via its LexA DNA-binding domain. Several resistant, positive clones were selected and sequenced. The resulting nucleotide sequences were analyzed using NCBI NBLAST. Transformants were grown on selective medium lacking leucine, tryptophan, histidine, and adenine, with addition of 20 mM 3-amino-1,2,4-triazole. Positive clones were sequenced by colony PCR using the primer set pPR3NFOR, 5′-GTCGAAAATTCAAGACAAGG-3′, and pPR3NREV, 5′-AAGCGTGACATAACTAATTAC-3′. Library plasmids were isolated from positive clones and retransformed into NMY51 to test bait dependency. Prey cDNAs, which activated the histidine and adenine reporters in the presence of Tmem176B and not pCCW-Alg5 or pBT3STE (negative control vectors), were considered true interactors.

Transfection of BMDC with stealth RNAi duplexes, activation, and MLR

Two nonoverlapping "stealth RNAi" (Stealth™ select RNAi, Invitrogen Life Technologies, Carlsbad, CA, USA) duplexes, specific for Tmem176B and

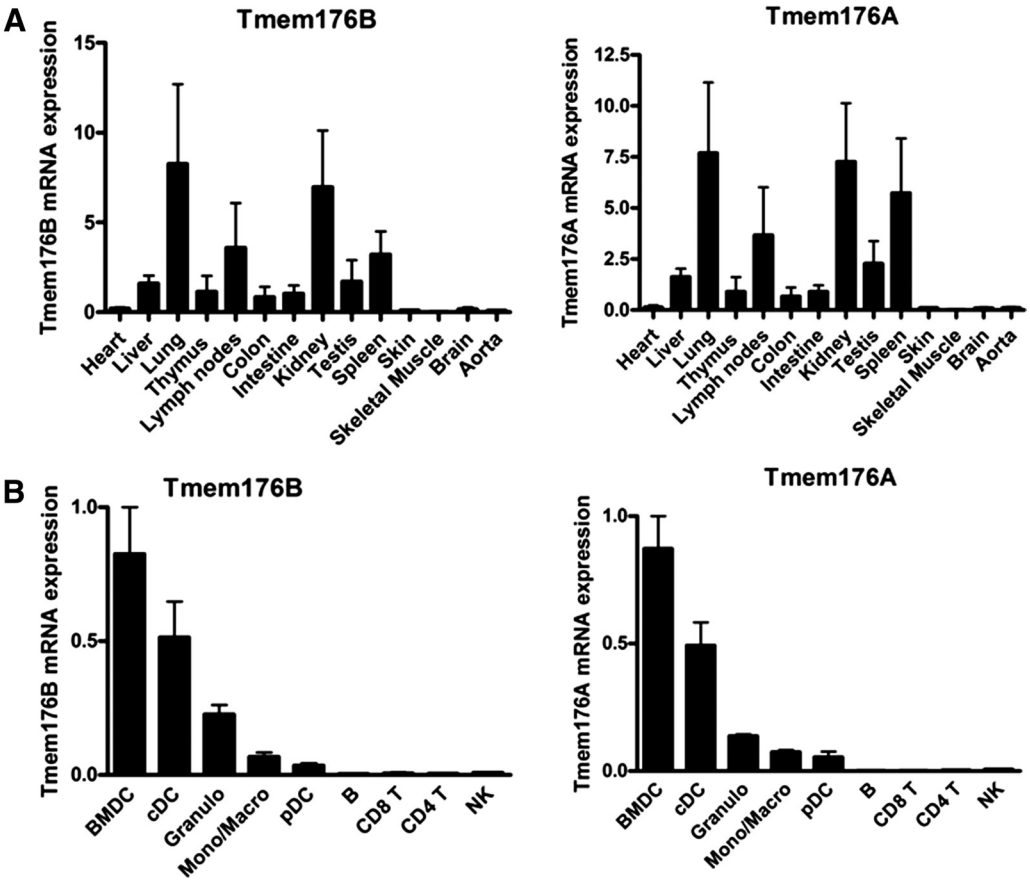

Figure 3. Expression of Tmem176B and Tmem176A in different organs and cell subtypes from naïve mice. (A) Tmem176B and Tmem176A expression was assessed by qRT-PCR in different organs from naïve mice; $n = 4$ in each group. Results are expressed in AU of Tmem176B or Tmem176A, normalized to HPRT ± SEM, compared with the reference thymus (value=1), or B-Tmem176B and Tmem176A expression was assessed by qRT-PCR in different, highly purified cells from naïve mice (n=3). Granulo, Ganulocytes; Mono/Macro, monocytes/macrophages; B, B cells; CD8 T or CD4 T, $CD8^+$ or $CD4^+$ T cells. Results are expressed in AU of Tmem176B or Tmem176A normalized to HPRT ± SEM.

