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TAO-kinase 3 governs the terminal differentiation of NOTCH2-dependent splenic conventional dendritic cells

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Antigen-presenting conventional dendritic cells (cDCs) are broadly divided into type 1 and type 2 subsets that further adapt their phenotype and function to perform specialized tasks in the immune system. The precise signals controlling tissue-specific adaptation and differentiation of cDCs are currently poorly understood. We found that mice deficient in the Ste20 kinase Thousand and One Kinase 3 (TAOK3) lacked terminally differentiated ESAM+ CD4+ cDC2s in the spleen and failed to prime CD4+ T cells in response to allogeneic red-blood-cell transfusion. These NOTCH2- and ADAM10-dependent cDC2s were absent selectively in the spleen, but not in the intestine of Taok3−/− and CD11c−cre Taok3fl/fl mice. The loss of splenic ESAM+ cDCs was cell-intrinsic and could be rescued by conditional overexpression of the constitutively active NOTCH intracellular domain in CD11c-expressing cells. Therefore, TAOK3 controls the terminal differentiation of NOTCH2-dependent splenic cDC2s.

Significance

Dendritic cells (DC) play a crucial role in the immune system by bridging innate and adaptive immunity. In the spleen, a specific subset of DCs accumulates around the blood-filtering marginal zone to capture particulate antigens such as red blood cells. We show here that these specialized DCs develop in response to NOTCH2 instruction in the spleen and are required for defense against attaching and effacing pathogens such as Citrobacter rodentium. Despite the clear role for NOTCH2 in terminal differentiation of cDCs, it is unclear how Notch pathway activation is regulated. Only a subset of cDCs becomes Notch-instructed, despite ubiquitous expression of NOTCH2 by all cDCs. Furthermore, although NOTCH2 ligation markedly improved the generation of CD103+ CD11b+ cDC2s in the intestinal lamina propria. These specialized cDC2s produce high amounts of IL-23 and are required for defense against attacking and effacing pathogens such as Citrobacter rodentium (26). Despite the clear role for NOTCH2 in terminal differentiation of cDCs, it is unclear how Notch pathway activation is regulated. Only a subset of cDCs becomes Notch-instructed, despite ubiquitous expression of NOTCH2 by all cDCs (27). Furthermore, although NOTCH2 ligation markedly improved the generation of cDC1s in vitro, the prototypical ESAM marker is not induced in cDC2s.
of the transcription factors IRF4 and IRF8 in cDC1s and cDC2s (Fig. 1D).

In secondary lymphoid organs, cDCs occupy specific anatomical locations crucial for their function (9). In the spleen, cDC2s have been shown to reside in the marginal zone and in bridging channels, which connect the T cell zone with the red pulp through interruptions in the ring of CD169+ macrophages surrounding the white pulp (37, 38). Immunostaining for CD11c and DCIR2 (33D1) allowed us to address the location of cDC2s in Taok3−/− mice (Fig. 1E). While DCIR2+ clusters were readily identified in bridging channels of wild-type (WT) littermates, they were absent in the spleen of Taok3−/− mice. Although the median DCIR2 expression was slightly lower on Taok3−/− cDC2s (Fig. 1D), this could not sufficiently explain the drastic loss of DCIR2 signal on histology. Accordingly, CD11c+ cells were scattered throughout the red pulp and did not aggregate in well-defined clusters at the bridging channels in Taok3−/− mice. In contrast, the staining of CD11c+ cells residing in the T cell zone was preserved in knockout mice, in line with the preserved cDC1 counts found by flow cytometry. Of note, no B220 staining was identified outside of the ring of the CD169+ marginal metallophilic macrophages, consistent with the absence of MZB cells in Taok3−/− mice (33).

cDC Development in Taok3−/− Mice. We wondered whether the loss of ESAM+ CD4+ cDC2s in Taok3−/− mice reflected defects in the development of cDCs from progenitors in the bone marrow. Committed precDCs have been identified in bone marrow and spleen (12, 39). In the bone marrow, the absence of Taok3 had no effect on the frequency of bipotent S Ig eH+ Ly6C− and S Ig eH+ Ly6C+ precDCs (gating strategy, SI Appendix, Fig. S2B). S Ig eH− Ly6C− CD24hi precDCs were equally represented in the bone marrow but increased in the spleen of Taok3−/− mice compared to littermate controls. The percentage of S Ig eH− Ly6C− precDCs, the direct precursors for mature cDC2s, was significantly elevated in bone marrow and spleen (Fig. 1F). These data suggest that the decreased cDC2 numbers in Taok3−/− mice are not due to defective development of DC progenitors. Rather, the accumulation of Taok3−/− precDCs implies a failure of these cells to undergo terminal differentiation.

To study whether the loss of cDC2s was unique to spleen, we examined the frequency of cDCs in other lymphoid and non-lymphoid organs. We found intact numbers of cDC2s in the lung, liver, and skin-draining lymph node (SLN) of Taok3−/− mice (Fig. 1G). However, subtle changes were noted in the phenotype of cDC2s across different tissues: the expression levels of F4/80 and CD172a were consistently lower in spleen, lung, liver, and lymph nodes. In addition, the MFI of CD11b significantly increased in the liver but not in the lung of Taok3−/− mice (SI Appendix, Fig. S3 A–E). We found a small but significant increase in the number of cDC1s in the lung, while resident cDC1s in the SLN decreased in knockout mice (Fig. 1G). Thus, loss of Taok3 is associated with an altered cDC2 phenotype across different tissues.

