



ROR γ t-expressing dendritic cells are functionally versatile and evolutionarily conserved antigen-presenting cells

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Conventional dendritic cells (cDCs) are potent antigen-presenting cells (APCs) that integrate signals from their environment allowing them to direct situation-adapted immunity. Thereby they harbor great potential for being targeted in vaccination, autoimmunity, and cancer. Here, we use fate mapping, functional analyses, and comparative cross-species transcriptomics to show that ROR γ t⁺ DCs are a conserved, functionally versatile, and transcriptionally distinct type of DCs. ROR γ t⁺ DCs entail various populations described in different contexts including Janus cells/ROR γ t-expressing extrathymic Aire-expressing cells (eTACs), subtypes of Thetis cells, ROR γ t⁺-DC (R-DC) like cells, cDC2C and ACY3⁺ DCs. We show that in response to inflammatory triggers, ROR γ t⁺ DCs can migrate to lymph nodes and in the spleen can activate naïve CD4⁺ T cells. These findings expand the functional repertoire of ROR γ t⁺ DCs beyond the known role of eTACs and Thetis cells in inducing T cell tolerance to self-antigens and intestinal microbes in mice. We further show that ROR γ t⁺ DCs with proinflammatory features accumulate in autoimmune neuroinflammation in mice and men. Thus, our work establishes ROR γ t⁺ DCs as immune sentinel cells that exhibit a broad functional spectrum ranging from inducing peripheral T cell tolerance to T cell activation depending on signals they integrate from their environment.

dendritic cells | ROR γ t | AIRE | innate lymphocytes | antigen presenting cells

cDCs are immune sentinels located in lymphoid and nonlymphoid tissues (1–3). They effectively sense pathogens and subsequently migrate to and initiate T cell responses in secondary lymphoid organs (1–3). Their functional versatility makes them attractive for being targeted in autoimmunity and vaccination against pathogens or cancer (2–5). Accordingly, considerable work has been invested in understanding the functional and ontogenetic diversity of cDCs, yet these cells remain ambiguous to define as fate mapping has recently uncovered novel populations with overlapping phenotype but distinct origin (2, 6–11).

Type 2 cDCs (cDC2) are potent regulators of CD4⁺ T cell responses (1–3) that entail Notch2-dependent ESAM^{high} or T-bet-expressing cDC2A and Notch2-independent ESAM^{low} or T-bet-negative cDC2B that derive from myeloid progenitors (12–14). Both subtypes can induce Th17 responses but cDC2A regulate T follicular helper cell (T_{fh}) responses (15), while cDC2B may better promote Th2 responses (16). Recently, fetal liver lymphoid progenitors and lymphoid-derived transitional DCs (tDCs) have been shown to differentiate into cells resembling cDC2A and cDC2B (8–10, 17), the functional consequences of which are still unclear. tDCs can also contribute to antiviral responses and inflammation induced-immunopathology (8, 9, 17). Additionally, DC3 arise from monocytic progenitors, resemble cDC2B but in vitro appear superior to cDC2B in polarizing producing Th17 cells (11, 18, 19). The above heterogeneity suggests a careful division of labor between DC subtypes which needs to be better defined to uncover the full functional spectrum of cDCs.

Next to cDCs, ROR γ t-expressing APCs have emerged as potent regulators of T cell-mediated tolerance (20–30). ROR γ t is a ligand-activated transcription factor encoded by the *Rorc* gene that differs from its isoform ROR γ by three amino acids at the amino terminus (31–33). ROR γ t-expressing APCs include subsets of ILC3s, ROR γ t⁺ extrathymic AIRE-expressing cells (eTACs—including Janus and AIRE⁺ ILC3-like cells), as well as other surfacing populations like Thetis cells (28–31, 34, 35). Their lineage relationships and functional specializations are ill-defined because ROR γ t-expressing APCs phenotypically resemble each other and share expression of CD11c and ZBTB46 with cDCs (25,

Significance

Antigen-presenting cells (APCs) orchestrate T cell immunity. Retinoic acid receptor-related orphan receptor- γ t (ROR γ t)-expressing APCs are heterogeneous regulators of T cell tolerance but their subtypes and lineage relationships are ill-defined. We report that ROR γ t⁺ dendritic cells (DCs) are evolutionarily conserved, exhibit wide tissue distribution and reconcile various ROR γ t⁺ APC populations known to promote peripheral T cell tolerance. We show that ROR γ t⁺ DCs can sense pathogens, migrate to lymph nodes, activate naïve CD4⁺ T cells, and accumulate in demyelinating neuroinflammation with a proinflammatory phenotype. Thus, ROR γ t⁺ DCs have a broad functional spectrum ranging from inducing T cell tolerance to T cell activation depending on signals they integrate from their environment. This highlights their therapeutic potential and their affiliation with DCs.

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29, 30, 35, 36). Janus cells express *Aire*, ROR γ t and the integrin b8 (*Itgb8*) but lack ILC3 markers CXCR6 and IL7R. They phenotypically and transcriptionally resemble CCR7 $^{+}$ migratory cDCs (29, 35) and have primarily been profiled from pooled cervical, brachial, axillary, inguinal, and mesenteric lymph nodes (mLN) (35). Thus, it is unclear if they exhibit site-specific heterogeneity. Thetis cells have first been described in neonatal mLN and resemble Janus cells although they are apparently absent from skin draining LN (30). Only a fraction of Thetis cells (TCI and TCIII) expresses *Aire*, while TCIV expresses *Itgb8* but lacks *Aire* (30). While Janus and Thetis cells in mice drive T cell tolerance, human R-DC-like cells that resemble Thetis cells can activate allogeneic T cells (37). Thus, the above cell types may function beyond T cell tolerance, highlighting the need for a more accurate alignment of Janus cells, Thetis cells, and ROR γ t $^{+}$ eTAC subsets, and the tissue-specific signals that regulate them.

cDC2B from adult mouse spleen show accessible chromatin at ROR response elements (RORE) in bulk ATAC sequencing, leading to the notion that cDC2B expresses ROR γ t (12, 31). However, active ROR γ t protein expression in cDC2B from adult mice has not been reported and only a minor fraction of cDC2B show ROR γ t expression history (10, 12). In contrast, we have demonstrated active ROR γ t protein expression in a fraction of cDC2-like cells in neonatal mouse spleen and Peyer's patches, that phenotypically and transcriptionally more closely resemble cDC2A than cDC2B (10, 38). These ROR γ t $^{+}$ cDC2-like cells in the neonatal spleen do not arise from *Clec9a*-expressing myeloid cDC progenitors (10), suggesting they could be a unique type of APC, possibly related to Thetis cell subsets, which resemble cDC2 in terms of CD11b expression (30).

