Published in final edited form as:

Nat Immunol. 2008 October ; 9(10): 1148–1156. doi:10.1038/ni.1648.

The calcium-activated nonselective cation channel TRPM4 is essential for the migration but not the maturation of dendritic cells

Gaëtan Barbet^{1,2,3,7}, Marie Demion^{1,2,3,7}, Ivan C Moura^{1,3,7}, Nicolas Serafini^{1,2,3}, Thibaut Léger^{1,2,3}, François Vrtovsnik^{1,3,4}, Renato C Monteiro^{1,3}, Romain Guinamard⁵, Jean-Pierre Kinet⁶, and Pierre Launay^{1,2,3}

¹Institut National de la Santé et de la Recherche Médicale U699, Paris F-75018, France.

²Equipe Avenir, Institut National de la Santé et de la Recherche Médicale, Paris F-75018, France.

³Université Paris 7-Denis Diderot, Faculté de Médecine, Site Xavier Bichat, Paris F-75018, France.

⁴Hôpital Bichat, Service de Néphrologie, Paris F-75018, France.

⁵Laboratoire de Physiologie Cellulaire, EA 3212, Université de Caen, Caen, F-14032, France.

⁶Department of Pathology, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, Massachusetts 02215, USA.

Abstract

Dendritic cell (DC) maturation and migration are events critical for the initiation of immune responses. After encountering pathogens, DCs upregulate the expression of costimulatory molecules and subsequently migrate to secondary lymphoid organs. Calcium (Ca²⁺) entry governs the functions of many hematopoietic cell types, but the role of Ca²⁺ entry in DC biology remains unclear. Here we report that the Ca²⁺-activated nonselective cation channel TRPM4 was expressed in and controlled the Ca²⁺ homeostasis of mouse DCs. The absence of TRPM4, which elicited Ca²⁺ overload, did not influence DC maturation but did considerably impair chemokine-dependent DC migration. Our results establish TRPM4-regulated Ca²⁺ homeostasis as crucial for DC mobility but not maturation and emphasize that DC maturation and migration are independently regulated.

Dendritic cells (DCs) are crucial in the initiation of adaptive immune responses. These 'professional' antigen-presenting cells, which are present in almost all peripheral tissues, capture and process antigens in the periphery before migrating to secondary lymphoid organs, where they prime naive T lymphocytes¹. DC-mediated modulation of immune responses depends on the DC maturation status. During maturation, DCs undergo several phenotypic

© 2008 Nature Publishing Group

Correspondence should be addressed to P.L. (pierre.launay@inserm.fr).

⁷These authors contributed equally to this work.

Note: Supplementary information is available on the Nature Immunology website.

AUTHOR CONTRIBUTIONS G.B. and I.C.M. were responsible for all experiments involving *in vivo* analysis of *Trpm4*^{-/-} and *Trpm5*^{-/-} mice; M.D. did the electrophysiological experiments in the whole-cell configuration; R.G. did the electrophysiological experiments in the 'inside-out' configuration; G.B. and M.D. did Ca²⁺ imaging; G.B. did the Transwell assays; M.D. did the time-lapse experiments; T.L. contributed to biochemical experiments; N.S. was responsible for quantitative RT-PCR; G.B. and N.S. were responsible for genotyping of mice; J.-P.K. was responsible for the generation of *Trpm4*^{-/-} mice, critical reading and comments; R.C.M. and F.V. provided experimental guidance; all authors critically reviewed and contributed to the manuscript; and P.L. directed and supervised all aspects of the study and the writing and editing of the manuscript.

Reprints and permissions information is available online at <http://npg.nature.com/reprintsandpermissions/>

Accession codes

UCSD-Nature Signaling Gateway (<http://www.signaling-gateway.org>): A003857 and A003767.

changes (such as up-regulation of the expression of costimulatory and major histocompatibility complex (MHC) class II molecules) that result in more potent antigen-presenting cell function. DC pattern-recognition receptors, including Toll-like receptors, are necessary for the sensing of pathogen-associated molecular patterns during microbial aggression. Hence, DCs act as 'sentinels', trafficking between tissues and lymph nodes to deliver signs of 'danger' to appropriate immune effector cells². In the absence of foreign antigens, DCs remain in an immature state but are able to present self antigen and thus are involved in peripheral tolerance³. DCs are heterogeneous and have been classified according to criteria such as their hematopoietic origin, surface marker expression, developmental stage, mobility and tissue localization⁴. For example, migratory DCs such as Langerhans cells and dermal DCs migrate from the peripheral tissues through the lymphatics to the draining lymphoid organs, whereas circulating DCs such as plasmacytoid DCs circulate in the bloodstream.

An increase in the cytosolic free calcium concentration ($[Ca^{2+}]_i$) is a ubiquitous signal that influences a wide range of hematopoietic cell functions, including cell cycle progression, adhesion, maturation and mobility⁵. Cytoplasmic increases in Ca^{2+} concentration induced by antigen receptor engagement modulate the initiation and maintenance of many cell functions^{6,7}. After bacterial or mechanical stimulation, Ca^{2+} fluxes are induced in DCs and between adjacent DCs by networks of nanotubes⁸. DC migration correlates with an influx of Ca^{2+} through Ca^{2+} release-activated Ca^{2+} channels^{9,10}, and the engagement of chemokine receptors such as CCR7 triggers DC trafficking by considerably increasing Ca^{2+} influx¹¹.

Thus, Ca^{2+} homeostasis seems to be a key determinant of DC activation, but the molecular mechanisms regulating the Ca^{2+} homeostasis of DCs are not well understood. The Ca^{2+} -activated nonselective (CAN) channel TRPM4 (transient receptor potential melastatin 4; A003857) has been detected in hematopoietic cells at both molecular and functional levels¹². The TRPM4 and TRPM5 (A003767) channels are the only CAN channels described so far in nonexcitable cells^{13,14}. In physiological conditions, TRPM4 channels allow massive entry of Na^+ , thereby inducing membrane depolarization and decreasing the driving force for Ca^{2+} entry¹⁵. TRPM4 can regulate the amount of Ca^{2+} entry and related physiological responses of T lymphocytes and mast cells^{15,16}.

To examine the function of Ca^{2+} homeostasis in DC biology, we generated and analyzed a TRPM4-deficient (*Trpm4*^{-/-}) mouse model. We found that TRPM4 was expressed in and controlled the Ca^{2+} homeostasis of DCs. TRPM4, but not TRPM5, acted as a Ca^{2+} 'gatekeeper', preventing Ca^{2+} overload in DCs. Ca^{2+} -dependent stimulation of *Trpm4*^{-/-} DCs promoted downregulation of the expression of phospholipase C- β 2 (PLC- β 2), which resulted in less subsequent Ca^{2+} mobilization in *Trpm4*^{-/-} cells. Furthermore, the migration of *Trpm4*^{-/-} DCs was profoundly impaired, but their maturation was not affected. Thus, our results show that the maturation and migration of DCs are regulated differently.

RESULTS

Fewer DCs in lymphoid organs of *Trpm4*^{-/-} mice

To assess the function of Ca^{2+} in DC biology, we generated mice in which exons 3–6 of *Trpm4* were removed by Cre recombinase-mediated excision. *Trpm4*^{-/-} mice lacked the targeted exons and TRPM4 protein (Supplementary Fig. 1 online) and were normal and fertile. The sizes of the thymus, spleen and lymph nodes of *Trpm4*^{-/-} mice were similar to those of their *Trpm4*^{-/-} littermates. Likewise, the numbers of CD4⁺ T cells, CD8⁺ T cells and B cells were similar in *Trpm4*^{-/-} and *Trpm4*^{-/-} mice (data not shown).