Altered Gene Expression Profile in Taok3−/− cDCs. To identify Taok3-regulated genes, we compared genome-wide expression profiles of splenic cDC1s and cDC2s from Taok3−/− mice and littermate controls. Principal component analysis demonstrated separation of cDC1 and cDC2 cell types along one direction, and separation of Taok3−/− and Taok3−/+ cells along another (SI Appendix, Fig. S3F).
**Fig. 1.** Taok3−/− mice lack ESAM+ CD4+ splenic cDCs. (A) The FlowSOM algorithm was run on single, live, lineage (CD3e, CD19, NK1.1, Ter119) splenocytes from Taok3−/− mice and littermate controls. A minimal spanning tree with 49 nodes and 20 metaclusters was generated by automated, unsupervised clustering. The average FlowSOM tree is displayed for all Taok3+/+ (Left) and all Taok3−/− (Right) mice. Node size corresponds to the frequency of that cell cluster among all analyzed cells. Pie charts indicate the mean expression of the given surface markers for each node (SI Appendix, Fig. S1A). In the Taok3+/+ tree, background colors denote the different metaclusters while, in the Taok3−/− tree, the background color highlights metaclusters that are significantly decreased (blue) or enriched (red) in Taok3−/− mice. Two-tailed Mann–Whitney U test with Bonferroni correction for multiple testing. Data are representative of two biologically independent experiments (n = 8 to 9 mice per group). The FlowSOM algorithm was run five times to ensure reproducibility of the results. (B) Representative contour plots of splenic cDCs from Taok3+/+ and Taok3−/− mice (gating strategy, SI Appendix, Fig. S2A). Numbers adjacent to the gates denote percentage of total cDCs. (C) Number of cDC1s, cDC2s, and ESAM+ CD4+ cDCs in Taok3+/+ (black) and Taok3−/− (blue) spleen. Conventional DC1s are defined as XCR1+ CD172a− cDCs, and cDC2s as XCR1− CD172a+ cDCs. Data pooled from two independent experiments (n = 8 to 9 mice per group), two-tailed Mann–Whitney U test. (D) Histograms indicating expression levels on the surface of splenic cDC1s (Top) and cDC2s (Bottom) of Taok3+/+ (black) and Taok3−/− (blue) mice. Data are representative of at least two independent experiments (n = at least 8 mice per group). (E) Confocal image of spleens from Taok3+/+ and Taok3−/− mice. (Magnification: 10×.) Green: CD169; white: DCIR2; magenta: CD11c; blue: B220. Data are representative of three independent experiments (n = 13 mice per group). (F) Frequency of precDCs (gating strategy, SI Appendix, Fig. S2B) among live cells in bone marrow and spleen of Taok3+/+ (black) and Taok3−/− (blue) mice. Data pooled from two independent experiments (n = 10 per group). Two-tailed Mann–Whitney U test. (G) Absolute number of cDC1s and cDC2s in SLN (Left), lung (Middle), and liver (Right) of Taok3+/+ (black) and Taok3−/− (blue) mice. “Res.” and “Migr.” indicate resident and migratory cDC populations, respectively. Data are representative of at least two independent experiments (n = 8 to 10 for lung and liver) or are representative of 4 to 5 mice per group (SLN). Two-tailed Mann–Whitney U test. (H and I) cDNA microarray on sorted splenic cDC1s and cDC2s from Taok3+/+ and Taok3−/− mice. (H) Volcano plot based on fold change and P value of all transcripts in cDC1s (Left) and cDC2s (Right). (I) Heatmap of differentially expressed genes between Taok3+/+ and Taok3−/− cDC1s (Top) or cDC2s (Bottom) for the individual samples. Dendritic cells were sorted from three mice per group. Bars indicate mean ± SD; *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.
Gene expression analysis revealed 8 differentially expressed (DE) genes in cDC1s and 101 DE genes in cDC2s between wild-type and knockout cells, while Taok3 was effectively knocked out in both cDC subsets (Fig. 1H). These results are consistent with the minor impact of Taok3 loss on cDC1 homeostasis compared to the effects observed in cDC2s. Top DE genes for cDC1s and cDC2s are listed in Fig. 1I. Based on gene ontology (GO) enrichment analysis, the processes associated with down-regulated genes concern mainly regulation of interleukin-2 (IL-2) biosynthesis and leukocyte differentiation.

In contrast, the main GO term associated with up-regulated genes in Taok3−/− cDC2s is regulation of cell adhesion. Indeed, many DE genes in cDC2s related to cell adhesion and migration such as L1cam, Epn2m, Vim, Ilgals1, and Lgals3 were up-regulated, while Mmp9 and Mmp12 were down-regulated in knockout cDC2s. Differentially expressed genes in cDC2s also comprised type 1 IFN-induced genes such as Ifit1 and Oasl2. In addition, the expression of several genes associated with small GTPases increased (Rhob) or decreased (Arhgap6 and Rasgrp3) in Taok3−/− cDC2s.