Tmem176A, were synthesized commercially by Invitrogen Life Technologies. Stealth Tmem176B RNAi 1 (5′-UGCAAUCAAGCAUGGACCCUGGGUA-3′), Tmem176B RNAi 2 (5′-CGGCCAAAGAGAAGAUCCCUGCUAU-3′), Tmem176A RNAi 1 (5′-GGACCCUUCUUGUGCUGGCAAGUUU-3′), and Tmem176A RNAi 2 (5′-GACUCUUCAUAUGGAUGGUCCACCA-3′) were designed to target different coding regions of the respective mouse mRNA sequence (GenBank Accession Nos. NM_023056 and NM_025326, respectively, at http://www.ncbi.nlm.nih.gov/GenBank/). A BLAST (NCBI database) search was carried out to confirm that the only targets of the two stealth RNAi duplexes were these molecules. At Day 8 of culture, 2 million adherent C57Bl/6 BMDCs were transfected with lipofectamine RNAiMAX (Invitrogen Life Technologies) and with 200 pmol control RNAi (medium GC content Stealth RNAi negative universal control, Invitrogen Life Technologies) or with RNAi specific for Tmem176B and Tmem176A. Cells were stimulated or not with poly I:C (12.5 μg/ml; Sigma-Aldrich) for 48 h.

Two days following transfection, BMDCs were harvested and stained using anti-CD11c, class I MHC, class II MHC, CD80, CD86, or CD40 mAb for flow cytometry analysis or were plated with Balb/C lymph node-derived T cells in complete RPMI medium (ratio 1:4). Alternatively, Balb/C lymph node-derived T cells were labeled with CFSE (Molecular Probes, Eugene, OR, USA). Three days later, cells were pulsed for 8 h with 0.5 μCi/well methyl-[3H] thymidine (Amersham, Les Ulis, France), and thymidine incorporation was measured using a scintillant counter (Top Count NXT, Perkin Elmer, Waltham, MA, USA). CFSE-labeled cells were stained with anti-TCR, anti-CD4, and anti-CD25 antibodies and were analyzed by flow cytometry at Day 3 of culture.

RNA extraction and real-time qRT-PCR

Total RNA from tissues or cells was prepared using Trizol (Invitrogen Life Technologies), according to the manufacturer's instructions. Real-time qPCR was performed as described previously using a GenAmp 7700 sequence detection system and SYBR Green PCR master mix (Applied Biosystems, Applera, Courtaboeuf, France) [9]. The oligonucleotides used in this study are described in Table 1. HPRT was used as endogenous control genes to normalize for variations in the starting amount of RNA. Relative expression was calculated using the 2-ΔΔ comparative threshold method and expressed in AU [9].

Cytokine assays

Supernatants from cultures were harvested at 48 h for BMDCs transfected with control RNAi or specific Tmem176B or Tmem176A RNAi. Cytokines were measured by a murine ELISA set (BD Biosciences), according to the manufacturer's instructions.

Statistical analysis

Statistical evaluation was performed using the Student's *t*-test for unpaired data, and results were considered significant if *P* values were <0.05. Data are expressed as mean ± SEM.

RESULTS

Determination of interacting partners of Tmem176B

We described previously Tmem176B as being highly expressed by immature myeloid cells in rat and human, but nothing was known about the signaling of this molecule or its interaction with other proteins. To determine interacting partners of Tmem176B, we applied a split-ubiquitin yeast two-hybrid system using Tmem176B as bait against a murine expression library. Among the positive clones (~150), which we obtained by this technique, we identified the cDNA encoding for Tmem176A (in 29% of the clones), for the β2-microglobulin (in 11% of the clones), and for Tmem176B (in 3% of the clones). The other cDNA sequenced were identified in <1% of the clones and were therefore considered as false-positives (data not shown). Then, we applied a bait dependency test to confirm the interaction of Tmem176B with Tmem176A with the β2-microglobulin and with itself. As shown in **Figure 1**, we observed that the growth of the clones on interaction selection media (–LWHA) with pSTE-Tmem176B was high at the third