However, we noted that the spleen of *Trpm4*^{-/-} mice consistently had about 50% fewer CD11b⁺CD11c⁺ DCs (Fig. 1a). The percentage and absolute number of plasmacytoid DCs in

Trpm4^{-/-} mice was normal (about 0.3%; data not shown). Further phenotypic analysis showed that this effect was limited to specifically the CD8α⁻ subset; absolute numbers of CD8α⁺ DCs were not affected (Supplementary Fig. 2a online). However, the steady-state maturation status of *Trpm4*^{-/-} and *Trpm4*^{+/+} DCs was identical (Fig. 1b) independently of CD8α expression (Supplementary Fig. 2b), as assessed by the expression of costimulatory and MHC class II molecules. We then assessed the maturation of DCs in inflammatory conditions in *Trpm4*^{-/-} mice *in vivo*. After subcutaneous injection of *Escherichia coli*, the expression of CD86 and MHC class II was similar on DCs from *Trpm4*^{-/-} and littermate control mice (Fig. 1c). However, the absolute number of mature DCs in draining lymph nodes was lower in *Trpm4*^{-/-} mice (Fig. 1d). Thus, we noted fewer splenic DCs in steady-state conditions and fewer migratory DCs in inflammatory conditions in TRPM4-deficient mice. However, the absence of TRPM4 did not affect DC maturation.

Impaired DC migration in *Trpm4*^{-/-} mice

Cell mobility requires an increase in intracellular Ca²⁺. As TRPM4 is involved in Ca²⁺ homeostasis, and as the number of mature DCs in inflamed lymph nodes was lower in *Trpm4*^{-/-} mice, we further analyzed the effect of *Trpm4* deletion on the homing of DCs to lymph nodes. After sensitization of skin with a proinflammatory agent, immature DCs begin to mature and migrate to the draining lymph nodes¹⁷. Thus, we painted the back skin of *Trpm4*^{-/-} mice and littermate control mice with a 1% solution of fluorescein isothiocyanate (FITC) 2 h before inoculating the mice with *E. coli* by scarification. We isolated cells from brachial and mesenteric lymph nodes at 12, 24 and 48 h after scarification and counted FITC⁺CD11c⁺ cells in lymph nodes by flow cytometry. We noted many fewer FITC⁺CD11c⁺ cells in draining (brachial) lymph nodes of TRPM4-deficient mice at all time points studied (Fig. 2a). We found no such cells in nondraining (mesenteric) lymph nodes. However, the frequency of CD11c⁺ cells was similar in the skin of *Trpm4*^{-/-} and *Trpm4*^{+/+} mice in steady-state conditions (Fig. 2b).

To confirm the defect in DC migration in *Trpm4*^{-/-} mice, we assessed the trafficking of adoptively transferred bone marrow-derived DCs (BMDCs) from *Trpm4*^{-/-} and *Trpm4*^{+/+} mice. We labeled immature BMDCs (iDCs) from *Trpm4*^{-/-} and *Trpm4*^{+/+} mice differentially with the cytosolic dye CFSE (*Trpm4*^{-/-}, CFSE^{lo}; *Trpm4*^{+/+}, CFSE^{hi}) and injected equal amounts of the cells together into footpads of recipient mice with the same genetic background (Fig. 2c). We induced DC migration by inoculating mice with *E. coli* at 2 h after BMDC transfer, then counted CFSE^{hi} and CFSE^{lo} DCs in popliteal lymph nodes 12, 24 and 48 h later. We detected significantly fewer *Trpm4*^{-/-} DCs in the popliteal lymph nodes at all time points (Fig. 2c). As expected, DC mobility was much lower in mice not inoculated with *E. coli* (Fig. 2c). These results collectively suggest that the impaired DC migration noted in TRPM4-deficient mice was due to a DC-intrinsic defect.

Absence of CAN current in *Trpm4*^{-/-} DCs

The regulation of Ca²⁺ is essential for DC function; however, only a few studies have examined ionic channels in DCs¹⁰. Furthermore, of 28 members of transient receptor potential channels discovered, none have been studied in DCs¹⁸. Thus, we used the patch-clamp technique to measure TRPM4 channel activity in BMDCs.

We first compared the electrophysiological profiles of *Trpm4*^{-/-} and *Trpm4*^{+/+} BMDCs using the whole-cell configuration of the patch-clamp technique. We used an [Ca²⁺]_i of 1 μM to promote the activation of CAN channels. We detected an outwardly rectifying current in wild-type but not in TRPM4-deficient BMDCs; in the latter, we detected only a residual inward-rectifying current (Fig. 3a). By subtracting the mean residual current-voltage relationships of *Trpm4*^{-/-} DC traces from those of *Trpm4*^{+/+} DCs, we identified a TRPM4-like current (Fig.

3a), as reported before for human embryonic kidney (HEK293) clones over-expressing TRPM4 (ref. 12).

We further characterized the TRPM4 current by studying cell-free excised membrane patches in the 'inside-out' configuration. We obtained typical single-channel recordings at several membrane potentials in symmetrical ionic conditions¹⁹ (Fig. 3b, inset). The corresponding current-voltage curve showed a linear conductance of 22.1 ± 0.6 pS ($n = 6$ cells; Fig. 3b). Changing the internal solutions showed that the channel did not discriminate between Na^+ and K^+ and did not conduct Cl^- (Fig. 3c). Channel activity was increased by membrane depolarization (Fig. 3d) and by an increase in $[\text{Ca}^{2+}]_i$, with a half-maximum effector concentration of $14 \mu\text{M}$ (Fig. 3e,f), but was decreased by the internal application of ATP (Fig. 3g). Thus, our electrophysiology studies established that TRPM4 was expressed and functional in *Trpm4*^{+/+} BMDCs (20 of 42 'inside-out' patches) but not *Trpm4*^{-/-} BMDCs (0 of 58 'inside-out' patches).

TRPM4 is the main CAN channel regulating Ca^{2+} entry in DCs

We next analyzed Ca^{2+} influx in *Trpm4*^{-/-} and *Trpm4*^{+/+} iDCs by activation of the cells with an *E. coli* supernatant (ECS), as reported before⁸. We noted higher Ca^{2+} influx in *Trpm4*^{-/-} iDCs than in *Trpm4*^{+/+} iDCs (Fig. 4a). To determine whether this was due to sustained Ca^{2+} entry or to the release of Ca^{2+} from internal stores, we incubated iDCs in Ca^{2+} -free medium, stimulated them with ECS and added a solution of $2 \text{ mM } \text{Ca}^{2+}$ 90 s later. We found no difference in Ca^{2+} release from internal stores (Fig. 4b), which suggested that the higher $[\text{Ca}^{2+}]_i$ in *Trpm4*^{-/-} DCs was due to enhanced Ca^{2+} entry.