![Fig. 2.](image-url) Loss of Taok3 hampers CD4+ T cell priming to allogeneic red blood cells. (A) Histograms of surface CD40, CD86, and CCR7 expression on splenic cDC2s from Taok3+/+ (black) and Taok3−/− mice (blue). Mice were injected intraperitoneally with 20 μg LPS in PBS (dotted line) or PBS only (solid line) and killed 8 h later. (B) Median fluorescence intensities of CD40, CD86, and CCR7 on cDC2s from A. One-way ANOVA with post hoc Tukey’s test. (C) Confocal images of spleens from Taok3−/− mice and littermate controls treated with LPS or PBS as in A. (Magnification: 10×) B220 in blue, CD169 in white, CD11c in orange. Data in A–C are representative of two independent experiments (n = 7 to 8 per group in total). (D–F) Taok3−/− and littermate control mice were injected with 5 × 10^5 CellTrace Violet-labeled OTII-specific CD45.1+ CD4+ T cells and transfused with 100 μL of HOD-RBCs (Materials and Methods) 1 day later. (E) Representative dot plots of OTII cells in the spleen and inginal lymph node (iLN) of Taok3+/+ (Left) and Taok3−/− (Right) acceptor mice 72 h after HOD-RBC transfusion. Cells are gated on live CD19− NK1.1− Ter119− CD3ε+ CD4+ CD45.1+ T cells. Numbers adjacent to the gates indicate the percentage of OTII cells. (F) Proliferation and expansion index of OTII cells in the spleen of the acceptors, according to the Flowjo proliferation algorithm. Two-tailed Mann–Whitney U test. Data in E and F are representative of two independent experiments (n = 14 to 15 acceptor mice per group in total). Bars indicate mean ± SD; ns, nonsignificant; *P < 0.05, **P < 0.001, and ****P < 0.0001.
Functional Responses of Taok3−/− cDCs. We reasoned that the defects in terminal differentiation might affect the maturation and function of Taok3-deficient cDCs. To assess their ability to mature and migrate in response to pathogen-associated molecular patterns, we injected mice with the TLR4 ligand lipopolysaccharide (LPS) intravenously (i.v.) (40). Upon exposure to LPS, cDCs from Taok3−/− mice up-regulated the costimulatory markers CD40, CD80 and CD86 to the same extent as littermate controls (Fig. 2 A and B). Mature Taok3−/− cDCs also up-regulated the chemokine receptor CCR7, albeit not to the same level as wild-type cDCs. To verify whether the difference in surface expression levels of CCR7 impacted the ability of DCs to migrate to the T cell zone, we assessed the position of CD11c+ cells in the spleen 8 h after LPS exposure in vivo (40). In wild-type mice, the CD11c+ clusters in the bridging channels had disappeared and the DCs had relocated to the T cell zone upon LPS injection. In Taok3−/− mice, similar accumulation of CD11c+ cells in the T cell zone was observed (Fig. 2C). Thus, Taok3 deficiency did not impair the normal maturation and migration of DCs to the T cell zone.

Next, we wanted to probe the capacity of Taok3−/− cDCs to prime CD4+ T cell immune responses in vivo. The role of splenic cDCs in the generation of CD4+ T cell responses against blood-borne antigens and apoptotic and damaged cells is well established (25, 37, 41, 42). More specifically, efficient CD4+ T cell priming successively depended on CD4+ cDCs in a model of alloigenic red-blood-cell transfusion (43). Taok3−/− mice and littermate controls received a transfusion of stored red blood cells (RBCs) from congenic B6 mice that express a membrane-bound mate controls received a transfusion of stored red blood cells (RBCs) from congenic B6 mice that express a membrane-bound antigens. Two hours after transfusion, both types of CD4+ T cells were enriched for the set of genes down-regulated in cDCs (43, 44). To address whether cDC homeostasis requires Taok3 expression in hematopoietic or stromal cells, we crossed Taok3−/− mice to Vav-cre mice that express the CRE recombinase constitutively in all hematopoietic cells. Because the gene trap construct of Taok3−/− mice contains loxP sites, Taok3 expression can be restored selectively by CRE-mediated recombination and gene deletion. We found that the percentage of ESAM+ CD4+ cDC2s was almost completely restored in the spleen of Taok3−/− x Vav-cre−/− mice, ruling out a role for stromal Taok3 in cDC2 development (SI Appendix, Fig. S4 A and B). Conceivably, the observed cDC phenotype of Taok3−/− mice could be secondary to their MZB cell deficit. Marginal zone B cells do occupy a similar niche as bridging channel cDCs, and regulatory roles of B cells in cDC homeostasis have been described (6). To test this hypothesis, we crossed Taok3−/− mice to Mb1-cre mice allowing selective gene trap reversal in B cells (45). While the number of CD21.35+CD23lo MZB cells was partially restored in Taok3−/− x Mb1-cre−/− mice, the number and phenotype of splenic cDCs did not differ from littermate Taok3−/− x Mb1-cre−/− controls (Fig. 3A and B). In order to address more directly whether the role of Taok3 in cDCs was cell-intrinsic, we generated Taok3−/− x Cd11c-cre mice. Strikingly, the number of ESAM+ CD4+ cDC2s in Taok3−/− x Cd11c-cre−/− mice was restored to wild-type levels (Fig. 3A and B). As expected, MZB cells were still completely absent in these mice. Thus, selective restoration of Taok3 expression in Cd11c+ cells was sufficient for ESAM+ CD4+ cDC2 development. We validated these findings in Taok3−/− x Cd11c-cre−/− x Cd11c-cre mice, where the CRE is expressed only after the precDC stage. While this still restored cDC2 numbers, it rescued ESAM and CD4 expression less efficiently than the earlier acting CD11c-cre, suggesting a role for Taok3 early in the commitment of precDC2s to cDCs (SI Appendix, Fig. S4 C and D).

Rescue of Taok3 expression in CD11c-positive cells was also sufficient to restore clustering of DCIR2+ dendritic cells at marginal zone bridging channels (Fig. 3C). Together, these data demonstrate independent and cell-intrinsic roles of Taok3 in DCs and MZB cells. Furthermore, they prove that the presence of splenic cDC2s and their correct positioning at bridging channels does not depend on the presence of marginal zone B cells.