Here, we show that ROR γ t $^{+}$ DCs are a transcriptionally distinct and evolutionarily conserved DC subtype that reconciles various previously described ROR γ t $^{+}$ APC populations, including ROR γ t $^{+}$ eTACs, Janus and Thetis cells, ROR γ t $^{+}$ cDC2-like cells, R-DC like cells, ACY3 $^{+}$ DCs (39) and PRDM16 $^{+}$ cDC2C (40). We demonstrate that ROR γ t $^{+}$ DCs are ontogenetically and transcriptionally distinct from other cDC subtypes, as well as from ILC3. Yet, ROR γ t $^{+}$ DCs bear hallmark features of cDCs, including migration to lymph nodes and ability to activate naïve CD4 $^{+}$ T cells in response to inflammatory stimuli that warrant their affiliation with DCs.

Results

ROR γ t $^{+}$ DCs Exist in the Murine Spleen Across Age. We first profiled spleens from ROR γ t GFP *Clec9a*^{Cre} *Rosa*^{Tom} and *Clec9a*^{Cre} *Rosa*^{Tom} mice systematically across age by flow cytometry. In these mice, Tomato expression tracks cells arising from committed *Clec9a*-expressing myeloid cDC progenitors (41), while GFP expression reports the ROR γ t-specific isoform encoded by *Rorc* (42). We first identified MHCII $^{+}$ ILC3s as CD90 $^{+}$ CD127 $^{+}$ MHCII $^{+}$ GFP $^{+}$ cells and then gated cDCs as CD11c $^{+}$ MHCII $^{+}$ cells lacking the canonical ILC3 markers CD90 and CD127—also known as interleukin-7 receptor (IL-7R) (Fig. 1A and SI Appendix, Fig. S1A). Within CD11c $^{+}$ MHCII $^{+}$ splenocytes, we detected GFP-expressing cells at all ages examined (Fig. 1A). Importantly, GFP $^{+}$ CD11c $^{+}$ MHCII $^{+}$ cells also stained with an anti-RORC antibody (Fig. 1E), confirming that GFP signal in ROR γ t GFP mice accurately reports ROR γ t protein expression and that anti-RORC antibody staining in CD11c $^{+}$ MHCII $^{+}$ cells predominantly identifies cells expressing the γ t isoform of RORC. Unbiased gating of GFP $^{+}$ cells showed that ROR γ t $^{+}$ CD11c $^{+}$ MHCII $^{+}$ cells lacked ILC3 markers CD127, CD90, and CXCR6 (Fig. 1B and C and SI Appendix, Fig. S1B and C). The frequency of ROR γ t $^{+}$ cells within CD11c $^{+}$ MHCII $^{+}$ cells declined with age; however, their absolute numbers correlated with organ size and increased until weaning age and remained constant

until adulthood (8 to 15 wk of age, Fig. 1F and SI Appendix, Fig. S1D).

We had previously found that CD11b $^{+}$ ROR γ t $^{+}$ cDC2-like cells were nearly undetectable by two weeks of age (10). Indeed, at one week of age, CD11c $^{+}$ MHCII $^{+}$ ROR γ t $^{+}$ cells uniformly expressed CD11b but lacked CD24. Starting at two weeks of age ROR γ t $^{+}$ cells showed a CD11b^{low} to negative phenotype, while retaining the cDC2 marker CD172a (10) (Fig. 1D), and stained positive for CD24 (Fig. 1A and B). At all ages examined ROR γ t $^{+}$ CD11c $^{+}$ MHCII $^{+}$ cells stained negative for the cDC1-specific marker XCR1 (Fig. 1B). Thus, the apparent lack of CD11b by two weeks of age explains why we had previously missed these cells in adult mice and suggests that these cells are either heterogeneous or change their phenotype with age. In line with our previous work (10), ROR γ t $^{+}$ CD11c $^{+}$ MHCII $^{+}$ cells lacked Tomato expression in *Clec9a*^{Cre} *Rosa*^{TOM} mice at all ages examined, supporting that they do not arise from *Clec9a*-expressing DC progenitors (10, 41). ROR γ t $^{+}$ CD11c $^{+}$ MHCII $^{+}$ cells were present in *Rag2*^{-/-} γ c^{-/-} mice, which lack lymphocytes, including ILC3s and their precursors (Fig. 1G and H) (43), but fate mapped prominently in lymphoid-specific hCD2^{iCre} *Rosa*^{YFP} lineage-tracer mice (SI Appendix, Fig. S1E). ROR γ t $^{+}$ CD11c $^{+}$ MHCII $^{+}$ cells were strongly reduced in mice deficient for *fms*-like tyrosine kinase 3 ligand (FLT3L) (Fig. 1I), a growth factor critical for the development of all DCs and ILCs (44, 45). Thus, ROR γ t $^{+}$ CD11c $^{+}$ MHCII $^{+}$ cells exist in the murine spleen across age and can either be detected using an anti-RORC antibody or by GFP signal in ROR γ t GFP mice (Fig. 1A and E). As these cells do not develop in FLT3L^{-/-} mice and phenotypically resemble DCs (10), we refer to these cells as ROR γ t $^{+}$ DCs from hereon in accordance with suggestions for DC and ILC nomenclature (1, 31, 46).

Single-Cell Multiomic Profiling Aligns Murine ROR γ t $^{+}$ DCs with Janus Cells, ROR γ t $^{+}$ eTACs and Thetis Cells. To gain insight into the transcriptional and regulatory relatedness of ROR γ t $^{+}$ DCs to cDCs and ILC3s across age, we performed paired single-cell RNA sequencing (scRNA-seq) and single-cell assay for transposase-accessible chromatin (scATAC) from the same cell using 10X multiomic profiling. ROR γ t $^{+}$ DCs and MHCII $^{+}$ ILC3s were sorted from spleens of two-week-old or adult ROR γ t GFP mice and mixed at a 1:1 ratio to enrich for ROR γ t $^{+}$ DCs. CD11c $^{+}$ MHCII $^{+}$ cDCs were added in 10-fold excess to capture their heterogeneity. After quality filtering, we retained chromatin accessibility and gene expression profiles from 9,899 and 11,980 nuclei respectively. Unsupervised clustering based on gene expression (RNA) and open chromatin (ATAC) profiles revealed 18 and 16 clusters, respectively, that were largely congruent and could be classified as cDC1, cDC2, migratory cDCs (migDCs), ILC3s, pDCs, and tDCs (Fig. 2A and SI Appendix, Fig. S2A–F and Table S1). cDC2 split into 5 clusters based on gene expression, of which clusters 1, 2, and 3 corresponded to cDC2A (12, 47) (SI Appendix, Fig. S2C). Of note, cDC2 clusters 1 and 2 showed uniform chromatin accessibility (SI Appendix, Fig. S2B and D), as discussed below. cDC2 clusters 4 and 5 transcriptionally resembled both cDC2B and DC3 and were denoted as cDC2B/DC3 as they could not confidently be delineated as either cell type (Fig. 2A and SI Appendix, Fig. S2C and E) (11, 12). pDCs showed uniform chromatin accessibility but split into two clusters based on gene expression (SI Appendix, Fig. S2A, B, and D). *Rorc* $^{+}$ cells distributed across 5 clusters, of which ILC3_1 and ILC3_2 scored high for ILC3 signature genes and expressed *Rora*, *Il7r*, *Cxcr6*, and one cluster transcriptionally resembled ex-ILC3 (48) (Fig. 2A and B and SI Appendix, Fig. S2C and Table S2). Additionally, one cluster expressed *Rorc*, *Prdm16*, and *Lingo4* (10) while lacking *Il7r*, *Rora*, and *Cxcr6* and transcriptionally resembled ROR γ t $^{+}$ cDC2-like cells from neonatal