We next sought to determine what type of receptors were involved in the *E. coli*-mediated Ca^{2+} response of DCs. We tested whether G protein-coupled receptors, which are involved in the stimulation of DCs by *Mycobacterium tuberculosis*²⁰, were involved in DC activation by *E. coli*. We incubated BMDCs overnight with pertussis toxin, an inhibitor of transduction through G protein-coupled receptors, then activated them with ECS. Ca^{2+} -mediated signals induced by *E. coli* were abolished in the presence of pertussis toxin (Fig. 4c), which confirmed the interaction between G protein-coupled receptors and *E. coli*. Among G protein-coupled receptors expressed on iDCs, chemokine receptors such as CCR5 are involved in Ca^{2+} signals²⁰ and cell mobility²¹. Therefore, we assessed whether CCR5 was triggered by *E. coli* in DCs. Indeed, treatment with TAK-779, a potent antagonist of CCR5, resulted in much lower ECS-mediated Ca^{2+} signals in iDCs (Fig. 4d). *E. coli* can also directly bind to receptors for the Fc fragment of immunoglobulin G type III (Fc γ III)²²; however, *E. coli*-induced Ca^{2+} signals in iDCs were not mediated by Fc receptors dependent on immunoreceptor tyrosine-based activation motifs, as iDCs from Fc γ -deficient mice had Ca^{2+} responses similar to those of control iDCs (Supplementary Fig. 3a online). These findings collectively indicate that *Trpm4*^{-/-} iDCs have a prolonged Ca^{2+} signal after *E. coli* triggering of G protein-coupled receptors, in particular chemokine receptors.

TRPM5 is closely related to TRPM4 in terms of electrophysiological properties, and we did detect TRPM5 transcripts by RT-PCR in DCs (Fig. 4e). However, we detected no other CAN current in *Trpm4*^{-/-} DCs. Quantitative RT-PCR showed that TRPM4 mRNA was almost 200 times more abundant than TRPM5 mRNA in wild-type DCs, but that TRPM5 expression was three times higher in BMDCs from *Trpm4*^{-/-} mice than in those from littermate control mice (Fig. 4f). We therefore generated double-knockout *Trpm4*^{-/-}*Trpm5*^{-/-} mice by mating our *Trpm4*^{-/-} mice with *Trpm5*^{-/-} mice²³. After being stimulated with ECS, iDCs from *Trpm5*^{-/-} mice had Ca^{2+} responses identical to those of iDCs from wild-type mice, whereas *Trpm4*^{-/-}*Trpm5*^{-/-} iDCs had a significantly higher Ca^{2+} flux than did *Trpm5*^{-/-} iDCs (Fig. 4g). *Trpm4*^{-/-}*Trpm5*^{-/-} mice also had fewer splenic DCs than did *Trpm5*^{-/-} mice (Fig. 4h).

These results show that TRPM4 is the main Ca^{2+} -activated nonselective channel involved in Ca^{2+} homeostasis in mouse DCs and that TRPM5 cannot compensate for the lack of TRPM4.

Enhanced Ca^{2+} influx affects only DC mobility

As *Trpm4*^{-/-} DCs had more Ca^{2+} influx after stimulation, we examined whether DC differentiation and maturation were also affected. We assessed the percentage of BMDCs (CD11c⁺CD11b⁺) generated by the addition of granulocyte-macrophage colony-stimulating factor *in vitro* every 2 d from day 4 to day 13. We noted similar differentiation of *Trpm4*^{-/-} and *Trpm4*^{+/+} BMDCs (Fig. 5a).

To determine whether the higher $[\text{Ca}^{2+}]_i$ permitted by TRPM4 deficiency affected DC maturation, we assessed maturation induced by a Ca^{2+} -independent stimulus (LPS) and a Ca^{2+} -dependent stimulus (paraformaldehyde-fixed *E. coli*). As reported before²⁴, activation with bacteria allowed Ca^{2+} influx, whereas LPS did not (Supplementary Fig. 3b,c). Each stimulus upregulated the expression of maturation markers (CD86, CD80, MHC class II and CCR7) to a similar extent in *Trpm4*^{-/-} and *Trpm4*^{+/+} BMDCs (Fig. 5b,c), which suggested that the enhanced Ca^{2+} influx due to *Trpm4* deletion did not affect DC maturation. We also tested the rate of *E. coli* phagocytosis and DC survival after incubation with fixed bacteria. Each was similar in BMDCs from *Trpm4*^{-/-} and *Trpm4*^{+/+} mice (Supplementary Fig. 4a,b online). Therefore, maturation, differentiation and phagocytosis were not affected by Ca^{2+} overload in *Trpm4*^{-/-} DCs.

Given the known function of Ca^{2+} in promoting cell mobility, the observed lower migration of *Trpm4*^{-/-} DCs, which had large amounts of Ca^{2+} , was unexpected. To further characterize the *Trpm4*^{-/-} DC mobility defect, we made time-lapse videos of *Trpm4*^{-/-} and control BMDCs after activation. We stimulated iDCs with ECS and measured their trajectories in two-dimensional x-y projections every 5 min for 4 h. Immature BMDCs from *Trpm4*^{-/-} mice were more mobile than were those from *Trpm4*^{+/+} control mice (Fig. 6a).

However, because we had found that DC migration was impaired in *Trpm4*^{-/-} mice *in vivo*, we then assessed oriented migration in response to the CCR7 ligand CCL21, which is involved in the trafficking of DCs to lymph nodes^{25,26}. In Transwell chambers, we compared chemotaxis of DCs matured for 24 h with fixed *E. coli* or with LPS. The chemotaxis of *Trpm4*^{-/-} mature DCs (mDCs) was significantly lower than that of *Trpm4*^{+/+} DCs when we used *E. coli* for cell maturation (Fig. 6b), but it was significantly higher than that of *Trpm4*^{+/+} DCs when we used LPS for maturation (Fig. 6c). This lower chemotaxis of *E. coli*-matured *Trpm4*^{-/-} DCs was conserved even at higher concentrations of CCL21 (Supplementary Fig. 5 online). In contrast, mDCs from *Trpm4*^{+/+} mice showed similar chemotaxis after stimulation with either *E. coli* or LPS (Fig. 6b,c).

Because the *in vitro* experiments showed greater mobility of *Trpm4*^{-/-} DCs after LPS maturation, we tested the effect of LPS on the migration of *Trpm4*^{-/-} iDCs *in vivo* by adoptive transfer assay. We differentially labeled *Trpm4*^{-/-} and *Trpm4*^{+/+} iDCs with CFSE as described above, then injected the cells into the footpads of mice 2 h before LPS injection (50 ng per footpad) and counted migratory CFSE⁺ DCs 24 h after LPS injection. We detected significantly more *Trpm4*^{-/-} DCs in the popliteal lymph nodes (Fig. 6d). These results collectively indicate that the alteration in Ca^{2+} homeostasis induced deregulation of DC migration but not DC maturation.

Ca^{2+} overload modulates PLC- β 2 expression in *Trpm4*^{-/-} mDCs

Cell migration to lymph nodes involves chemokine receptors coupled to G proteins. One important component of chemokine receptor signaling pathways is PLC²⁷. PLC produces

inositol-1,4,5-triphosphate, which, by binding to its receptor on the endoplasmic reticulum, causes rapid release of Ca^{2+} from endoplasmic reticulum stores. As reported above, maturation of *Trpm4*^{-/-} DCs with *E. coli* resulted in less CCL21-mediated chemotaxis. Similarly, we found that Ca^{2+} entry after ECS stimulation was lower in mDCs from *Trpm4*^{-/-} than in those from *Trpm4*^{+/+} mice (Fig. 7a). We also noted this lower Ca^{2+} mobilization after CCL21 stimulation (Fig. 7b). The use of Ca^{2+} -free medium showed that the defect in ECS-induced Ca^{2+} flux in *Trpm4*^{-/-} mDCs was due to impaired release of Ca^{2+} from internal stores (Fig. 7c). Furthermore, the release of Ca^{2+} stores induced by thapsigargin was identical in *Trpm4*^{-/-} and *Trpm4*^{+/+} DCs (data not shown). These findings suggest that *Trpm4*^{-/-} mDCs have defects in signaling pathways ‘upstream’ of the release of Ca^{2+} stores.