To reveal even more subtle intrinsic differences in DC homeostasis and development, we also set up competitive bone marrow chimeric experiments, where cells of wild-type or Taok3−/− origin need to compete in the same host. Lethally irradiated wild-type mice were reconstituted with CD45.2 Taok3−/− and CD45.1 wild-type bone marrow in equal parts (Fig. 3D). After reconstitution, MZB cells were almost uniquely derived from the WT compartment, while bone marrow of both genotypes contributed equally to the follicular B-cell population in the spleen (Fig. 3E), confirming earlier results (33). Interestingly, Taok3 expression was not intrinsically required in cDC1s or in DC progenitors in spleen and bone marrow. In contrast, wild-type ESAM+ CD4+ cDC2s outcompeted Taok3−/− counterparts by a ratio of more than 10:1. This effect was less pronounced on the total cDC2 population, as Taok3-deficient bone marrow still gave rise to ESAM+ cDC2s. We finally also created conditional Taok3−/−/− mice and crossed them to CD11c-cre mice to generate Taok3−/− cDCs. We confirmed a significant decrease in the number and percentage of cDC2s in the spleen of Taok3−/− cDCs (Fig. 3 F–H). Again, this reduction was specific for a subpopulation of cDCs that expressed high levels of CD4, ESAM, and F4/80. Despite having decreased numbers of ESAM+ CD4+ cDC2s, Taok3−/− cDCs were still able to induce CD4+ T cell responses to allogeneic HOD-RBCs (SI Appendix, Fig. S4 E and F), implying that additional defects contribute to the impaired T cell priming in Taok3−/− mice. While the frequency of cDC1s was comparable to littermate controls, the MFI of CD8α and CD205 was slightly lower in Taok3−/− mice (Fig. S5G), supporting our earlier findings. Based on these results, we conclude that Taok3 plays a nonredundant, cell-intrinsic role in the terminal differentiation of ESAM+ CD4+ cDCs. Impaired Notch Signaling in Taok3−/− cDCs. The homeostasis of ESAM+ CD4+ cDC2s requires trophic signals through the lymphotixin β receptor (LTβR) (6, 20, 26). We hypothesized that TAOK3 deficiency in DCs (20, 26). Moreover, we previously demonstrated that Notch instruction is hampered in Taok3−/− deficient mice is highly reminiscent of the phenotype of mice lacking Notch signaling in DCs (20, 26). Moreover, we previously demonstrated that Notch instruction is hampered in Taok3−/− B cells due to the lack of surface ADAM10 in transitional B cells, the immediate precursors of MZB cells (33). To assess whether loss of Taok3 also affected the NOTCH2 pathway in cDCs, we performed gene set enrichment analysis (GSEA) on the gene expression data from Taok3−/− mice using published gene expression profiles of Notch2+/− cDCs (26). We found that the down-regulated genes in Taok3−/− cDCs and cDCs were significantly enriched for the set of genes down-regulated in Notch2+/− cDCs and cDCs, respectively. Equally, the set of genes up-regulated in Notch2+/− cDCs and cDCs was strongly
Fig. 3. The role of Taok3 in splenic cDC2s is cell-intrinsic. (A) Representative contour plots of cDCs (Top row) and marginal zone B cells (Bottom row) in the spleen of Taok3−/−, Taok3+/+, Taok3−/− x CD11cIcre (where Taok3 expression is selectively restored in CD11c-expressing cells) and Taok3−/− x Mb1cre mice (where Taok3 expression is selectively restored in Mb1-expressing cells). The ESAM+ CD4+ cDC gate was pregated on cDCs, and the MZB cell gate was pregated on live CD19+, CD93− cells. (B) Frequency of the indicated cDC subsets among all cDCs (Top Left) and their absolute number (Bottom Left). Number of marginal zone B cells (Bottom Right). One-way ANOVA with Tukey's post hoc test. Data in A and B are representative of two independent experiments (n = 7 to 8 per group). (C) Representative confocal images of spleens from Taok3+/+ (Left), Taok3−/− (Middle), and Taok3−/− x CD11cIcre mice (Right). (Magnification: 10x.) White: DCIR2; blue: B220; red: CD3e. Data are representative of two independent experiments (n = 4 to 8 per group). (D) Schematic representation of CD45.1 WT:CD45.2 Taok3−/− mixed BM chimeras. (E) Contribution of donor cells to immune cell populations 10 wk after transplantation in BM and spleen. Results are expressed as the ratio between CD45.1 (WT) and CD45.2 (Taok3−/−) cells. Ratios of CD45.1/CD45.2 origin along cDC1 and cDC2 development are compared to follicular (FoB) and marginal zone (MZB) B cells. Unless otherwise indicated, cell populations originate from the spleen. The same Ly6C− SiglecH+ and Ly6C+ SiglecH+ BM precDC populations are depicted twice. One-way ANOVA with Bonferroni correction for multiple testing. Data representative of two independent experiments (n = 13 mice). (F) Representative contour plot of ESAM and CD4 expression on splenic cDCs from Taok3ΔDC mice. (G) Histograms illustrate surface expression of the indicated markers on splenic cDC1s (Top) and cDC2s (Bottom) from Taok3ΔDC mice (blue) and Cre-negative littermate controls (black). (H) Quantification of splenic cDC subsets as a percentage of all cDCs (Left) and in absolute numbers (Right). Two-tailed Mann–Whitney U test. Data in F and H are representative of four mice per group. Numbers adjacent to the gates (A and F) indicate the percentage of the parent population. Bars indicate mean ± SD; *P < 0.05, **P < 0.01, ****P < 0.0001.
overrepresented among the up-regulated genes in Taok3Δ/Δ cDC1s and cDC2s, respectively (SI Appendix, Fig. S6 A–D). These findings reveal considerable overlap between the gene expression signatures of Notch2−/− and Taok3Δ/Δ dendritic cells.

Recently, a method was described to generate CD8α− cDC1s and cDC2s in vitro on DLL1-expressing fibroblasts in a NOTCH2-dependent manner (28, 29). To further assess the ability of Taok3Δ/Δ precDCs to undergo Notch instruction, we cultured Taok3Δ/Δ and Taok3Δ/Δ−/− bone marrow-derived DCs (BMDCs) on OP9 cells transduced with DLL1 or with a GFP construct as a control. We confirmed that WT BMDCs cultured on OP9-DLL1 fibroblasts generated higher numbers of DC1s and DC2s and lower numbers of plasmacytoid DCs (pDCs) than did OP9-GFP cocultures (SI Appendix, Fig. S6E). In line with previous findings (29), OP9-DLL1 strongly induced the expression of CD8α and CD205 on BM-derived cDCs but failed to induce relevant levels of ESAM expression on DCs. Compared to littermate controls, cocultures of Taok3Δ/Δ−/− BMDCs with OP9-DLL1 fibroblasts generated fewer XCR1+ DC1s and CD127α+ DC2s (SI Appendix, Fig. S6E). Additionally, loss of Taok3 markedly reduced the expression of levels of CD8α and CD205 on DCs (Fig. 4 A and B).