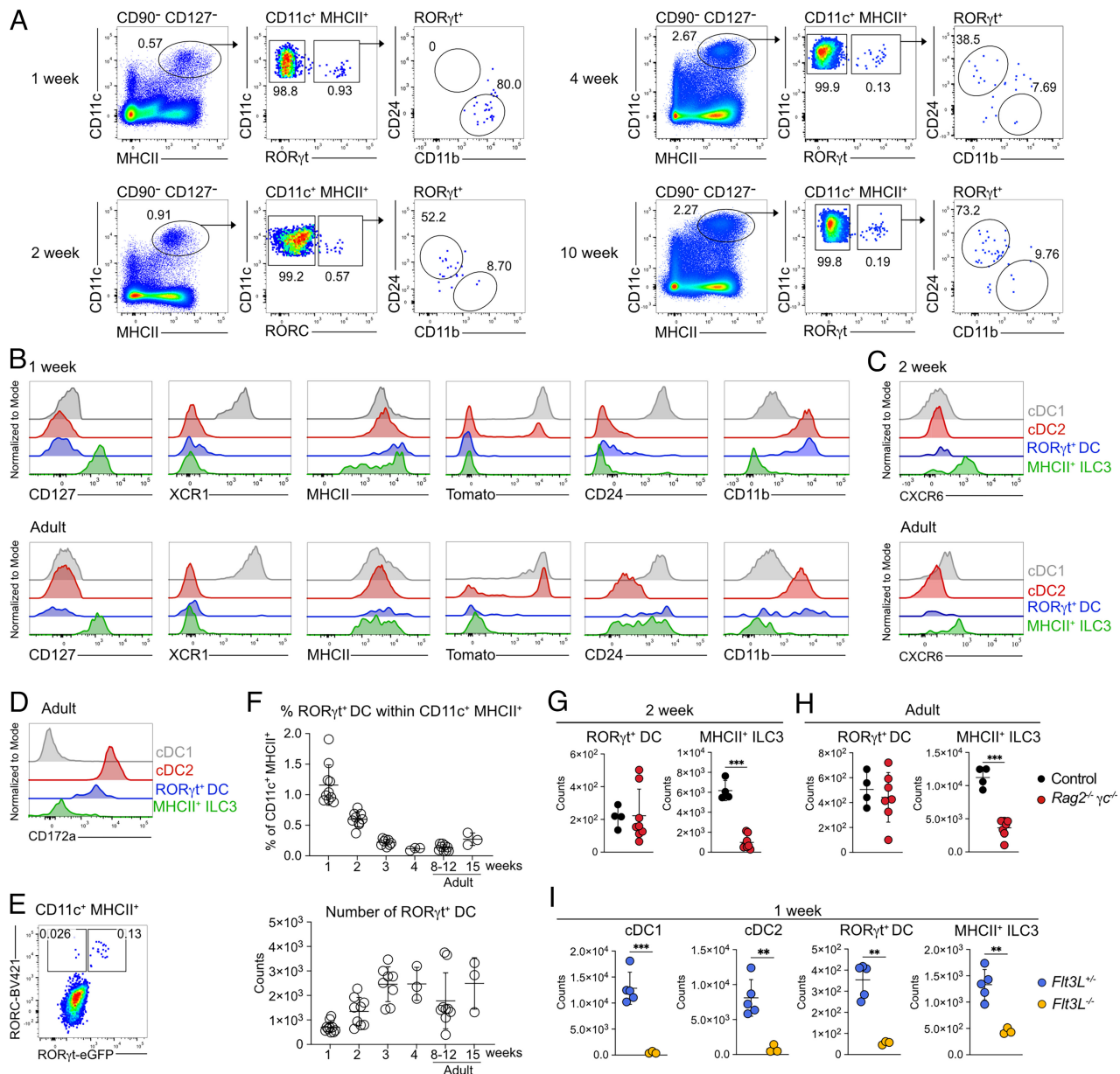


Fig. 1. RORγt⁺ DCs exist in the murine spleen across age. (A and B) Splenocytes from *Clec9a^{Cre}Rosa^{Tom}* or *Clec9a^{Cre}Rosa^{Tom}RORγt^{GFP}* mice at the indicated ages were analyzed by flow cytometry. (A) Live CD90⁺CD127⁻CD11c⁺MHCII⁺ cells were gated and RORγt⁺ cells revealed by GFP or anti-RORC intranuclear staining (2-wk time point). GFP⁺ or RORC⁺ cells were further analyzed for CD11b and CD24 expression. (B–D) Expression of the indicated surface markers in cDC1 (gray), cDC2 (red), MHCII⁺ ILC3 (green), and CD11c⁺MHCII⁺RORγt⁺ cells (blue) from spleens of mice at the indicated ages. Gating strategy *SI Appendix, Fig. S1A*. (E) CD11c⁺MHCII⁺ cells from *RORγt^{GFP}* mice were stained using an anti-RORC antibody to demonstrate that anti-RORC staining and RORγt driven GFP are largely congruent. (F) Frequency of RORγt⁺ cells within CD11c⁺MHCII⁺ cells and number of RORγt⁺ DCs in spleens of the indicated ages (1-wk-old n = 10; 2-wk-old, n = 9; 3-wk-old n = 8, 4-wk-old n = 3; 8 to 12-wk-old n = 6; 15-wk-old n = 3). Data are pooled from 1 to 2 independent experiments; each data point represents a biological replicate. (G and H) MHCII⁺ ILC3 and RORγt⁺ DCs were quantified in spleens from 2-wk-old (G) and adult *Rag2^{-/-}γc^{-/-}* mice (H) and littermate controls. Each data point represents an individual mouse from two independent experiments. (I) Quantification of cDC1, cDC2, RORγt⁺ DC, and MHCII⁺ ILC3 in spleens from 1-wk-old *Flt3L^{-/-}* and *Flt3L^{+/+}* littermate controls. RORγt⁺ DC quantified by intranuclear staining against RORC. Each dot represents one mouse, horizontal bars represent mean, error bars represent SD. **P (0.0021) ***P (0.0002). Statistical analyses in (G–I) were performed using two-tailed Welch's t test.

mouse spleen (10), establishing it as RORγt⁺ DCs (Fig. 2 B–D and *SI Appendix, Table S1*). This cluster expressed higher levels of *Ccr6*, *Cd200*, and *Epcam* compared to other DC subtypes and we could confirm higher expression of these markers on RORγt⁺ DCs compared to cDC1 and cDC2 by flow cytometry (Fig. 2F).