To determine if initial augmented Ca^{2+} entry during the maturation process was responsible for the lower Ca^{2+} flux noted during subsequent cell stimulations, we incubated iDCs with *E. coli* and 2.1 mM EGTA to decrease the Ca^{2+} concentration in the cell culture medium from 2 mM to 1 μM . Viability and maturation were similar for *Trpm4*^{-/-} and *Trpm4*^{+/+} DCs after overnight treatment with 2.1 mM EGTA (data not shown). However, EGTA treatment resulted in identical Ca^{2+} responses induced by subsequent CCL21 stimulation of *Trpm4*^{-/-} and *Trpm4*^{+/+} mDCs (Fig. 7d). In addition, when we matured DCs with LPS in presence of 2.1 mM EGTA, *Trpm4*^{-/-} DCs still showed subsequent higher Ca^{2+} influx than that of *Trpm4*^{+/+} DCs (Supplementary Fig. 6a online). As expected, by decreasing the external Ca^{2+} concentration to 1 μM during Transwell assays, we abolished the CCL21-induced chemotaxis of LPS-matured DCs (Supplementary Fig. 6b). These data collectively emphasize the function of the initial Ca^{2+} influx in the alteration of subsequent CCL21-mediated responses (Supplementary Fig. 7 online).

We next assessed which ‘upstream’ signaling molecule were involved in this regulatory process. As PLC- γ 2 regulates the migration of B cells²⁸ and PLC- γ 1 is involved in the unresponsiveness of T cells^{29,30}, we tested various PLC isoforms for their involvement in the Ca^{2+} -mediated regulation of DCs. We noted reproducibly lower PLC- β 2 expression in *E. coli*-matured *Trpm4*^{-/-} BMDCs (Fig. 7e). The proteasome inhibitor epoxomicyn had no effect on the lower PLC- β 2 expression in *E. coli*-matured *Trpm4*^{-/-} BMDCs (Supplementary Fig. 8 online). Moreover, overnight incubation with ionomycin, which mimics the Ca^{2+} overload noted in *Trpm4*^{-/-} DCs, triggered similarly lower PLC- β 2 expression in *Trpm4*^{+/+} DCs (Fig. 7e). This result suggests that Ca^{2+} overload is sufficient to induce PLC- β 2 downregulation in mDCs independently of proteasome degradation. Our data are consistent with the consequences of Ca^{2+} overload induced by ionomycin in T cells²⁹, in which PLC- γ 1 degradation leads to unresponsiveness of T cells. However, the intensity of the bands corresponding to PLC- γ 1 and PLC- γ 2 was similar in immature and mature DCs from *Trpm4*^{-/-} and *Trpm4*^{+/+} mice (Fig. 7f). Therefore, sustained entry of Ca^{2+} into *Trpm4*^{-/-} DCs resulted in lower PLC- β 2 expression and thus less Ca^{2+} mobilization during subsequent stimulations.

DISCUSSION

Ca^{2+} homeostasis is tightly regulated in hematopoietic cells, and the importance of Ca^{2+} influx in cell activation is widely acknowledged. DCs are central to the immune system, but little is known about the molecular mechanisms that control Ca^{2+} entry in DCs. To address the role of Ca^{2+} in DC function, we generated an animal model in which Ca^{2+} homeostasis was impaired by genetic ablation of Trpm4 channels. Trpm4 is a CAN channel that regulates Ca^{2+} influx by depolarizing the membrane and decreasing the Ca^{2+} driving force through store-operated channels¹⁵. In this study, we have shown that stimulation of DCs lacking Trpm4 led to intracellular Ca^{2+} overload with detrimental consequences for DC migration but not maturation.

Although we noted impaired migration of *Trpm4*-deficient DCs to lymph nodes, DC maturation was not altered in *Trpm4*-deficient mice; thus, DC functions are differentially sensitive to $[Ca^{2+}]_i$ variations. Indeed, the maturation induced by a Ca^{2+} -independent stimulus (LPS) and a Ca^{2+} -dependent stimulus (*E. coli*) was identical for *Trpm4*^{-/-} and *Trpm4*^{+/+} DCs. This indicated that the process of maturation in DCs is not directly affected by sustained Ca^{2+} entry.

Unlike maturation, the migration of DCs was highly dependent on variations in $[Ca^{2+}]_i$. In absence of *Trpm4*, DCs matured in a Ca^{2+} -independent way (by LPS) had more chemotaxis and homing to the draining lymph nodes. In contrast, Ca^{2+} -dependent (bacterial) maturation of *Trpm4*^{-/-} DCs led to intracellular Ca^{2+} overload and impaired migration. Thus, in inflammatory conditions and during DC activation with pathogens, *Trpm4* is needed to regulate initial Ca^{2+} influx. This 'fine tuning' of Ca^{2+} entry allows DCs to respond adequately to subsequent stimuli (such as chemokines). Therefore, in contrast to the common paradigm that an increase of Ca^{2+} influx is associated with an increase in cell activity, our results lead us to postulate that Ca^{2+} uptake must be tightly regulated to avoid DC unresponsiveness and that TRPM4 is essential to prevent such detrimental overactivation.

The relationship between $[Ca^{2+}]_i$ and cell mobility has also been studied in T lymphocytes. T cell immobilization correlates with an increase in intracellular Ca^{2+} that seems to induce a 'stop' signal³¹. T cell mobility has been shown to be decreased by sustained Ca^{2+} influx. In a nonselective environment, immature thymocytes move rapidly while maintaining low intracellular Ca^{2+} concentrations. Conversely, an increase in $[Ca^{2+}]_i$ is necessary and sufficient to abolish thymocyte mobility³². The Ca^{2+} ionophore ionomycin has been used in T cells to assess molecular pathways affected by Ca^{2+} overload³⁰. Such results show that in these cells, subsequent steps of stimulation are responsible for the degradation of PLC- γ 1, which drives them into an unresponsive state²⁹. In B lymphocytes, another PLC isoform, PLC- γ 2, is involved in the regulation of cell migration and homing. B cells from PLC- γ 2-deficient mice show impaired migration controlled by CXC chemokines²⁸. We found that neither PLC- γ 1 nor PLC- γ 2 was affected by Ca^{2+} overload in DCs. The β -isoforms of PLC are expected to be essential for DC migration, as chemokine receptors that guide DCs to secondary lymphoid organs^{33, 34} involve G_α protein family members that activate the β -isoforms of PLC enzymes⁶. We suggest that in inflammatory conditions, the downregulation PLC- β 2 induced by Ca^{2+} overload abrogates the homing of *Trpm4*^{-/-} DCs to secondary lymphoid organs. Thus, specific PLC isoforms seem to be important in Ca^{2+} -mediated regulation in a cell type-specific way.

TRPM5, the channel the most closely related to TRPM4, is also a CAN cation channel³⁵. On the basis of our electrophysiological data, we estimate that the number of TRPM4 channels expressed at the cell surface is around 1,000 molecules per DC. Quantitative PCR showed that TRPM4 expression was almost 200 times higher than TRPM5 expression; thus, we estimate that each DC expresses only 5 or 6 TRPM5 molecules. Furthermore, we detected no CAN current in *Trpm4*^{-/-} DCs. In addition, TRPM5 is not inhibited by the internal application of ATP³⁵, but we found that ATP suppressed DC CAN activity in 'inside-out' patch-clamp experiments. To further rule out the possibility of a contribution of TRPM5 channels to the Ca^{2+} homeostasis of DCs, we generated *Trpm4*^{-/-}*Trpm5*^{-/-} double-knockout mice. Phenotypic and physiological analysis of *Trpm4*^{-/-}*Trpm5*^{-/-} DCs showed that they were not different from *Trpm4*^{-/-}*Trpm5*^{+/-} DCs. Therefore, our data suggest that TRPM4 is the only CAN channel involved in the control of DC Ca^{2+} homeostasis.