We next interrogated how Notch signaling might be affected by loss of Taok3. Expression of the NOTCH2 receptor was intact in Taok3−/− cDCs at protein and messenger RNA levels (SI Appendix, Fig. S6 F and G). Activation of the canonical Notch pathway is regulated by specific posttranslational cleavage events that occur upon ligand binding (46). ADAM10 is a crucial NOTCH2 protease in vivo and has been shown to control the development of marginal zone B cells (34, 35). By analogy with transitional B cells, we wondered whether surface expression of ADAM10 was altered in Taok3−/− DCs or their progenitors. However, despite extensive efforts, we failed to demonstrate detectable levels of specific surface staining of ADAM10 in wild-type DCs or precDCs by flow cytometry (SI Appendix, Fig. S6H). To nevertheless assess a possible role of ADAM10 in the development of Taok3−/− and Notch2-dependent DCs, we crossed conditional Adam10floxed mice to CD11c-cre mice. In the spleen of these Adam10floxed/+ mice, ESAM+ CD4+ cDC2s were completely absent (Fig. 4 C and D), and clustering of DCIR2α+ cDC2s at the bridging channels was impaired (Fig. 4E). To address whether the role of Adam10 in cDCs was cell-intrinsic, we generated mixed bone marrow chimeras. We lethally irradiated wild-type mice and reconstituted them with CD45.1+ BM and CD45.2+ Adam10 Δ/Δ−/− bone marrow in a 1:1 ratio (Fig. 4F). After reconstitution, we found equal contributions of both donors to the follicular B-cell pool, while MZB cells were preferentially of WT origin (Fig. 4G). This suggests off-target effects of CD11c-cre in MZB cells, a finding supported by the reduced MZB rim in Adam10 Δ/Δ−/− found on histological examination (Fig. 4E). Along the developmental continuum of cDC2s, a step-up in the WT/Adam10Δ/Δ−/− ratio was seen from the precDC2 stage to the mature cDC2 stage. An additional step-up was seen in the ratio of ESAM+ CD4+ cDC2s, which were almost exclusively derived from Adam10−/−- sufficient bone marrow. In contrast, mature cDC1s and precDCs were equally derived from wild-type and Adam10Δ/Δ−/− bone marrow. The defect in ESAM+ CD4+ cDC2s in Adam10Δ/Δ−/− mice was of functional importance, as the proliferation of OVA-specific CD4+ T cells upon transfusion of HOD-RBCs was significantly reduced (SI Appendix, Fig. S7 A and B). In conclusion, these observations underscore a cell-intrinsic role of Adam10 in ESAM+ CD4+ cDC2s. Furthermore, the highly similar phenotypes of Taok3−/−, Adam10−/−, and Notch2-conditional knockout mice suggest regulation by a common molecular pathway, involved in MZB and cDC2 development in the splenic marginal zone, although the nature of the interaction remains elusive.

Taok3 Is Redundant for the Development of CD103+/− CD11b+ cDC2s in the Small Intestine. It has been reported that NOTCH2 also controls the expression of ESAM in a subset of cDC1s in the spleen and in resident cDC2s in SLN and mesenteric lymph nodes (MLN) (26). Upon careful examination, we confirmed ESAM expression on ±20% of cDC1s in wild-type spleen. Remarkably, this fraction was significantly reduced in Taok3Δ/Δ mice (SI Appendix, Fig. S8 A and B). Similarly, the percentage of ESAM-expressing cells decreased in resident cDC2s in SLN and MLN (SI Appendix, Fig. S8 C and D). Notch2−/− conditional knockout mice also lack a subset of cDC2s in the small intestine that express CD103. Given the importance of Taok3 for the development of Notch2-dependent cDC2s in spleen, we further investigated whether Taok3 is equally required for CD103+/− CD11b+ cDCs in the small intestine. The percentage of CD103+/− cDC2s in mesenteric lymph nodes and in the lamina propria of the small intestine was unaltered in Taok3−/− mice (SI Appendix, Fig. S9 A–C) and Taok3Δ/Δ mice (SI Appendix, Fig. S9D) compared to littermate controls. Next, we analyzed the contribution of Taok3-sufficient and Taok3−/− bone marrow to intestinal cDC subsets in mixed bone marrow chimeras. The WT:Taok3Δ/Δ origin ratio for CD103+/− CD11b+ cDC2s was not different from the ratio of precDC2s in bone marrow (SI Appendix, Fig. S9E). Together, these data suggest that Taok3 is redundant for the development of CD103+/− cDC2s in the small intestine.

Rescue of Taok3−/− Phenotype by NICD Overexpression. We finally reasoned that if Taok3 controlled NOTCH2 signaling in cDCs, forced overexpression of the downstream constitutively active notch intracellular domain (NICD) might override the dependency on Taok3. To test this hypothesis, we crossed Rosa26-LSL-NICD mice (47) onto a Taok3Δ/Δ and Taok3−/− background. In these mice, NICD is overexpressed from the Rosa26 locus only after Cre-mediated recombination and removal of a transcriptional stop site. Because the original Taok3−/− mice contained a floxed gene trap, which would also be excised by Cre-mediated recombination, we first had to generate another Taok3 knockout mouse by removing exon 6 with CRISPR-Cas9 technology. We confirmed that the cDC phenotype of these Taok3Δ+−/− mice was identical to that of the original Taok3−/− mice (SI Appendix, Fig. S10A). In wild-type littermates, CD11c-cre-driven NICD overexpression increased the fraction of ESAM+ CD4+ and ESAM− CD4− cDCs, confirming the role of NOTCH2 signaling in these cDC subsets. Overexpression of NICD in Taok3Δ/Δ−/− cDCs significantly rescued the development of cDC2s. In particular, the percentage of cDCs expressing ESAM and CD4 increased more than five-fold (Fig. 4 H and J). In cDC1s, NICD overexpression restored the expression of CD8α to levels comparable to wild-type mice (Fig. 4 I and K). Accordingly, a normal proportion of cDC1s expressed ESAM again in Taok3Δ+−/− NICDΔ11c mice (SI Appendix, Fig. S10 B and C).