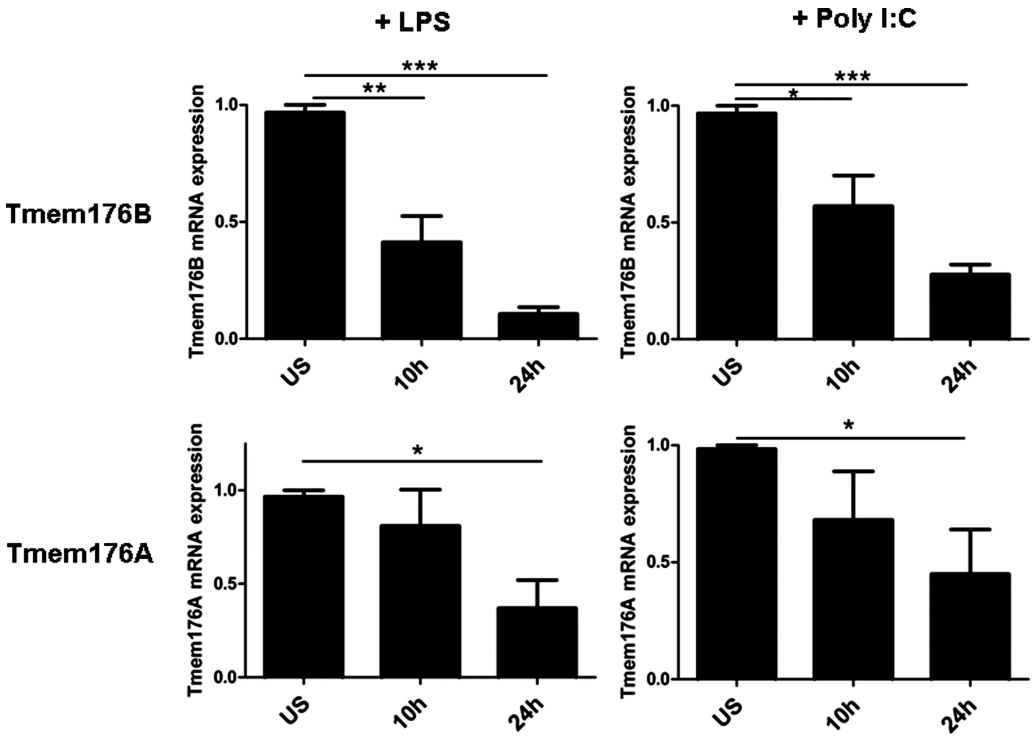

Figure 4. Regulation of Tmem176B and Tmem176A expression in DCs. Mice BMDCs were cultured for 10 or 24 h with TLR4-L (LPS, 0.5 μg/ml) or TLR3-L (poly I:C, 12.5 μg/ml). Tmem176B and Tmem176A expression was assessed by qRT-PCR, and results are expressed in AU of Tmem176B or Tmem176A normalized to HPRT ± SEM, compared with the reference control RNAi (value=1; n=3; *, $P<0.05$; **, $P<0.01$, ***, $P<0.001$). US, Unstimulated.

and fourth dilution rate of the cotransformed yeast for the Tmem176A and Tmem176B tests and was positive compared with the negative control (pSTE null). In contrast, for the β2-microglobulin, it was not more positive at the third/fourth dilution rate than the cotransformation with the negative control (pSTE null). Therefore, these data confirmed, by the bait-dependency test, the interaction of Tmem176B with Tmem176A and with itself, whereas the β2-microglobulin was a false-positive of the screening.

Structures of Tmem176B and Tmem176A

By yeast two-hybrid screening, we identified Tmem176A as a protein interacting with Tmem176B. We have suggested previously that Tmem176B and Tmem176A may belong to the same family of genes, as their structures were very similar (28.5% identity of the protein structure in rat) [4]. Moreover, we have shown that Tmem176B and Tmem176A proteins exhibit some similarities of structure with the proteins of the MS4A family (10–20% identity of the protein structure) with a similar intron/exon organization and the four transmembranous domains [4]. However, unlike Tmem176A, the genes of the MS4A family are not located on the same chromosome as Tmem176B, suggesting that Tmem176B and Tmem176A may in fact constitute a different kind of family than the MS4A family.

In mice, we also observed that Tmem176B and Tmem176A molecules exhibit a similar protein structure with four transmembranous domains [32% of identity using Psi-Blast (NCBI); **Fig. 2**]. Interestingly, the two genes encoding these proteins are located adjacent to each other on the same chromosome but in an opposite direction (5′–3′ sense), suggesting that these two genes are closely related. We demonstrated previously in the rat that Tmem176B was located in intracellular structures of the cells [4].

Taken together, the high similarity of protein structure, the chromosomal colocalization, and the fact that Tmem176B and Tmem176A can form multimers suggested that these molecules may be part of an intracellular, membranous complex.

Expression of Tmem176B and Tmem176A in murine organs and immune cells

Tmem176A was identified previously together with immunity-related genes, as expressed in mouse kidney proximal tubules exposed to proteinuria and as a possible autoantigen in hepatocellular carcinoma [10, 11]. However, no functional studies were described for this molecule.

To better characterize these two molecules, we analyzed their expression in different organs and in different isolated cell subpopulations from naïve mice by qRT-PCR. We observed a high mRNA expression of Tmem176B in lung, kidney, lymph nodes, and spleen, a low expression in liver, thymus, testis, colon, and intestine, and a weak expression in heart, skin, muscle, brain, and aorta (**Fig. 3A**). This profile of expression in mice is similar to the one that we described previously in rat [4]. Interestingly, we observed exactly the same pattern of expression for Tmem176A in the organs, with the highest expression in lung, kidney, and spleen (Fig. 3A).