Some migratory DCs expressed *Aire* (Fig. 2 B and D), as expected (35, 49) but we could also identify a distinct cluster of cells expressing *Aire*, *Rorc*, and *Prdm16*, which we termed RORγt⁺ eTACs. RORγt⁺ DCs and RORγt⁺ eTACs most closely resembled each other and migratory cDCs transcriptionally and based on chromatin accessibility (Fig. 2E). While RORγt⁺ eTACs and RORγt⁺ DCs showed similar chromatin accessibility, they somewhat diverged based on

gene expression profile (Fig. 2A). The *Aire* locus, including the conserved noncoding sequence 1 (CNS1) region (35) showed accessible chromatin peaks in migDCs, RORγt⁺ DCs and RORγt⁺ eTACs. Highest accessibility of the CNS1 region was observed in RORγt⁺ eTACs and RORγt⁺ DCs; however, some cell type-specific peaks were observed close to the transcriptional start site of *Aire* only in RORγt⁺ eTACs (Fig. 2G). The RORγt⁺ DC cluster was distinguished from other clusters by expression of genes including *Prdm16*, *Col17a1*, *Pigr*, *Ltb*, and *Lingo4*, of which *Prdm16* and *Col17a1* expression were shared with RORγt⁺ eTACs (Fig. 2D and *SI Appendix, Table S2*). RORγt⁺ eTACs showed specific expression of *Aire*, *Tnfrsf11a* (RANK), and *Itgb8* (Fig. 2D). Flow cytometry in

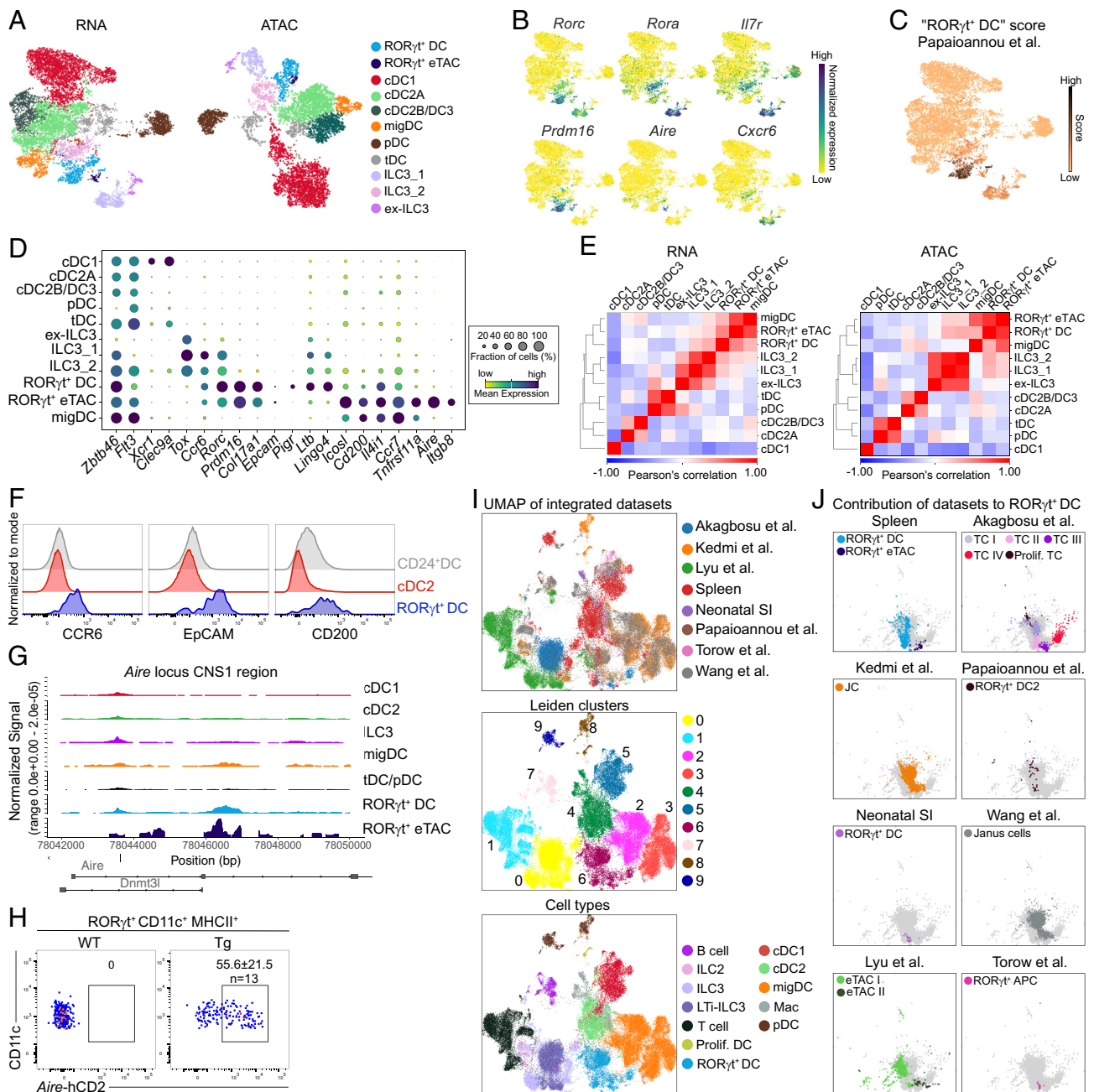


Fig. 2. Integrative transcriptional analyses align RORγt⁺ DCs with Janus cells, RORγt⁺ eTACs and Thetis cells. (A–E) Single-cell multiomic profiling (paired scATAC and scRNA-seq) of RORγt⁺ DCs, RORγt^{neg}CD11c⁺MHCII⁺ cells, and MHCII⁺ ILC3 from spleens of two-week-old ($n = 2$) or adult ($n = 3$) *RORγt^{GFP}* mice was performed. (A) RNA-based and ATAC-based UMAP of 11,980 nuclei annotated by cell type (see also *SI Appendix, Fig. S2*). (B) Expression of indicated genes on the RNA-based UMAP. (C) RNA-based UMAP depicting enrichment score for genes that distinguished RORγt⁺ cDC2-like cells in the neonatal spleen (10). (D) Bubble plot of select cell type defining markers. (E) Pearson's correlation to show the similarity between clusters identified based on RNA and ATAC profiles. (F) Staining of the indicated surface markers on RORγt⁺ DCs (blue), cDC2 (red), and CD24⁺ DCs (gray). (G) Chromatin accessibility at the CNS1 region of *Aire* locus in the indicated cell types. (H) AIRE expression in RORγt⁺CD11c⁺MHCII⁺ cells revealed by anti-hCD2 staining in the spleen of adult *Aire^{hCD2}* mice. (I) The indicated murine datasets were integrated. UMAP of the integrated datasets colored by dataset and by Leiden clusters. (J) Zoomed-in display of the RORγt⁺ DC cluster from the UMAP in (I) with all cells contributing to the cluster in gray. The contribution of the specified populations from the indicated publications is shown.