Based on the results from in vivo and in vitro experiments, we conclude that Taok3 controls terminal differentiation of NOTCH2- and ADAM10-dependent cDC2s in the spleen.

Discussion

In contrast to cDC1s, cDC2s harbor striking heterogeneity, determined at least partially by tissue-specific imprints. This allows cDC2s at the bridging channels was impaired (Fig. 4...
Fig. 4. Defective NOTCH signaling in Taok3−/− cDCs. (A) Representative contour plots of bone marrow-derived dendritic cells from Taok3−/− mice or littermate controls cocultured with OP9-GFP or OP9-DL1 cells. Plots are pregated on live GFP− lineage− B220− CD64− CD11c+ MHCII+ cells. (B) Median fluorescence intensity of CD8α and CD205 on the surface of cDC1s from Taok3−/− or Taok3+/+ BM-derived DCs cocultured with OP-DL1 fibroblasts. Two-tailed Mann–Whitney U test. Data in A and B are representative of two independent experiments (nine biological replicates per group). (C) Representative contour plot of ESAM and CD4 expression on splenic cDCs from Adam10ΔDC mice and Cre-negative littermate controls. (D) Absolute number of splenic cDC2s (Top) and percentage of ESAM+ CD4+ cells (Bottom) among cDCs in Adam10ΔDC mice. Two-tailed Mann–Whitney U test. Data in C and D are representative of four independent experiments (n = 15 to 16 per group). (E) Confocal image of spleens from Adam10ΔDC (Right) and littermate control (Left) mice. (Magnification: 10×.) White: DCIR2; blue: B220; magenta: CD11c. (F) Schematic representation of WT: Adam10ΔΔ mixed bone marrow chimeras. (G) Contribution of donor cells to several cell populations during cDC1 and cDC2 development, 10 wk after mixed BM transplantation. Results are expressed as the ratio between CD45.1 (WT) and CD45.2 (Adam10ΔDC) cells. Unless otherwise indicated, cell populations originate from spleen. Ly6C− SiglecH+ and Ly6C+ SiglecH+ BM precDCs are depicted twice. Data are representative of five acceptor mice. (H and I) Representative contour plots of splenic cDCs from Taok3−/− NIKDΔ94 CD11ccre−/− (Taok3−/− control), Taok3−/− NIKDΔ94 CD11ccre+/− (Taok3−/− NICDΔ1011c), Taok3−/− NIKDΔ94 CD11ccre+/+ (Taok3−/− NICDΔ1011c control), and Taok3−/− NIKDΔ94 CD11ccre+/+ (Taok3−/− NICDΔ1011c). (J) Frequency of ESAM+ CD4+ cells among cDC2s in spleen. (K) Frequency of CD8α+ cells among cDC1s in spleen. One-way ANOVA with Tukey’s post hoc test (J and K). Data in H–K are representative of two independent experiments (n = 6 to 9 per group). Numbers adjacent to the gates in A, C, H, and I represent the percentage of total cDCs. Bars indicate mean ± SD; *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.
The function of Taok3 upstream of the Notch2 pathway is supported by several lines of evidence. First, we found that both genes control the development of an identical population of splenic cDCs that is characterized by high levels of surface ESAM, CD4, and F4/80. Interestingly, loss of Taok3 affected the expression of F4/80 and CD172a across different tissues, implying that some of its effects might extend to all cDCs. Whether the expression of F4/80 and CD172a is also affected by Notch2 deficiency is not known. Second, Taok3 controlled ESAM expression in a fraction of cDC1s and resident lymph node cDCs, subpopulations that were selectively ablated in Notch2-deficient mice (26). Using gene set enrichment analysis, we found a significant overlap between the gene expression signatures from Taok3−/− and Notch2−/− cDCs and identified a set of core genes regulated by both Taok3 and Notch2 in cDC1s and cDCs. Next, Taok3−/− bone marrow generated fewer DC1s and DC2s in an in vitro model of NOTCH-driven dendritic cell differentiation (29). Moreover, surface expression of CD8α and CD205, target genes of the NOTCH pathway, was lower in Taok3−/− cDC1s in vitro and in vivo. Finally, we demonstrated how conditional overexpression of the NICD rescued development of ESAM-expressing cDC1s and cDC2s in Taok3−/− mice, indicating a function of Taok3 upstream of NICD.

Surprisingly, loss of Taok3 did not affect the differentiation of NOTCH2-dependent CD103+ cDC2s in the small intestine. Similarly, Taok3−/− cDC1s did not exactly phenocopy the effect of Notch2 deficiency (20). In contrast to the pronounced and cell-intrinsic defect of cDCs in Notch2fl/fl x CD11cCre mice, the number of CD8α+ cDC1s did not significantly decrease in Taok3−/− or Taok3 ΔDC mice. In addition, no intrinsic effects were observed in competitive bone marrow chimeras. These findings imply that Taok3 might differentially regulate NOTCH signaling in different subtypes of cDCs. Interestingly, no effect on splenic cDC1s and intestinal CD103+ cDC2s was observed in RBPjkop x CD11cCre mice, suggesting that in these cells NOTCH2 might signal through a noncanonical pathway that is not controlled by Taok3. Nonredundant roles for noncanonical NOTCH signaling have previously been demonstrated in immune cells, although the mechanisms remain unclear (49–51). To validate the coexistence of both pathways in cDCs, it would be interesting to assess whether RBPjk controls ESAM expression in cDC1s and whether cDC2s require Notch2 for ESAM transcriptional program, that could explain why key NOTCH target genes such as Hes1 and Delta1 were not differentially expressed in Taok3−/− cDC2s. Alternatively, these target genes might be expressed only briefly during a specific developmental window before the mature cDC stage. Nevertheless, the fact that Notch2−/− mice are embryonically lethal (52) while Taok3−/− mice are not further supports the idea that Taok3 only controls Notch signaling under highly specific conditions.