Similarly, in different immune cell subtypes from naive mice, we observed a high expression of Tmem176B and Tmem176A in BMDCs and in cDC (Fig. 3B). The expression of these two molecules decreases, respectively, in granulocytes, monocytes, and pDCs, and no expression was detected in resting NK, $CD4^+$ T, $CD8^+$ T, or B cells (n=3; Fig. 3B).

Taken together, these data demonstrate in mice, similarly to what we observed previously in rat, that Tmem176B is expressed preferentially in lung, kidney, and spleen and in myeloid cells [4]. Moreover, we showed that Tmem176A, a protein with similar structure, which interacts with Tmem176B, exhibits an identical expression profile.

Regulation of Tmem176B and Tmem176A expression in murine DCs

We demonstrated previously in rat and human that expression of Tmem176B was associated with the immature/resting state

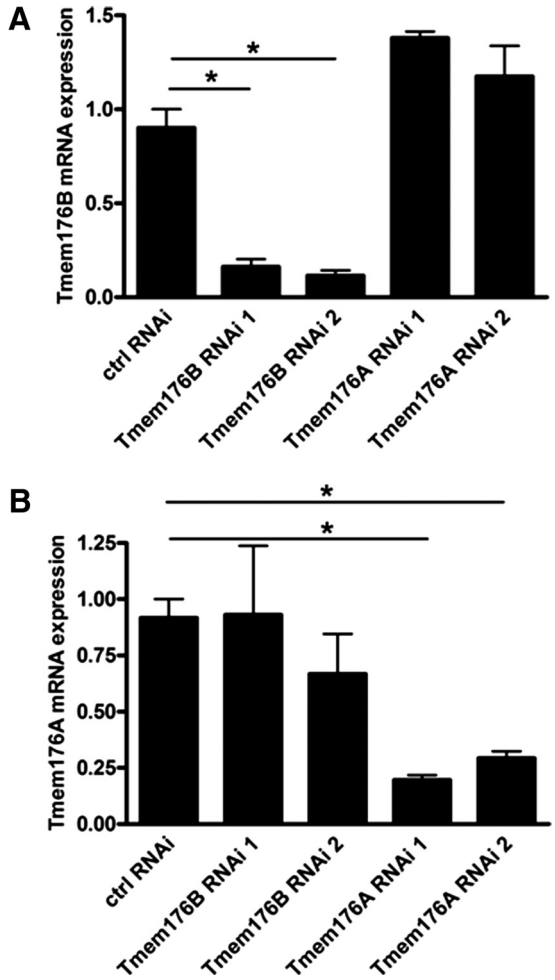

Figure 5. Inhibition of Tmem176AB and Tmem176A by RNAi. Mice BMDCs were transfected with control RNAi or (A) with two Tmem176B- or (B) with two Tmem176A-specific RNAi for 48 h. Tmem176B and Tmem176A expression was assessed by qRT-PCR, and results are expressed in AU of Tmem176B or Tmem176A, normalized to HPRT ± SEM, compared with the reference control RNAi (value=1; n=3; *, P<0.05).

of myeloid cells and that activation with proinflammatory stimuli strongly reduced expression of the transcripts [4]. Here, we investigated the regulation of expression of Tmem176B but also of Tmem176A in mice BMDCs in response to TLR triggering.

Mice BMDCs were stimulated with LPS (TLR4-L) or poly I:C (TLR3-L) for 10 or 24 h, and Tmem176B and Tmem176A mRNA expression was analyzed by qRT-PCR. We observed that LPS or poly I:C stimulation significantly down-regulated the expression of Tmem176B at 10 or 24 h of stimulation (n=3; *, P<0.05; **, P<0.01; ***, P<0.001; **Fig. 4**). Interestingly, we observed that the mRNA expression of Tmem176A was also down-regulated at 24 h of stimulation but at a lower extent than Tmem176B (n=3; *, P<0.05, Fig. 4). These results demonstrate in mice, similar to what we observed previously in rat and human, that Tmem176B expression in DCs is down-regulated following inflammatory stimulation [4]. In addition, we show that expression of its interacting protein, Tmem176A, is down-regulated following inflammatory stimulation.