Aire^{hCD2} reporter mice (50) confirmed that a fraction of RORγt⁺ DCs indeed express AIRE (Fig. 2H) but *Aire* deficiency did not influence the development of RORγt⁺ DCs in mixed bone marrow chimeras (*SI Appendix, Fig. S2G*). AIRE⁺ RORγt⁺ DCs stained slightly higher for CCR7, CD24, MCHII, and CD200 but resembled AIRE^{neg} RORγt⁺ DCs in terms of EpCAM, ICOSL, and CCR6 expression (*SI Appendix, Fig. S2H*). Altogether, these data suggest that RORγt⁺ eTACs are a transcriptional state of RORγt⁺ DCs.

Some of the aforementioned genes (*Prdm16*, *Col17a1*, *Pigr*, *Ltb*, *Aire*, *Tnfrsf11a*) have been reported in Thetis cells and Janus cells/

eTACs (29, 30, 34, 35). Therefore, we performed data integration of the above dataset, together with published scRNA-seq datasets of DCs from neonatal murine spleen (10), RORγt⁺ APCs from neonatal Peyer's patch (38), Thetis cells from neonatal mLN (30), Janus cells from mLN (*SI Appendix, Fig. S3 C and D*) and sILN (29, 35), and RORγt⁺ eTACs from mLN (23). We additionally integrated scRNA-seq data from neonatal small intestine in which we identified a cluster of RORγt-expressing DCs (*SI Appendix, Fig. S3 A and B*). After unsupervised dataset integration using scVI (51) cells from individual datasets were distributed evenly across

Leiden clusters (Fig. 2I). Cells originally annotated as the same broad cell types in the individual datasets (cDC1, cDC2, migDCs, pDCs, ILC3) clustered together in the integrated analysis irrespective of study and tissue of origin, and Leiden clusters corresponded well to the different cell types included (Fig. 2I and *SI Appendix, Fig. S2I*). We found that cells originally annotated as eTACs, Janus cells, Thetis cells, or ROR γ ⁺ APCs clustered together with ROR γ ⁺ DCs identified in our study, into a transcriptionally distinct cluster (Leiden 6), termed ROR γ ⁺ DCs (Fig. 2J and *SI Appendix, Fig. S2J*). Of note, Thetis cells from neonatal mLN also show heterogeneity in terms of *Aire* expression (*SI Appendix, Fig. S2J*) (30), supporting that ROR γ ⁺ DCs entail distinct transcriptional states. Together, this comprehensive multiomic profiling and meta-analysis establishes ROR γ ⁺ DCs as a transcriptionally distinct cell type with strong resemblance to ROR γ ⁺ eTACs, Janus and Thetis cells.

Comparative Transcriptomics Reveals ROR γ ⁺ DCs are Evolutionarily Conserved. To investigate whether ROR γ ⁺ DCs exist in the human spleen, we analyzed a scRNA-seq dataset of human spleen cDCs from a 61-year-old patient (12). 11 of 12 identified clusters corresponded to the previously identified clusters, including cDC1, CLEC10A⁺ cDC2A, and CLEC10A⁺ cDC2B, DC progenitor-like cells (pre-DC/ASDCs), CCR7⁺ migratory DCs (CCR7⁺ DCs), and one cluster of dividing cells (Fig. 3A and *SI Appendix, Fig. S4A–C*). Cluster 11 expressed *RORC* and *PRDM16*, but not the ILC3 genes *RORA* and *IL7R* (*SI Appendix, Fig. S4C*), and had been excluded from the original analyses as putative ILC3 contamination. Indeed, this *RORC*⁺ cluster scored high for expression of ILC3 signature genes (Fig. 3B) but also for genes that distinguished ROR γ ⁺ DCs in multiome and scRNA-seq analyses from murine spleen (10) (Fig. 3B and *SI Appendix, Table S1*). From the Tabula Sapiens human reference atlas, we selected spleen cells annotated as CD1c⁺ and CD141⁺ myeloid DCs, pDCs, and ILCs (52), which formed 8 clusters corresponding to cDC1 (cluster 2), cDC2 (cluster 4), ILC3 (cluster 1, 0, 5), and pDCs (clusters 6 and 3) (Fig. 3C and D and *SI Appendix, Fig. S4D–F*). Cells in cluster 7 expressed *RORC* and *PRDM16*, but not *RORA* and *IL7R* and scored highest for genes distinguishing ROR γ ⁺ DCs in mouse spleen, whereas ILC3s scored highest for ILC3 signature genes (Fig. 3D and *SI Appendix, Table S1*). This *RORC*⁺ cluster contained cells from multiple donors aged 59, 61, and 69 y and did not segregate by the sequencing method or cell cycle phase, validating it as a bona fide population (*SI Appendix, Fig. S4F*). After integration, *RORC*⁺ cells from both datasets clustered together and away from DCs and ILCs (Fig. 3E and F). Comparative gene expression analyses identified conserved markers, including *PRDM16*, *UBE2E2*, *IL4I1*, *PIGR*, and *LTB*, that distinguished ROR γ ⁺ DCs in murine spleen and the *RORC*⁺ clusters in the human spleen (Fig. 3G). Thus, ROR γ ⁺ DCs also exist in the human spleen.

We also identified *RORC*- and *PRDM16*-expressing cells that lacked *RORA* and resembled the *RORC*⁺ cluster in the human spleen in single-cell RNA-seq data from small and large intestines from healthy adults (25 to 75 y old) (53) (*SI Appendix, Fig. S5A–D* and *Table S5*), as well as in small intestine of healthy pediatric donors and pediatric Crohn's disease patients (*SI Appendix, Fig. S5E*). These data indicate that ROR γ ⁺ DCs are conserved in the human intestine across age. In the Tabula Sapiens lymph node dataset (52), we also detected *RORC*-, *PRDM16*-, and *LTB*-expressing cells, some of which expressed *AIRE* (*SI Appendix, Fig. S5F–I*), and that derived from multiple donors and anatomically distinct LNs (*SI Appendix, Fig. S5F and H*) and have recently been dubbed cDC2C (40).

By performing comparative gene expression analyses of ROR γ ⁺ DCs in each dataset described above and identifying commonalities, we revealed a set of genes, including *RORC*, *PRDM16*, *LTB*,

PIGR, and *IL4I1*, that reliably distinguished ROR γ ⁺ DCs across tissues and species in all of the above mouse and human datasets (Fig. 3H and I and *SI Appendix, Tables S1 and S5* and *Fig. S6E and F*). R-DC-like cells from tonsil, which correspond to Thetis cells (37) showed higher expression of these genes compared to other populations in the same dataset (Fig. 3H), indicating that these cells are homologous to ROR γ ⁺ DCs. Additionally, we could align ROR γ ⁺ DCs with PRDM16⁺ cDC2C from glioblastoma (GBM) (40, 54) and ACY3⁺ DCs from cerebral spinal fluid (CSF) of patients with multiple sclerosis (39) (Fig. 3H and *SI Appendix, Fig. S6A and B*) and identified ROR γ ⁺ DCs in two additional datasets from CSF of patients with demyelinating disease (55, 56) (*SI Appendix, Fig. S6C–E*). This comprehensive meta-analysis establishes ROR γ ⁺ DCs as a transcriptionally distinct and evolutionarily conserved cell type that encompasses previously described populations including ROR γ ⁺ eTACs, Janus cells, Thetis cells, R-DC-like cells, cDC2C and ACY3⁺ DCs.