The role of Taok3 and NOTCH signaling seems to concentrate on the terminal differentiation of cDCs. The precDC1s and precDC2s numbers increased in the spleen of Taok3−/− mice, implying that the transition to mature cDCs requires Taok3. However, the effects of CD11c-cre–mediated gene trap reversal were more pronounced than those of the later-acting CD11c-cre–GFP, indicating that Taok3 activity in precDCs might already determine their late differentiation. In line with this, NOTCH also controlled target gene expression in ESAM−/− cDC2s (26). Analogous to our findings in MZB cell development (33), we hypothesized that Taok3 controlled the action of ADAM10 by regulating its translocation to the cell surface. However, we failed to identify a convincing ADAM10 signal on cDCs or precDCs by flow cytometry. The reasons behind this observation remain unclear. It is possible that the surface levels on precDCs are lower than those on transitional B cells or that ADAM10 is more rapidly down-regulated ex vivo. Alternatively, ADAM10 might be expressed only transiently during a brief developmental window. Yet, the drastic reduction in NOTCH2-dependent cDC1s and cDC2s in conditional ADAM10 knockout mice strongly suggests that ADAM10 is the essential NOTCH2 sheddase in DCs. In support of our findings, a role of dendritic cell ADAM10 in the differentiation of cDC2s and the generation of Th2 responses was previously reported by us and others (53, 54). Although off-target effects of the CD11c-driven Cre have been described (55, 56), our mixed bone marrow experiments clearly confirmed the effects of ADAM10 to be cell-intrinsic. Still, it remains unclear whether ADAM10 bioactivity in cDCs is controlled by TAOK3 and how this process is regulated at the molecular level. To advance our understanding of the role of TAOK3 in controlling ADAM10 localization and/or activity in cDCs, the development of additional tools such as reporter mice and in vivo assays for proteolytic activity is warranted.

The capacity of Taok3−/− mice to mount CD4+ T cell responses to transfused allogeneic red blood cells was impaired. This was not caused by an inability of cDCs to undergo normal maturation and migrate to the T cell zone upon TLR activation. Conceivably, the remaining Taok3−/− cDCs might be unable to process or present antigens efficiently, a conclusion not supported by in vitro findings (25). Alternatively, the defective clustering of cDCs in marginal-zone bridging channels could limit the exposure of Taok3-deficient cDCs to antigens. Indeed, the precise location of cDC2 in bridging channels, which is controlled by sphingosine-1-phosphate (SIP) and by gradients of dihydroycholesters acting on the chemokine receptor EB2 (GPR183), (37, 41, 57, 58) is required for efficient T cell priming to circulating particulate antigens (37, 41, 59). It is unclear whether EB2 signals and Notch instruction are interrelated or simply control different aspects of cDC2 function. It has been reported that Gpr183−/− cDC2s still express ESAM and can be rescued by treatment with an LITJR agonist (37). These findings sharply contrast with the phenotype of Taok3−/− cDCs, implying that the effects of Taok3 cannot be completely explained by interference with the EB2 pathway. Why CD4+ T cell priming was similarly impaired in Adam10ΔDC mice but not in Taok3ΔDC mice remains unclear. We speculate that some functional collaboration exists between marginal zone B cells, cDC2s, and macrophages in priming the T cell response to allogeneic RBCs. Still, it remains unclear whether ADAM10 bioactivity in cDCs is controlled by sphingosine-1-phosphate (SIP) and by gradients of dihydroycholesters acting on the chemokine receptor EB2 (GPR183), (37, 41, 57, 58) or that the effects of ADAM10 to be cell-intrinsic. Why CD4+ T cell priming was similarly impaired in Adam10ΔDC mice but not in Taok3ΔDC mice remains unclear. We speculate that some functional collaboration exists between marginal zone B cells, cDC2s, and macrophages in priming the T cell response to allogeneic RBCs. Still, it remains unclear whether ADAM10 bioactivity in cDCs is controlled by sphingosine-1-phosphate (SIP) and by gradients of dihydroycholesters acting on the chemokine receptor EB2 (GPR183), (37, 41, 57, 58) or that the effects of ADAM10 to be cell-intrinsic.

Materials and Methods

Mice. The WT C57BL/6J mice were obtained from the Janvier laboratory. Taok3−/− mice were described previously (33). In brief, a loxP–flanked splicing acceptor was inserted as a gene trap in the intronic region between exon 1 and exon 2 of the Taok3 gene, leading to premature transcriptional termination. Conditional Taok3fl/fl mice were generated in-house with the Easy-CRISPR method (64). The guide RNAs (gRNAs) were designed using the CRISPOR webtool to target a region in intron 3 (5′-GCCTGGTGGTCTGACAT AT 3′) and a region in intron 6 (5′-GGAGGCTGAGGCGGAACCAA 3′) in order to introduce loxP sites flanking exon 6 (ENSMMUSE00000373662.1) of the Taok3 gene. A long single-stranded DNA repair template containing exon 6,
upstream and downstream intronic region with loxP sites inserted, was acquired as described above. Automated analysis of flow cytometry samples was conducted in a LightCycler 480 (Roche) using the SensiFAST sybr no-ROX mix (Bioline). The following primer pairs were used: Gadph (5′ GGGTGC- GAAACTCTGCTTCC-3′), Taok3 (5′ TTGTCTCACTGACC-3′/5′ TTGTCTCACTGACC-3′), and Hoc1 (5′ TTGTCTCACTGACC-3′/5′ TTGTCTCACTGACC-3′). Images were acquired with a Zeiss LSM710 confocal microscope equipped with 488-, 561-, and 633-nm lasers and with a tunable two-photon laser. Emissions were recorded in three separate channels. Digital pictures at 1,024×1,024-pixel density and 8-bit depth were acquired with Zen software (Zeiss). Images were analyzed with Imaris software.