The factors governing the migration of DCs from skin to lymph nodes have been studied extensively, but because of the difficulty in tracking subsets of migrating cells and DC precursors *in vivo*, less is known about DC trafficking in steady-state conditions^{36,37}. Studies have shown that in addition to bone marrow, spleen is a lymphoid organ where DC precursors

can seed and differentiate^{38,39}. Also, findings indicate that mice lacking the receptor tyrosine kinase Flt3 have impaired DC development⁴⁰. This defect in peripheral DC homeostasis seems to be dependent on both progenitor migration and in situ conventional DC division⁴⁰. Our data have shown that TRPM4-deficient mice had constitutively fewer splenic CD8 α ⁻ DCs. These lower numbers of DCs could be the result of impaired migration and/or development of *Trpm4*^{-/-} DC precursors. Further detailed studies must be done to distinguish between these two possibilities.

Matured monocyte-derived DCs are increasingly being used as cancer immunotherapy treatments³. As successful priming of tumor-specific T cells necessitates that such mature DCs generated *ex vivo* have the ability to migrate to lymphoid organs and thus to respond to specific chemokines, our data suggest that care should be taken to avoid Ca²⁺ overload during *ex vivo* maturation procedures^{41,42}. In summary, our results have shown that TRPM4 prevents Ca²⁺ overload and thus permits the homing of DCs to draining lymph nodes. In addition, our findings emphasize that DC migration and maturation are independently regulated.

METHODS

Mice

Trpm4^{-/-} mice were viable and fertile. Pups were born at the expected mendelian ratio. Double-knockout mice were generated by intercrossing of *Trpm4*^{-/-} and *Trpm5*^{-/-} mice (provided by C.S. Zuker). Double-knockout mice were viable and fertile and had no noteworthy abnormalities. In all experiments, mice were 6–14 weeks of age and controls were littermates. Mice were housed in specific pathogen-free conditions and were handled in accordance with French and European directives.

BMDC cultures

Bone marrow cells were extracted from femurs and tibias. Cells were cultured at 37 °C and 5% CO₂ at a density of 0.5×10^6 cells per ml in Iscove's modified Dulbecco's medium (Sigma-Aldrich) containing 10% (vol/vol) FCS (PAA Laboratories), 1 mM glutamine (Invitrogen), 10 μ M β -mercaptoethanol (Sigma-Aldrich) and 20 ng/ml of granulocyte-macrophage colony-stimulating factor from supernatants of J558 cells (mouse plasma-cytoma cell line of BALB/c origin transfected with the gene encoding granulocyte-macrophage colony-stimulating factor). DCs were matured for 24 h by the addition of LPS (10 ng/ml; Sigma-Aldrich) or paraformaldehyde-fixed *E. coli* (K12 strain; 1×10^6 bacteria per ml) to the culture medium. Where indicated, cells were matured in presence of 2.1 mM EGTA to chelate free Ca²⁺ in medium from 1.92 mM to 1 μ M. *E. coli* fixation was done on bacteria growing in logarithmic phase with 1% (vol/vol) paraformaldehyde.

Flow cytometry

Cells were immunostained with fluorochrome-conjugated monoclonal antibodies to mouse CD11c (HL-3), CD11b (M1/70), CD8 α (53-6.7), I-Ab (AF6-120.1), CD80 (16-10A1) and CD86 (GL1; all from Becton Dickinson). Cells were stained for CCR7 with antibody to CCR7 (anti-CCR7; 4B12; Serotec); as described⁴³. Data were acquired with a FACSCalibur (Becton Dickinson) and analyzed with FlowJo 8.3.3 software (TreeStar).

In vivo maturation assays

Mice were subcutaneously injected in the footpads with 1×10^8 *E. coli* in PBS. At 24 h after injection, popliteal lymph nodes were extracted and were treated with DNase and collagenase. DCs were purified with CD11c microbeads according to the manufacturer's protocol (Miltenyi Biotec) and were analyzed by flow cytometry.

Homing assays

Mice anesthetized with xylasin and ketamin (Sigma-Aldrich) were shaved and depilated. Mice were then painted with 150 μ l of a 1% (wt/vol) solution of FITC (isomer I; Sigma-Aldrich) in acetone and dibutylphthalate (1:1). At 2 h after skin painting, mice were scarified with *E. coli*. Cells from draining and nondraining lymph nodes were analyzed by flow cytometry 12, 24 or 48 h after scarification.

Migration of CFSE-labeled BMDCs

Immature BMDCs from TRPM4-deficient or control mice were loaded with different concentrations of CFSE (carboxyfluorescein diacetate succinimidyl diester; Sigma-Aldrich). Cells in suspension (1×10^8 cells per ml) were labeled by the addition of either 500 μ M (CFSE^{hi}) or 50 μ M (CFSE^{lo}) CFSE in PBS with 0.1% (wt/vol) BSA. *Trpm4*^{-/-} and *Trpm4*^{+/+} BMDCs were then mixed in equivalent proportions, and 0.5×10^6 cells were injected in the footpads of recipient mice 2 h before injection of *E. coli* (5×10^6 bacteria per footpad) or LPS (50 ng per footpad). Single-cell suspensions from draining and nondraining lymph nodes were incubated with anti-CD11c and were analyzed by flow cytometry.

Immunoblot

The expression of TRPM4 (from intestine) and PLC (from iDCs and mDCs after incubation for 1 h at 37 °C at a density of 3×10^6 cells per ml, as reported²⁹) was analyzed with 200 μ g or 25 μ g of total protein, respectively (concentration determined by Bio-Rad protein assay). Proteins were separated by 8% or 10% SDS-PAGE and were transferred to polyvinylidene difluoride membranes (Amersham). Protein expression was assessed by immunoblot analysis with the primary antibodies anti-PLC- γ 1 (2822; Cell signaling), anti-PLC- γ 2 (sc-407; Santa Cruz Biotechnology), anti-PLC- β 2 (sc-206; Santa Cruz Biotechnology), anti- β -actin (ACTN05 (C4); AbCam) and polyclonal anti-TRPM4 (produced with Proteogenix). Horseradish peroxidase-conjugated antibodies were anti-rabbit (NA934; Amersham) and anti-mouse (115-035-068; Jackson ImmunoResearch Laboratories).

Ca²⁺ measurement

Cells were loaded and acquired as described¹⁵ (Supplementary Methods online). For flow cytometry, cells were loaded for 45 min with pluronic acid and a probe of the acetoxymethyl ester of the fluorescent Ca²⁺ indicator Fluo-4 (1 μ M; Invitrogen) in Kreb's solution (145 mM NaCl, 5 mM KCl, 1 mM Na₂HPO₄, 5 mM glucose, 1 mM CaCl₂, 0.5 mM MgCl₂, 10 mM HEPES and 0.1% (wt/vol) BSA, pH 7.4). Individual fluorescence values were then analyzed with FlowJo and Origin software to normalize the fluorescence with the first value according to the equation $(F/F_0) - 1$, where 'F' is the fluorescence at specific time point and 'F₀' is the fluorescence at time 0. The area under the curve was calculated for each cell. Where specified, cells were treated for 18 h with pertussis toxin (1 μ g/ml; Sigma-Aldrich) or for 5 min with TAK-779 (500 nM; National Institutes of Health AIDS Reagent Program).