Tmem176B and Tmem176A do not regulate expression of each other

We then investigated whether expression of one of the molecules regulates the expression of the other. To test this possibility, we used specific reformed-type small interfering RNA (stealth RNAi), which allows long-term and efficient inhibition with low, nonspecific activation [12]. Two nonoverlapping stealth RNAi specific for Tmem176B and Tmem176A (medium GC content) were used as well as a universal stealth control RNAi (medium GC content) in mice BMDCs, and the

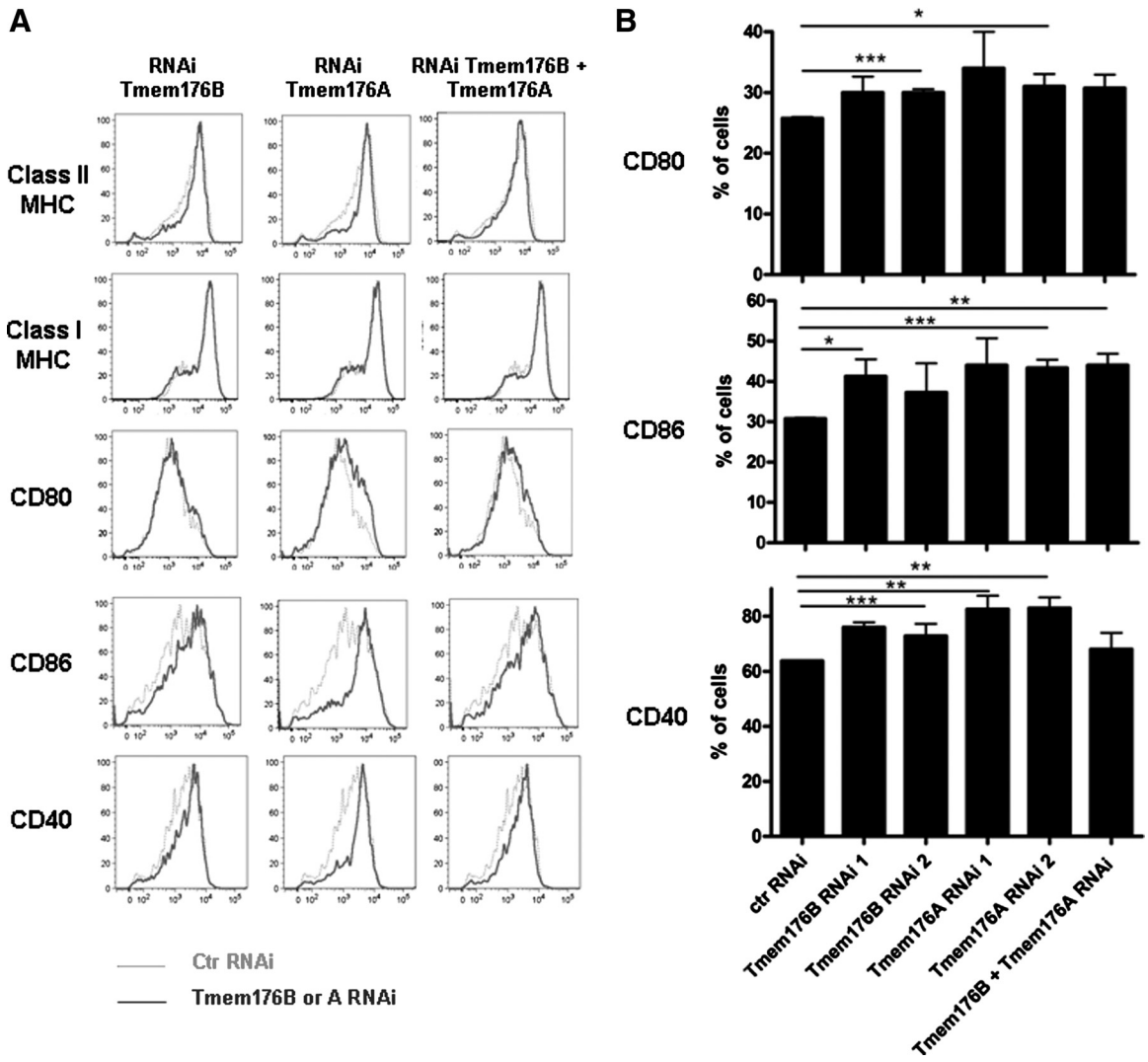

Figure 6. Effect of Tmem176B or Tmem176A inhibition on DC phenotype. Mice BMDCs were transfected with control (ctr) RNAi, with two Tmem176B- or two Tmem176A-specific RNAi, or with a mix of both (Tmem176B+Tmem176A) for 48 h. BMDCs were analyzed by flow cytometry. (A) Representative analysis histograms of BMDCs for class II MHC, class I MHC, CD80, CD86, or CD40. The same data were obtained with the two different RNAi and are representative of three independent experiments. (B) Histograms representing the percentage of BMDCs that express at high level CD80, CD86, or CD40 (n=4; *, P<0.05; **, P<0.01; ***, P<0.001).

level of expression of these molecules was analyzed by qRT-PCR. We observed in **Figure 5** that Tmem176B and Tmem176A RNAi suppressed the expression of their respective mRNA transcripts efficiently (>70% of inhibition; $n=3$; *, $P<0.05$; Fig. 5, A and B, respectively). Moreover, we observed that inhibition of Tmem176B or Tmem176A by specific RNAi did not modulate the respective expression of Tmem176A or Tmem176B in BM-DCs (Fig. 5).