Distribution and Phenotype of ROR γ ⁺ DCs Across Lymphoid and Nonlymphoid Tissues in Mice. We next assessed the tissue distribution of ROR γ ⁺ DCs across age in ROR γ ^{GFP} mice. Migratory cDCs in nonlymphoid tissues express CD127 (25, 57) (*SI Appendix, Fig. S7A*). Thus, we gated cDCs irrespective of CD127 as CD90⁺ CD11c⁺ MHCII⁺ cells negative for the monocyte marker CD64. In mLN, we detected ROR γ ⁺ DCs at all ages examined and found their frequency was highest in neonatal mLN and declined with age (Fig. 4A). As reported (30), we consistently detected the highest numbers of ROR γ ⁺ DCs at two weeks of age (Fig. 4A and *SI Appendix, Fig. S7B*). In small intestinal lamina propria (siLP) the frequency of ROR γ ⁺ DCs within CD11c⁺ MHCII⁺ cDCs was highest in neonates and absolute numbers peaked at two weeks of age (Fig. 4B). Of note, for technical reasons, Peyer's patches (PP) were removed from SI only for mice older than two weeks (Fig. 4B). ROR γ ⁺ DCs were present in the colon, which lacks PP, of neonatal and adult mice (Fig. 4C). ROR γ ⁺ DCs differed phenotypically from MHCII⁺ ILC3s (CD90⁺ CD127⁺ MHCII⁺ GFP⁺ cells, *SI Appendix, Fig. S7A, C, E, and F*) and in mLN lacked the ILC3 marker CXCR6 (*SI Appendix, Fig. S7D*). In mLN and siLP from one-week-old, but not adult mice, ROR γ ⁺ DCs expressed CD11b (*SI Appendix, Fig. S7C and F*). In both tissues ROR γ ⁺ DCs expressed high levels of MHCII, consistent with a migratory DC phenotype and lacked the cDC1 marker XCR1, although some ROR γ ⁺ DCs expressed XCR1 in adult mLN (*SI Appendix, Fig. S7C and F*). In the lung, ROR γ ⁺ DCs were least frequent and their numbers peaked at two weeks of age (Fig. 4D and *SI Appendix, Fig. S8A–C*). Pulmonary ROR γ ⁺ DCs lacked XCR1 but expressed CD11b and were distinguished from ILC3s by expression of CD11c, MHCII and lack of CD90 (*SI Appendix, Fig. S8A and B*). Importantly, we also detected ROR γ ⁺ DCs with high MHCII expression in skin draining lymph nodes (Fig. 4E and *SI Appendix, Fig. S8D–F*).

Transcriptional Regulation of ROR γ ⁺ DCs. We next investigated the transcriptional regulation of ROR γ ⁺ DCs in the spleen (Fig. 2). While cells from different ages distributed evenly across ATAC and RNA clusters, RNA cluster cDC2_2 was dominated by cells from the adult time point (*SI Appendix, Fig. S9A*). Because cDC2 clusters 1 and 2 showed uniform chromatin accessibility but split into two clusters based on gene expression (*SI Appendix, Fig. S2A, B, and D*), these data indicate that while cDC2 chromatin identity is conserved environmental signals may shape gene expression in cDC2 across age, as reported (10, 38, 58). Accordingly, we identified 687 differentially expressed genes between the cDC2 metacluster across age (*SI Appendix, Table S3*). cDC2 from adult mice were enriched for genes downstream of IFN γ , tumor