Transwell assays

Chemotaxis was assessed in 8- μ m Transwell chambers (Corning). Matured BMDCs (1×10^5 cells) were loaded into the upper chamber previously coated for 1 h with Iscove's modified Dulbecco's medium and 1% (wt/vol) BSA, then CCL21 (25 ng/ml; R&D systems) was added to lower chamber (in Iscove's modified Dulbecco's medium and 1% (wt/vol) BSA), followed by incubation for 2 h at 37 °C and 5% CO₂. The chemotaxis index was calculated, with normalization of values to those obtained in conditions without CCL21.

Additional methods

Information on PCR (primers, Supplementary Table 1 online), electrophysiology, cell-mobility assays and skin staining is available in the Supplementary Methods.

Statistical analysis

Statistical significance was calculated with the one-way analysis of variance test. *P* values of less than 0.05 were considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank S.Y. Lin (ABgenomics) and B. Koller (University of North Carolina) for help with generation of *Trpm4*^{-/-} mice; C.S. Zuker (University of California, San Diego) for *Trpm5*^{-/-} mice; E. Ferrary for help with the patch-clamp setup; M. Benhamou and U. Blank for advice and critical reading of the manuscript; and J. Bex, A. Bouhalfaia and E. Couchi for help in animal care. Supported by the Association pour la Recherche sur le Cancer (G.B.), Institut National de la Santé et de la Recherche Médicale (M.D. and P.L.), Ministère de l'Enseignement Supérieur et de la Recherche, Université Paris 7 (N.S.), Fondation pour la Recherche Médicale (T.L. and P.L.) and Action Concertée Incitative Jeunes Chercheurs (P.L.).

References

1. Steinman RM, Hawiger D, Nussenzweig MC. Tolerogenic dendritic cells. *Annu. Rev. Immunol* 2003;21:685–711. [PubMed: 12615891]
2. Bonasio R, von Andrian UH. Generation, migration and function of circulating dendritic cells. *Curr. Opin. Immunol* 2006;18:503–511. [PubMed: 16777395]
3. Banchereau J, Palucka AK. Dendritic cells as therapeutic vaccines against cancer. *Nat. Rev. Immunol* 2005;5:296–306. [PubMed: 15803149]
4. Naik SH. Demystifying the development of dendritic cell subtypes, a little. *Immunol. Cell Biol* 2008;86:439–452. [PubMed: 18414430]
5. Lewis RS. Calcium signaling mechanisms in T lymphocytes. *Annu. Rev. Immunol* 2001;19:497–521. [PubMed: 11244045]
6. Feske S. Calcium signalling in lymphocyte activation and disease. *Nat. Rev. Immunol* 2007;7:690–702. [PubMed: 17703229]
7. Scharenberg AM, Humphries LA, Rawlings DJ. Calcium signalling and cell-fate choice in B cells. *Nat. Rev. Immunol* 2007;7:778–789. [PubMed: 17853903]
8. Watkins SC, Salter RD. Functional connectivity between immune cells mediated by tunneling nanotubules. *Immunity* 2005;23:309–318. [PubMed: 16169503]
9. Partida-Sanchez S, et al. Regulation of dendritic cell trafficking by the ADP-ribosyl cyclase CD38: impact on the development of humoral immunity. *Immunity* 2004;20:279–291. [PubMed: 15030772]
10. Hsu S, et al. Fundamental Ca²⁺ signaling mechanisms in mouse dendritic cells: CRAC is the major Ca²⁺ entry pathway. *J. Immunol* 2001;166:6126–6133. [PubMed: 11342632]
11. Scandella E, et al. CCL19/CCL21-triggered signal transduction and migration of dendritic cells requires prostaglandin E₂. *Blood* 2004;103:1595–1601. [PubMed: 14592837]
12. Launay P, et al. TRPM4 is a Ca²⁺-activated nonselective cation channel mediating cell membrane depolarization. *Cell* 2002;109:397–407. [PubMed: 12015988]
13. Nilius B, et al. Regulation of the Ca²⁺ sensitivity of the nonselective cation channel TRPM4. *J. Biol. Chem* 2005;280:6423–6433. [PubMed: 15590641]
14. Prawitt D, et al. TRPM5 is a transient Ca²⁺-activated cation channel responding to rapid changes in [Ca²⁺]_i. *Proc. Natl. Acad. Sci. USA* 2003;100:15166–15171. [PubMed: 14634208]
15. Launay P, et al. TRPM4 regulates calcium oscillations after T cell activation. *Science* 2004;306:1374–1377. [PubMed: 15550671]

16. Vennekens R, et al. Increased IgE-dependent mast cell activation and anaphylactic responses in mice lacking the calcium-activated nonselective cation channel TRPM4. *Nat. Immunol* 2007;8:312–320. [PubMed: 17293867]
17. Macatonia SE, Knight SC, Edwards AJ, Griffiths S, Fryer P. Localization of antigen on lymph node dendritic cells after exposure to the contact sensitizer fluorescein isothiocyanate. Functional and morphological studies. *J. Exp. Med* 1987;166:1654–1667. [PubMed: 3119761]
18. Clapham DE. SnapShot: mammalian TRP channels. *Cell* 2007;129:220. [PubMed: 17418797]
19. Demion M, Bois P, Launay P, Guinamard R. TRPM4, a Ca^{2+} -activated non-selective cation channel in mouse sino-atrial node cells. *Cardiovasc. Res* 2007;73:531–538. [PubMed: 17188667]
20. Floto RA, et al. Dendritic cell stimulation by mycobacterial Hsp70 is mediated through CCR5. *Science* 2006;314:454–458. [PubMed: 17053144]
21. Sanchez-Sanchez N, et al. Chemokine receptor CCR7 induces intracellular signaling that inhibits apoptosis of mature dendritic cells. *Blood* 2004;104:619–625. [PubMed: 15059845]
22. Pinheiro da Silva F, et al. CD16 promotes *Escherichia coli* sepsis through an FcR γ inhibitory pathway that prevents phagocytosis and facilitates inflammation. *Nat. Med* 2007;13:1368–1374. [PubMed: 17934470]
23. Zhang Y, et al. Coding of sweet, bitter, and umami tastes: different receptor cells sharing similar signaling pathways. *Cell* 2003;112:293–301. [PubMed: 12581520]
24. MacAry PA, et al. HSP70 peptide binding mutants separate antigen delivery from dendritic cell stimulation. *Immunity* 2004;20:95–106. [PubMed: 14738768]
25. Forster R, et al. CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. *Cell* 1999;99:23–33. [PubMed: 10520991]
26. Gunn MD, et al. Mice lacking expression of secondary lymphoid organ chemokine have defects in lymphocyte homing and dendritic cell localization. *J. Exp. Med* 1999;189:451–460. [PubMed: 9927507]
27. Hubbard KB, Hepler JR. Cell signalling diversity of the Gq family of heterotrimeric G proteins. *Cell. Signal* 2006;18:135–150. [PubMed: 16182515]
28. de Gorter DJ, et al. Bruton's tyrosine kinase and phospholipase C γ 2 mediate chemokine-controlled B cell migration and homing. *Immunity* 2007;26:93–104. [PubMed: 17239630]
29. Heissmeyer V, et al. Calcineurin imposes T cell unresponsiveness through targeted proteolysis of signaling proteins. *Nat. Immunol* 2004;5:255–265. [PubMed: 14973438]
30. Macian F, Im SH, Garcia-Cozar FJ, Rao A. T-cell anergy. *Curr. Opin. Immunol* 2004;16:209–216. [PubMed: 15023415]
31. Donnadieu E, Bismuth G, Trautmann A. The intracellular Ca^{2+} concentration optimal for T cell activation is quite different after ionomycin or CD3 stimulation. *Pflügers Arch* 1995;429:546–554. [PubMed: 7617445]
32. Bhakta NR, Oh DY, Lewis RS. Calcium oscillations regulate thymocyte motility during positive selection in the three-dimensional thymic environment. *Nat. Immunol* 2005;6:143–151. [PubMed: 15654342]
33. Randolph GJ, Ochando J, Partida-Sanchez S. Migration of dendritic cell subsets and their precursors. *Annu. Rev. Immunol* 2008;26:293–316. [PubMed: 18045026]
34. Sanchez-Sanchez N, Riolo-Blanco L, Rodriguez-Fernandez JL. The multiple personalities of the chemokine receptor CCR7 in dendritic cells. *J. Immunol* 2006;176:5153–5159. [PubMed: 16621978]
35. Ullrich ND, et al. Comparison of functional properties of the Ca^{2+} -activated cation channels TRPM4 and TRPM5 from mice. *Cell Calcium* 2005;37:267–278. [PubMed: 15670874]
36. Lindquist RL, et al. Visualizing dendritic cell networks *in vivo*. *Nat. Immunol* 2004;5:1243–1250. [PubMed: 15543150]
37. Ohl L, et al. CCR7 governs skin dendritic cell migration under inflammatory and steady-state conditions. *Immunity* 2004;21:279–288. [PubMed: 15308107]
38. Naik SH, et al. Intrasplenic steady-state dendritic cell precursors that are distinct from monocytes. *Nat. Immunol* 2006;7:663–671. [PubMed: 16680143]
39. Naik SH, et al. Development of plasmacytoid and conventional dendritic cell subtypes from single precursor cells derived *in vitro* and *in vivo*. *Nat. Immunol* 2007;8:1217–1226. [PubMed: 17922015]