These results demonstrate that Tmem176B and Tmem176A do not regulate expression of each other.

Function of Tmem176B and Tmem176A in mice DCs

To determine the function of these two molecules, we transfected mice BMDCs with two different RNAi, specific for Tmem176B, Tmem176A, or both. We evaluated the effect of inhibition of expression on DC maturation and on the capacity to prime allogeneic T cells. To test the effect on matured DCs, we used poly I:C activation, which in contrast to LPS, decreases at a lower extent the expression of these two molecules (Fig. 4). We observed in immature BMDCs that inhibition of expression of Tmem176B, Tmem176A, or both increased the expression of the costimulatory molecules CD86 and CD40 compared with the control RNAi (**Fig. 6A**, representative histograms; Fig. 6B, histograms representing the percent of cells expressing these molecules at the highest level). No difference was observed in the expression of class II or class I MHC. In contrast, when the cells where matured with poly I:C, which induced an increase in the expression of costimulatory molecules, we did not retrieve the effect of the inhibition of expression of these molecules on the expression of CD80, CD86, or CD40 (data not shown). The increased expression of costimulatory molecules by poly I:C stimulation may be too high to observe an additional effect by the inhibition of Tmem176B or Tmem176A molecules.

Therefore, these results strongly suggest that Tmem176B and Tmem176A play a similar role in the maintenance of the immature state of the DC (e.g., ensuring a lower level of expression of costimulatory molecules).

We then analyzed the effect of the inhibition of Tmem176B or Tmem176A on the secretion of cytokines by unstimulated or poly I:C-stimulated DC. We observed no IL-6 secretion at all by unstimulated cells (data not shown) and no modification of IL-6 secretion by poly I:C-stimulated cells when these molecules were inhibited (**Fig. 7**; no p70 IL-12 or IL-10 was detectable; data not shown). The absence of effect on the expression of costimulatory molecules and on cytokine secretion in fully activated DCs suggests that these two molecules play their role only in very immature DCs.

We then analyzed the effect of the inhibition of Tmem176B, Tmem176A, or both on the capacity of DCs to promote allogeneic T cell priming. We observed that their inhibition in immature BMDCs increased proliferation of allogeneic lymph node cells (MLR) significantly ($n=3$; *, $P<0.05$; **Fig. 8A**). As the DCs are immature in these culture conditions, the differentiation of allogeneic effector cells is relatively low. Nevertheless, we confirmed these results using CFSE staining with enhanced, allogeneic $CD4^+$ T cell proliferation when expression of Tmem176B, Tmem176A, or both was inhibited in DCs. Indeed, more allogeneic $CD4^+$ T cells expressing CD25 (marker of T cell activation) proliferated in response to DCs, in which expression of Tmem176B, Tmem176A, or both had been down-regulated [results are expressed in percent or AU compared to control siRNA; $n=3$, *$P<0.05$ (Fig. 8); representative dot plots (Fig. 8B); histograms Fig. 8C)]. Again, this effect on allogeneic $CD4^+$ T cell activation was not retrieved with poly I:C-activated DCs (Fig. 8C). Taken together, these data demonstrate that Tmem176B and Tmem176A play a role in the maintenance of the immature state of the DCs by maintaining a lower expression level of costimulation molecules. However, when the DCs are more matured, these molecules are no longer influential.

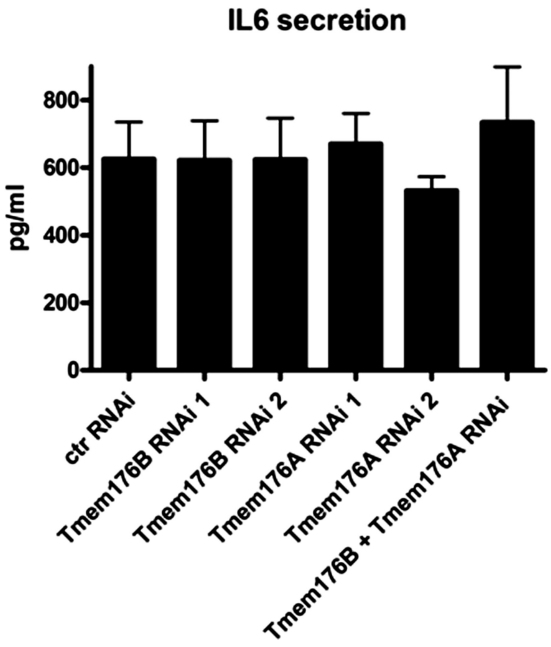

Figure 7. Effect of Tmem176B or Tmem176A inhibition on IL-6 secretion by DCs. Mice BMDCs were transfected with control RNAi, with two Tmem176B- or two Tmem176A-specific RNAi, or with a mix of both (Tmem176B+Tmem176A) for 48 h and stimulated with TLR3-L (poly I:C, 12.5 μg/ml) for 48 h. IL-6 production was assessed in supernatants by ELISA ($n=3$).