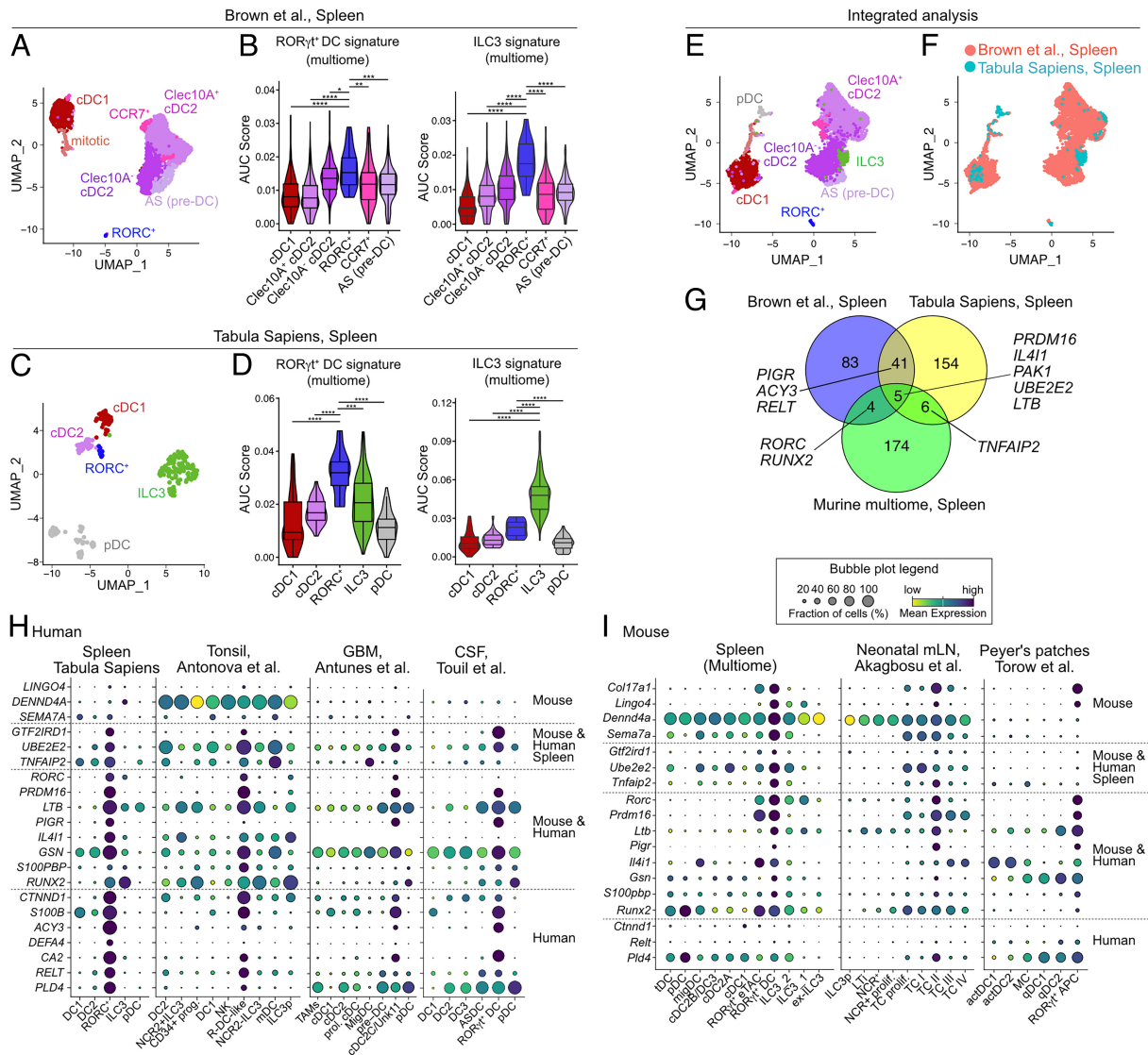


Fig. 3. Comparative transcriptomics reveals RORγt⁺ DCs are evolutionarily conserved. (A) Annotated UMAP of 4,717 cells from scRNA-seq dataset of human splenic DCs. (B) Enrichment scores for the RORγt⁺ DC and ILC3 signatures from the murine multiome dataset were calculated for each cluster. (C) Annotated UMAP of 262 cells from scRNA-seq dataset of human spleen. (D) Enrichment scores for the RORγt⁺ DC signature or ILC3 signature from the murine multiome dataset were calculated for each cluster. (E and F) Annotated UMAP (E) of integrated scRNA-seq datasets generated using Seurat Integration of scRNA-seq datasets pipeline, and colored by dataset (F). (G) Venn diagram showing the overlap of genes distinguishing RORγt⁺ DCs in the indicated mouse and human scRNA-seq datasets. (H and I) Bubble plots of selected genes deduced from comparative gene expression analyses that distinguish RORγt⁺ DCs in various human (H) and murine (I) scRNA-seq datasets. Genes are ordered according to the species and organs they were found in to differentiate RORγt⁺ DCs from other cell types. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. Statistical analysis was performed using the Wilcoxon nonparametric ranked sum test.

necrosis factor-α (TNF-α), IL-2, and IFNα signaling (SI Appendix, Fig. S9B), corroborating our previous findings (10), and validating the suitability of our dataset to compare populations across age. Contrary to cDC2, RORγt⁺ DC clusters had equal contribution of cells from both ages and only 29 genes were differentially expressed between RORγt⁺ DCs across age (SI Appendix, Fig. S9A and Table S3). Thus, RORγt⁺ DCs are transcriptionally stable between 2 wk of age and adulthood.

Focused multiomic analysis of the RORγt⁺ DC and RORγt⁺ eTAC clusters confirmed that RORγt⁺ eTACs form a separate cluster from RORγt⁺ DCs in ATAC and mRNA profiles (Fig. 5A and B and SI Appendix, Fig. S9C). *Aire*^{neg}RORγt⁺ DCs could further be divided into 3 clusters (Fig. 5A and B and SI Appendix, Fig. S9C). Of note, *Itgb8* expression, which promotes Treg differentiation (29, 30) was restricted to the RORγt⁺ eTAC cluster (Fig. 5B). RORγt⁺ DC cluster b showed lower expression of *Rorc* than the other two clusters (Fig. 5B), suggesting that RORγt⁺ DCs can lose RORγt expression. Indeed, in spleens from two-week-old and adult

RORγt^{Cre}*Rosa*^{YFP}*RORγt*^{GFP-knockin} reporter mice cells with RORγt expression history (YFP) outnumbered those with active RORγt expression (GFP; Fig. 5C) although these cells were otherwise phenotypically similar (SI Appendix, Fig. S9D). Similar observations were made in the lung, mLN and siLP (Fig. 5D and SI Appendix, Fig. S9E). Especially in lung and mLN from two-week-old mice, cells with *RORγt*^{Cre} expression history largely outnumbered those actively expressing RORγt (Fig. 5D and SI Appendix, Fig. S9E).

Finally, combining chromatin accessibility and gene expression profiles from individual cells we used SCENIC+ (59) to identify cell type-specific enhancer-driven gene regulatory networks (eRegulons). Dimensionality reduction based on target gene and target region enrichment scores of eRegulons separated the same main cell states as identified above (Fig. 5E). Of note, RORγt⁺ DCs and RORγt⁺ eTACs were similar in this analysis suggesting that they are closely related cell states. SCENIC+ identified well-known master regulators of cDC1 (*Irf8*), cDC2 (*Runx3*), pDCs (*Tcf4/E2-2*, *Spib*), and ILC3 (*Rora*, *Irfz1*, *Rorc*), confirming the