40. Waskow C, et al. The receptor tyrosine kinase Flt3 is required for dendritic cell development in peripheral lymphoid tissues. *Nat. Immunol* 2008;9:676–683. [PubMed: 18469816]
41. Mailliard RB, et al. α -type-1 polarized dendritic cells: a novel immunization tool with optimized CTL-inducing activity. *Cancer Res* 2004;64:5934–5937. [PubMed: 15342370]
42. Ten Brinke A, Karsten ML, Dieker MC, Zwaginga JJ, van Ham SM. The clinical grade maturation cocktail monophosphoryl lipid A plus IFN γ generates monocyte-derived dendritic cells with the capacity to migrate and induce Th1 polarization. *Vaccine* 2007;25:7145–7152. [PubMed: 17719152]
43. Del Prete A, et al. Regulation of dendritic cell migration and adaptive immune response by leukotriene B4 receptors: a role for LTB4 in up-regulation of CCR7 expression and function. *Blood* 2007;109:626–631. [PubMed: 16985179]

Figure 1.

DC populations in TRPM4-deficient and littermate control mice. **(a)** Staining profiles (left) and absolute numbers (right) of splenic CD11c⁺CD11b⁺ DCs from TRPM4-deficient mice (*Trpm4*^{-/-}) and control mice (*Trpm4*^{+/+}). Numbers adjacent to outlined areas (left) indicate percent CD11c⁺CD11b⁺ cells. Each symbol (right) represents an individual mouse; small horizontal lines indicate the mean. **(b)** Flow cytometry of the expression of costimulatory markers on spleen DCs from TRPM4-deficient and control mice. **(c)** Flow cytometry of the expression of CD86 and MHC class II molecules (MHCII) on popliteal lymph node DCs in steady-state conditions (filled histograms) and 24 h after subcutaneous injection of *E. coli* (bold lines). Numbers above bracketed lines indicate percent CD86⁺ cells (left) or MHC class II-high cells (right). **(d)** Absolute numbers of CD11c⁺, MHC class II-high (MHCII^{hi}) DCs in popliteal lymph nodes 24 h after subcutaneous injection of *E. coli* into TRPM4-deficient and control mice. *, *P* < 0.01. Data are representative of ten **(a,b)** or six **(c,d)** independent experiments (error bars **(d)**, s.e.m.).

Figure 2.

Impaired migration of DCs in *Trpm4*^{-/-} mice. **(a)** Flow cytometry of CD11c⁺ gated cells from lymph nodes of *Trpm4*^{-/-} and littermate control mice painted with a 1% solution of FITC on the back skin 2 h before the addition of *E. coli* by scarification. Left, numbers above outlined areas indicate percent FITC⁺CD11c⁺ cells in draining (brachial) lymph nodes 12 h after scarification. FSC, forward scatter. Right, absolute numbers of FITC⁺CD11c⁺ cells at 12, 24 and 48 h in draining (brachial) lymph nodes and nondraining (mesenteric) lymph nodes. **(b)** Immunohistochemistry of uninfamed skin from *Trpm4*^{-/-} and control mice stained with anti-CD11c and counterstained with hematoxylin. Arrowheads indicate CD11c⁺ cells. Original magnification, ×400. **(c)** Flow cytometry of a 1:1 mixture of iDCs from *Trpm4*^{-/-} mice (CFSE^{lo}) and control mice (CFSE^{hi}) before and after injection into the footpads of recipient mice at 2 h before *E. coli* inoculation (top row); far right, BMDCs detected in popliteal lymph nodes of recipients at 12 h after *E. coli* footpad injection. Number adjacent to outlined area (far left) indicates percent CFSE⁺ cells from popliteal lymph nodes; numbers above bracketed lines (middle) and below outlined areas (right) indicate percent CFSE^{lo} cells (left) or CFSE^{hi} cells (right). SSC, side scatter. Below, absolute numbers of migrated BMDCs in popliteal lymph nodes of mice with (left) or without (right) *E. coli* injection. *, *P* < 0.05; **, *P* < 0.01. Data are representative of at least three independent experiments (error bars (a,c), s.e.m.).

Figure 3.

A TRPM4-like current is present in *Trpm4*^{+/+} BMDCs but not in *Trpm4*^{-/-} BMDCs. **(a)** Current-voltage relationships recorded in *Trpm4*^{+/+} and *Trpm4*^{-/-} BMDCs in the whole-cell patch-clamp configuration. The pipette solution contained mainly potassium glutamate (K-glut) with 1 μ M Ca^{2+} ; the external solution contained mainly sodium gluconate (Na-gluc). The reversal potential was -43.98 ± 15.72 mV in *Trpm4*^{+/+} DCs and -79.72 ± 5.08 mV in *Trpm4*^{-/-} DCs ($P = 0.032$). The diamond curve results from subtraction of the *Trpm4*^{+/+} values from the *Trpm4*^{-/-} values. HP, holding potential; V_M , membrane potential. **(b-g)** Inside-out recordings. **(b)** Current-voltage curve obtained with both pipette and bath containing a standard solution of 145 mM NaCl with 1 mM CaCl_2 . Points were fitted by linear regression to determine conductance (g 22.1 ± 0.6 pS). Inset, current traces at various voltages; dashed line, closed channel. **(c)** Current-voltage curves in 145 mM KCl (conductance, 22.3 ± 1.4 pS; reversal potential, 1.7 ± 1.2 mV; K^+ permeability/ Na^+ permeability, 0.95 ± 0.04) or in 42 mM NaCl (open circles); points were fitted by the Goldman-Hodgkin-Katz equation (reversal potential, 22.3 ± 1.5 mV; Cl^- permeability/ Na^+ permeability, 0.11). **(d)** Channel voltage dependence, showing the open probability (P_o) determined at various voltages in experiments identical to that in **(b)**. **(e)** Open probability at various $[\text{Ca}^{2+}]_i$ values ($V_M +40$ mV). Points were fitted with the Hill equation, which provided a half-maximum effector concentration of 14 μ M. **(f)** Effect of $[\text{Ca}^{2+}]_i$ on unitary channel activity ($V_M +40$ mV). c, closed state of the channel; o, open state of the channel. **(g)** Effect of internal ATP on an inside-out patch. o1, open state of one channel; o2, open state of two channels. Right, effect of ATP on the open probability. *, $P < 0.01$. Data are representative of at least five **(a,d)** or four **(b,c,e-g)** independent experiments (error bars **(c-e,g)**, s.e.m.).