DISCUSSION

We previously identified a new gene, Tmem176B (alias, TORID in rat, clast-1 in mice, and LR8 in human), of unknown function as strongly overexpressed in a model of allograft tolerance in rats [4]. We showed that Tmem176B possesses four transmembrane domains and was expressed in intracellular structures [4]. Moreover, we demonstrated that Tmem176B was highly expressed by immature monocytes and DCs in rat and human and that its expression was strongly down-regulated following inflammatory stimulation. In addition, we showed that overexpression of Tmem176B by viral transduction rendered rat DCs more immature and resistant to the maturation induced by LPS [4]. Collectively, these data sug-

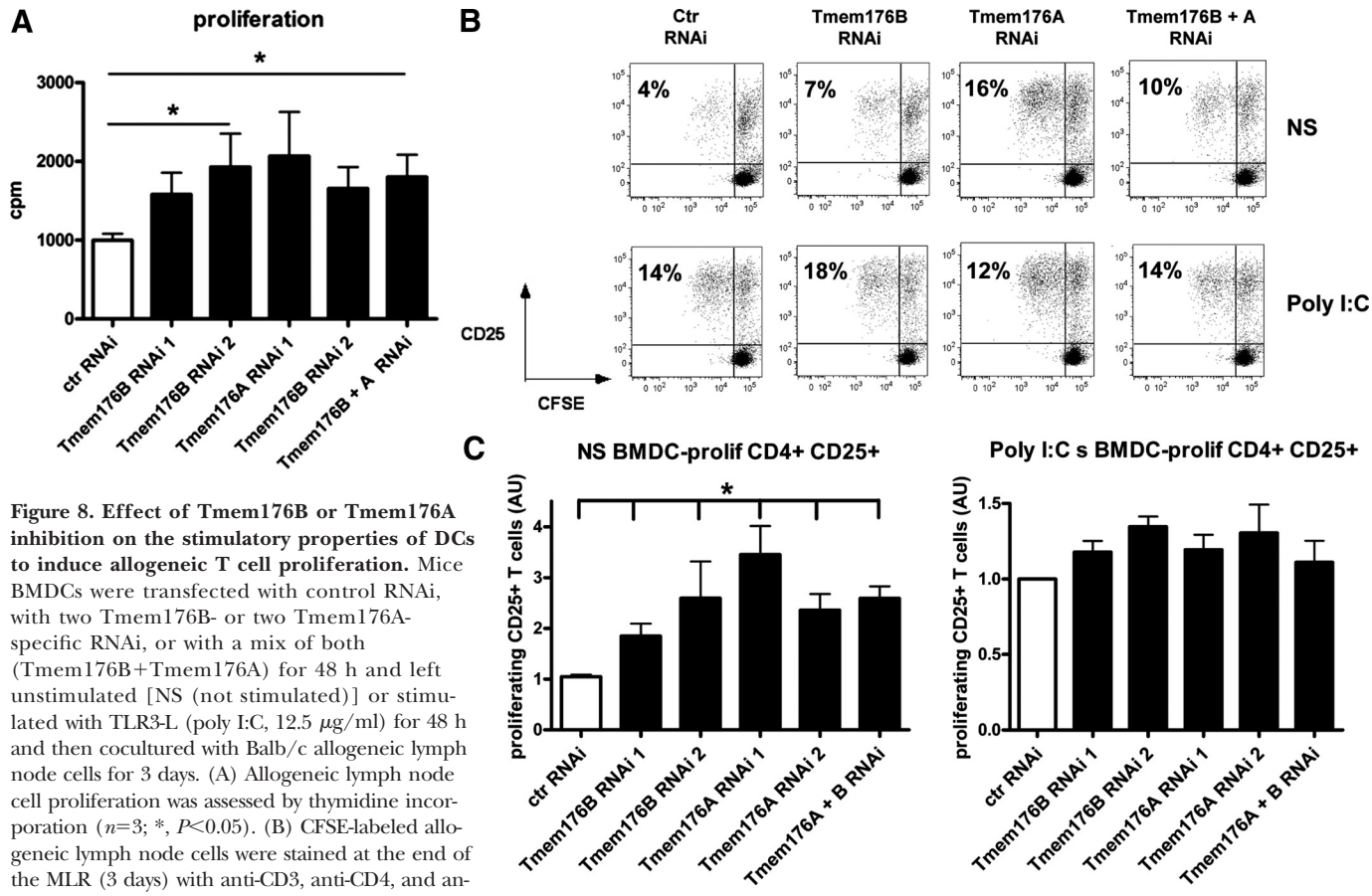

Figure 8. Effect of Tmem176B or Tmem176A inhibition on the stimulatory properties of DCs to induce allogeneic T cell proliferation. Mice BMDCs were transfected with control RNAi, with two Tmem176B- or two Tmem176A-specific RNAi, or with a mix of both (Tmem176B+Tmem176A) for 48 h and left unstimulated [NS (not stimulated)] or stimulated with TLR3-L (poly I:C, 12.5 μg/ml) for 48 h and then cocultured with Balb/c allogeneic lymph node cells for 3 days. (A) Allogeneic lymph node cell proliferation was assessed by thymidine incorporation (n=3; *, P<0.05). (B) CFSE-labeled allogeneic lymph node cells were stained at the end of the MLR (3 days) with anti-CD3, anti-CD4, and anti-CD25 antibodies and were analyzed by flow cytometry. (B) Representative dot plots (data are representative of three independent experiments), and (C) histograms represent the percentage of proliferating CD25$^+$ T cells compared with the reference control RNAi (value=1; n=3; *, P<0.05).

gested a role for Tmem176B in the maintenance of the immature state of the DCs. To gain insight into the functional role of this molecule, we searched for proteins interacting with Tmem176B using the yeast two-hybrid system. Interestingly, we observed that Tmem176B can interact with itself and with Tmem176A (alias, hca112), a gene that possesses similar structure to Tmem176B [10]. The Tmem176A protein also contains four transmembranous domains and has a similar intron/exon organization to Tmem176B. The similarity between the two proteins is 32% of identity in mice. Interestingly, the Tmem176A gene is located in the immediate vicinity of the Tmem176B gene on the same chromosome. These two genes are coded in the direction opposite to that of the overlapping 5′-untranslated regions in the putative first introns. The high homology of structure and the proximity on the chromosome suggest that these two genes may exert similar functions and may be controlled by similar regulations. We investigated the profile of expression of Tmem176A in mice and observed that this molecule displays a similar profile of expression and regulation to Tmem176B, with an expression in immature monocytes and DCs and a down-regulation following proinflammatory stimulation. We observed that these two molecules are poorly expressed in skin, an organ rich in Langherans DCs, and in pDCs and NK, B, and T cells. These data suggest that Tmem176B and Tmem176A are preferentially expressed in cells of the myeloid lineage. Tmem176A has been