RNA Extraction and qRT-PCR. Whole-spleen tissue was homogenized with the TissueLyser II (Qiagen) prior to RNA extraction using TRIzol reagent (Roche). Splenic cDNAs and cDNAs were sorted directly into RNAeasy lysis buffer, and RNA was extracted with the RNeasy Mini kit (Qiagen) according to the manufacturer’s protocol. The complementary DNA (cDNA) was synthesized using the Sensifast cDNA synthesis kit (Bioline). qRT-PCR reactions were conducted in a LightCycler 480 (Roche) using the SensiFAST sbyr no-ROX mix (Bioline). The following primer pairs were used: Gsp (5′ GGCGT- TGGTTCAGTACGAGC, 3′ TGGATGATGCTGCCCACTTTCA), Ubc (5′ AAAACCTCCCACT- TCTCCGACG, 5′ CGTATGTCCTCCTCCAGGAAG), Serotec Bio-Rad. PE-conjugated DCIR2 (33D1), eFluor660-conjugated CD11c (N418), and eFluor450-conjugated CD11c (N418) antibodies were obtained from eBioscience. APC-conjugated CD4 (G4-210), PE-conjugated CD4 (G4-210), and eFluor450-conjugated CD11c (N418) antibodies were obtained from eBioscience. Sections were stained with the primary antibodies diluted in PBT (phosphate-buffered saline) containing 1% bovine serum albumin (Sigma) (PBT buffer) for 30 min. After washing, the cells were incubated with PerCP-eFluor710-conjugated B220 (RA3-6B2) antibodies and with eFluor450-conjugated CD93 (AA4.1), CD45.1 (B6.SJL-129S7ScN-Ptcr−/+), and eFluor660-conjugated CD11c (N418) antibodies. After washing, the cells were incubated for 30 min at 4°C with a mixture of viability dye (eFluor506 or eFluor780 from eBioscience) to identify dead cells and with an FcγRIII antibody (2.4G2) for 30 min to limit aspecific binding. After washing, the cells were incubated for 30 min at 4°C with a mixture of fluochrome- or biotin-labeled antibodies. The following antibodies were used: B220 (RA3-6B2), B220 (RA3-6B2), CD3 (145-2C11), CD4 (GK1.5), CD8 (53-6.7), CD45.1 (B6.SJL-129S7ScN-Ptcr−/+), CD11c (N418), CD11b (M1/70), CD11c (N418), CD19 (1D3), CD21 (53.6.7), CD23 (B23), CD24 (B23), M246 (2.145L), CD25 (3/23), CD43 (56), CD45 (30-5F1), CD61 (5B4.1A3), CD68 (16-10A1), CD86 (PO3), CD93 (AA4.1), CD103 (2-E7), CD135 (A2F10), CD172a (P84), CD122a (P84), CD205 (205yekta), DCIR2 (33D1), ESAM (1G8), F4/80 (BM8), IgM (II/41), LT (R), Ly6C (HK1.4 or AL-21), Ly6G (1A8), MHCII (M5/114.15.2), NK1.1 (PK136), Siglec H (ebio440c), Ter119 (TER-119), and XCR1 (ZEN).
Culture of Bone Marrow-Derived Dendritic Cells on OP9-DL1 Cells. OP9 cell lines transduced with GFP- or DL1-encoding retroviruses were cultured in MEM medium containing 20% fetal calf serum (FCS, Bodinco), 100 μg/mL genticyn (Gibco), and 1% GlutaMAX (Gibco) at 37 °C and 5% CO2. Single-cell suspensions of bone marrow were generated by crushing tibias and femurs and filtering them over a 70-μm nylon sieve. After erythrocyte lysis, cells were resuspended in complete RPMI-1640 medium supplemented with 10% FCS (Bodinco), 1.1 mg/mL 1-mercaptoethanol, 50 μg/mL genticyn, 1% GlutaMAX, and 250 ng/mL HFL3T. The BM cells were plated at 2 × 10^6 per well in 24-well plates. On day 3 of the primary BM culture, half of the cells from every well were transferred to a single well that contained a mono-layer of OP9-GFP or OP9-DL1 cells. OP9 cells were pretreated with 10 μg/mL mitomycin C for 2 h at 37 °C and plated on 24-well plates 1 d before the start of the coculture. Cell cultures were analyzed on day 7.

Microarray and GSEA. Dendritic cells were sorted from the spleen of Taok2<sup>−/−</sup> and Taok3<sup>−/−</sup> animals by fluorescence-activated cell sorting. Within the conventional dendritic cell gate, cDC1s were defined as XCR1<sup>+</sup> CD172α<sup>−</sup> and cDC2s as XCR1<sup>−</sup> CD172α<sup>+</sup>. RNA was extracted using the QiAshredder spin column (Qiagen) and RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol and sent to the nucleomics facility, Flemish Institute for Biotechnology (VIB), Leuven, Belgium, where the microarrays were performed using the GeneChip Mouse Gene 1.0 ST arrays (Affymetrix). Samples were subsequently analyzed using R/Bioconductor. All samples passed quality control, and the Robust Multiarray Average (RMA) procedure was used to normalize data within arrays (probe set summarization, background correction, and quantile normalization). Array data were subsequently analyzed using R/Bioconductor. All samples passed quality control, and the Robust Multiarray Average (RMA) procedure was used to normalize data within arrays (probe set summarization, background correction, and quantile normalization). Array data were subsequently analyzed using R/Bioconductor. All samples passed quality control, and the Robust Multiarray Average (RMA) procedure was used to normalize data within arrays (probe set summarization, background correction, and quantile normalization). Array data were subsequently analyzed using R/Bioconductor. All samples passed quality control, and the Robust Multiarray Average (RMA) procedure was used to normalize data within arrays (probe set summarization, background correction, and quantile normalization).


53. S. R. Damle et al., ADAM10 and Notch1 on murine dendritic cells control the development of type 2 immunity and IgE production. Allergy 73, 125–136 (2018).