Figure 4.

Trpm4 is the main DC CAN channel regulating Ca²⁺ signaling. (a) Entry of Ca²⁺ into iDCs after activation by ECS, assessed in *Trpm4*^{+/+} BMDCs (*n* = 46 cells) and *Trpm4*^{-/-} BMDCs (*n* = 51 cells). Area under the curve in arbitrary units (AU): 22.23 ± 1.82 AU, *Trpm4*^{+/+}; 33.58 ± 2.36 AU, *Trpm4*^{-/-}; *P* = 0.0003. Iono, ionomycin. (b) Ca²⁺ signaling in *Trpm4*^{+/+} iDCs (*n* = 54) and *Trpm4*^{-/-} iDCs (*n* = 104) after ECS stimulation in Ca²⁺-free solution (5 mM EGTA) with the subsequent addition of 2 mM Ca²⁺. Area under the curve: 102.38 ± 9.86 AU, *Trpm4*^{+/+}; 139.71 ± 7.42 AU, *Trpm4*^{-/-}; *P* = 0.003. (c) Ca²⁺ signals in *Trpm4*^{+/+} BMDCs with or without pretreatment with pertussis toxin: 14.07 ± 2.52 AU with pertussis toxin (*n* = 28 cells); 35.33 ± 3.51 AU without pertussis toxin (Control; *n* = 26 cells); *P* = 0.00001. (d) Ca²⁺ signals in *Trpm4*^{+/+} BMDCs with or without TAK-779 treatment: 15.15 ± 2.48 AU with TAK-779 (*n* = 43 cells); 27.6 ± 2.52 AU without TAK-779 (Control; *n* = 32 cells); *P* = 0.0009. (e) RT-PCR of the expression of *Trpm4* and *TRPM5* mRNA in BMDCs. Rps15, small ribosomal protein (positive control); H₂O, no DNA. bp, base=pairs. (f) Quantitative RT-PCR of the expression of *Trpm4* and *Trpm5* in *Trpm4*^{+/+} and *Trpm4*^{-/-} BMDCs, presented relative to the expression of Rps14 (which decodes ribosomal protein 14). (g) Ca²⁺ signals in *Trpm4*^{+/+}*Trpm5*^{-/-} BMDCs (25.6 ± 4 AU; *n* = 24 cells) and *Trpm4*^{-/-}*Trpm5*^{-/-} BMDCs (38.6 ± 2.3 AU; *n* = 28 cells); *P* = 0.006. *P* = 0.375, wild-type (*Trpm4*^{+/+}*Trpm5*^{+/+}) versus *Trpm4*^{+/+}*Trpm5*^{-/-}; *P* = 0.168, *Trpm4*^{-/-}*Trpm5*^{+/+} versus *Trpm4*^{-/-}*Trpm5*^{-/-}. (h) Flow cytometry of steady-state splenic DCs. Numbers above outlined areas indicate percent CD11c⁺CD11b⁺ cells. Data are representative at least three independent experiments (error bars (f), s.e.m.).

Figure 5.

Identical differentiation and maturation of *Trpm4*^{-/-} and *Trpm4*^{+/+} BMDCs. **(a)** Generation of CD11c⁺-CD11b⁺ BMDCs during *in vitro* culture. Gray shading indicates the culture period during which BMDCs were used for experiments. **(b,c)** Flow cytometry of the surface phenotypes of *Trpm4*^{+/+} and *Trpm4*^{-/-} BMDCs before (iDC) and after (mDC) 24 h of stimulation with LPS **(b)** or fixed *E. coli* **(c)**. Data are representative of three independent experiments (error bars **(a)**, s.e.m.).

Figure 6.

Greater mobility but less chemotaxis of *Trpm4*^{-/-} iDCs. (a) Time-lapse video microscopy (4 h) of the mobility of *Trpm4*^{+/+} iDCs ($n = 26$) and *Trpm4*^{-/-} iDCs ($n = 25$) after stimulation with fixed *E. coli* (left); colored lines indicate the movement of single cells every 5 min. Scale bars, 100 μm . Right, mean distance. (b,c) Transwell migration of *Trpm4*^{+/+} and *Trpm4*^{-/-} BMDCs triggered by CCL21 (25 ng/ml) after 24 h of maturation with fixed *E. coli* (b) or LPS (c), assessed as the ratio of chemotaxis with CCL21/chemotaxis without CCL21. $P = 0.0008$, fixed *E. coli* versus LPS for *Trpm4*^{-/-} BMDCs; $P = 0.087$, fixed *E. coli* versus LPS for *Trpm4*^{+/+} BMDCs. (d) *In vivo* migration of *Trpm4*^{-/-} (CFSE^{lo}) and control (CFSE^{hi}) iDCs injected as a 1:1 mixture into the footpads of recipient mice 2 h before LPS injection (50 ng/footpad), presented as the absolute number of migrated BMDCs of each genotype in popliteal (Draining) and brachial (Nondraining) lymph nodes 24 h after LPS injection. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Data are representative of at least three independent experiments (error bars, s.e.m.).

Figure 7.

PLC-β2 downregulation induced by Ca^{2+} overload in *Trpm4*^{-/-} DCs. **(a)** Ca^{2+} signals after ECS stimulation of *Trpm4*^{+/+} BMDCs (122.82 ± 12.43 AU; $n = 57$ cells) and *Trpm4*^{-/-} BMDCs (68.93 ± 6.13 AU; $n = 64$ cells) matured by stimulation with fixed *E. coli*. $P = 0.0001$. **(b)** Ca^{2+} signals after CCL21 stimulation of *Trpm4*^{+/+} and *Trpm4*^{-/-} BMDCs matured with fixed *E. coli* (19.31 ± 4.59 AU, *Trpm4*^{+/+}; 8.58 ± 6.49 AU, *Trpm4*^{-/-}; $P = 0.038$). **(c)** Ca^{2+} signals after ECS stimulation of *Trpm4*^{+/+} mDCs (80.9 ± 10.5 AU; $n = 70$ cells) and *Trpm4*^{-/-} mDCs (48.6 ± 8.6 AU; $n = 57$ cells) in the absence of external Ca^{2+} . $P = 0.0007$. **(d)** CCL21-mediated Ca^{2+} responses of *Trpm4*^{+/+} and *Trpm4*^{-/-} BMDCs matured with *E. coli* in the presence of EGTA. **(e)** PLC-β2 protein expression in *Trpm4*^{+/+} (+/+) and *Trpm4*^{-/-} (-/-) BMDCs before (iDC) and after *E. coli* maturation or after 24 h of incubation with ionomycin (1 μM). Right, PLC-β2 expression relative to that of β-actin, normalized to iDC values. *, $P < 0.05$; **, $P < 0.01$. **(f)** Expression of PLC-γ1 and PLC-γ2 in *Trpm4*^{+/+} and *Trpm4*^{-/-} BMDCs before and after Ca^{2+} -dependent maturation. β-actin serves as a loading control. Data are representative of three **(a,c,f)**, at least five **(b,d)**, or eleven **(e)** independent experiments (error bars **(e)**, s.e.m.).