described poorly so far. This gene has been identified to be overexpressed in mouse kidney proximal tubules exhibiting proteinuria, together with genes involved in metabolism, degradation pathway, or regulator of inflammation [10]. Moreover, Tmem176A was identified as a tumor autoantigen in patients with hepatocellular carcinoma, suggesting a possible role for this protein in the transformation, metastasis, or immune evasion of tumors [11]. These data also suggest a role for this molecule in immune activation of the cells. We demonstrate here that Tmem176A interacts with Tmem176B possibly to form multimers and that these two molecules exhibit similar profiles of expression and regulation. In addition, we showed by RNAi that inhibition of Tmem176A or Tmem176B molecules increased the expression of costimulatory molecules and the subsequent activation of allogeneic T cells. These data suggest that these two molecules are part of a complex located in intracellular, membranous structures, which may play a role in the maturation of the DCs. Interestingly, Tmem176B KO mice have been generated and reported to develop sporadic ataxia as a result of a defect in the generation of the cerebellar granule cell [6]. No functional study about immune cells has been reported, and unfortunately, we cannot have access to these mice. Interestingly, the same phenotype of ataxia is retrieved in mice deficient in ion channels [13]. For example, a defect

in the maturation of granule cells has also been observed in mice deficient in Numb [14, 15], which is a molecule that regulates cell differentiation, endocytic recycling, and intracellular trafficking by a mechanism involving Ca^{2+} influx and MAPK activation by specific channels [14–17]. Ion channels have been shown recently to play important roles in immune function of various types of cells by regulating their activation, proliferation, or migration [18–22]. Interestingly, it has been shown recently that DCs express numerous ion channels, which were described initially to be highly expressed in the nervous system [23]. Moreover, a switch of ion channel expression in DCs during the maturation step has been shown, which regulates their functional activities [20, 23]. As Tmem176B and Tmem176A exhibit their structure in four transmembranous domains, and these two molecules can interact together, this suggests that these two molecules may also be part of a membrane ion channel. By influencing intracellular signaling events, these ion channels may play a determinant role in the nervous system but also in the fine-tuning of DC maturation.

The molecular mechanisms by which these two molecules exert their function remain to be elucidated but may help to determine new targets for the therapeutic modulation of DC maturation.

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AUTHORSHIP

T.C., L.LT., D.H., and A.L. performed the experiments. M.H., F.H., S.C., H.W., and M.-C.C. were responsible for intellectual and technical input. E.C. was responsible for the concept, experimental design, and supervision; and for preparation of the manuscript.

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KEY WORDS:
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