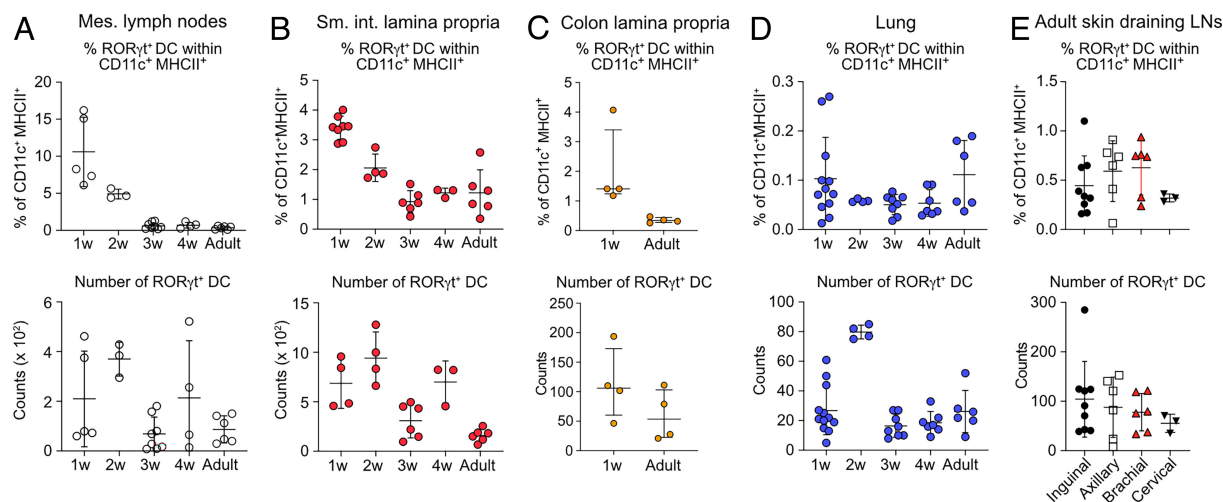


Fig. 4. RORγt⁺ DCs exist across lymphoid and nonlymphoid tissues in mice and humans. (A–C) RORγt⁺ DCs were quantified in mLN (A), siLP (B) colon LP (C), lung (D), and skin draining lymph nodes (E) of RORγt^{GFP} reporter mice (representative gating in *SI Appendix, Fig. S7* and *S8*). (A) Frequency of RORγt⁺ cells within CD11c⁺MHCII⁺ cells (Upper plot) and number of RORγt⁺ cells (Lower plot) in mLN of mice of indicated ages (1 wk n = 5; 2 wk n = 3; 3 wk n = 8, 4 wk n = 4; Adult n = 6). (B–D) Frequency of RORγt⁺ cells within CD11c⁺MHCII⁺CD64⁺ cells (Top) and number (Bottom) in siLP (1-wk-old n = 4 to 8; 2-wk-old n = 4; 3-wk-old n = 6, 4-wk-old n = 3; Adult n = 6), colon (1-wk-old n = 4; adult n = 4), and lung (1-wk-old n = 11; 2-wk-old n = 4; 3-wk-old n = 7, 4-wk-old n = 6; Adult n = 6) from mice of indicated ages. (E) RORγt⁺ DCs from axil, brachial, cervical, and inguinal skin draining LN were gated as in *SI Appendix, Fig. S8D* and quantified. The frequency of RORγt⁺ cells within CD11c⁺MHCII⁺ cells (Top) and number of RORγt⁺ DCs (Bottom) is shown.

validity of our approach (*SI Appendix, Fig. S9F*). RORγt⁺ DCs and RORγt⁺ eTACS shared regulatory networks *Nfat5* and *Relb* with migDCs and *Rel* with cDC2 and migDCs, aligning them with DCs (Fig. 5F and *SI Appendix, Fig. S9F*). SCENIC+ predicted RORγt⁺ DCs and RORγt⁺ eTACS to be specifically regulated by *Prdm16*, which is also specifically expressed in these cells (Figs. 2 B and D and 5F and *SI Appendix, Fig. S9F*). eRegulons of the nuclear receptors *Thrb/Nr1a2*, *Rorc*, and *Ppara* were equally predicted by SCENIC+ to regulate RORγt⁺ DCs (Fig. 5G and *SI Appendix, Fig. S9F*). PRDM16 is a master regulator of brown adipogenesis that in adipocytes directly controls expression of *Ppara*, a nuclear receptor that, like RORγt, integrates signals from free fatty acids and is involved in lipid metabolism (60, 61). Visualization of the eRegulons formed by *Rorc* and *Prdm16* in RORγt⁺ DCs suggests cooperativity between these factors in RORγt⁺ DCs (Fig. 5G). Downstream target genes of PRDM16 showed higher expression in RORγt⁺ DCs and RORγt⁺ eTACS than in other cell types and some of these genes, including *Ltb*, appear coregulated by *Prdm16* and *Rorc* (*SI Appendix, Fig. S10B*). SCENIC+ predicted *Rorc* to regulate expression of *Pigr*, *Gtf2ird1*, and *Col17a1* (Fig. 5G and *SI Appendix, Fig. S10B*), suggesting that RORγt shapes the identity of RORγt⁺ DCs (Fig. 5G). *Prdm16* was predicted to regulate expression of *Tlr12* and *Il23a*, which showed highest expression in RORγt⁺ DCs over other clusters (Fig. 5G and *SI Appendix, Fig. S10B*). RORγt⁺ DCs did not show enrichment of genes reported to promote Tregs in Thetis cells (*SI Appendix, Fig. S10A*) (30), except for expression of *Itgb8* in a subset of RORγt⁺ DCs expressing *Aire*. Importantly, RORγt⁺ DCs showed comparable expression of genes involved in antigen processing and presentation to cDCs and migratory DCs (*SI Appendix, Fig. S10C*) and flow cytometry confirmed that RORγt⁺ DCs express costimulatory molecules, including CD80, CD86, PD-L1, and CD83. Notably, RORγt⁺ DCs expressed highest levels of CD40 and CCR7 when compared to cDC1 and cDC2 (*SI Appendix, Fig. S10D*), altogether suggesting that RORγt⁺ DCs have the necessary machinery to serve as APCs.

RORγt⁺ DCs are Bona Fide DCs. The capacity to activate naïve T cells is a hallmark feature of cDCs (2). We, thus, sort-purified RORγt⁺ DCs from spleens of two-week-old and adult RORγt^{GFP} mice, pulsed them with Ovalbumin peptide 323 to 339 (OVA₃₂₃₋₃₃₉)

and cultured them with cell trace violet (CTV)-labeled naïve OTII transgenic T cells from adult mice with or without T cell polarizing cytokines. cDC2 and MHCII⁺ILC3 from the same mice were used as controls (*SI Appendix, Fig. S11A*). As expected, cDC2 from two-week-old and adult mice induced T cell proliferation under all conditions tested, while MHCII⁺ILC3s were poor inducers of OTII proliferation (Fig. 6 A and E and *SI Appendix, Fig. S11 C, D, F, and G*). Under all conditions tested RORγt⁺ DCs from two-week-old and adult mice stimulated the proliferation of naïve T cells, although to a lower extent than cDC2, and supported the differentiation of OTII cells into effector T cells, as measured by intracellular cytokine and FOXP3 staining (Fig. 6 A–H). While RORγt⁺ DCs supported the differentiation of FOXP3⁺ Tregs equally or better than cDC2, the induction of Th17 cells by RORγt⁺ DCs was reduced compared to cDC2 (Fig. 6 C, D, G, and H). Thus, RORγt⁺ DCs can activate naïve CD4⁺ T cells and induce their effector differentiation with qualitative and quantitative differences compared to cDC2. In contrast, MHCII⁺ ILC3 induced little if any T cell proliferation and may require cytokine stimulation to achieve their full APC potential (62).

We next asked whether RORγt⁺ DCs have the capacity to process and present antigen. Specific expression of CLEC4A4 on cDC2 and RORγt⁺ DCs from neonatal and adult mice (10), but not on cDC1 or MHCII⁺ ILC3 (Fig. 6I) (63), suggested that anti-CLEC4A4-OVA (63) may allow to deliver antigen to RORγt⁺ DCs in vivo. We thus injected adult RORγt^{GFP} mice with anti-CLEC4A4-OVA or isotype-matched control antibody in the presence of the adjuvant CpG-B (Fig. 6 J–L), which did not alter the activation status of RORγt⁺ DCs relative to cDC1 or cDC2 (Fig. 6M and *SI Appendix, Fig. S11H*). 12 h later, we sorted 300 cDC1, cDC2, RORγt⁺ DCs and MHCII⁺ILC3s and cocultured them with 3000 naïve CTV-labeled OTII T cells (Fig. 6 K and L). cDC2 and RORγt⁺ DCs induced OTII proliferation after targeting with anti-CLEC4A4-OVA but not isotype-matched control antibody (Fig. 2 K and L), demonstrating successful targeting. In contrast, cDC1 and MHCII⁺ ILC3s did not induce T cell proliferation demonstrating cell type-specific antigen targeting (Fig. 2 K and L). OTII proliferation induced by RORγt⁺ DCs appeared marginally lower than that achieved by cDC2, which could be due to differences in CLEC4A4 targeting efficiency, responsiveness to CpG-B,

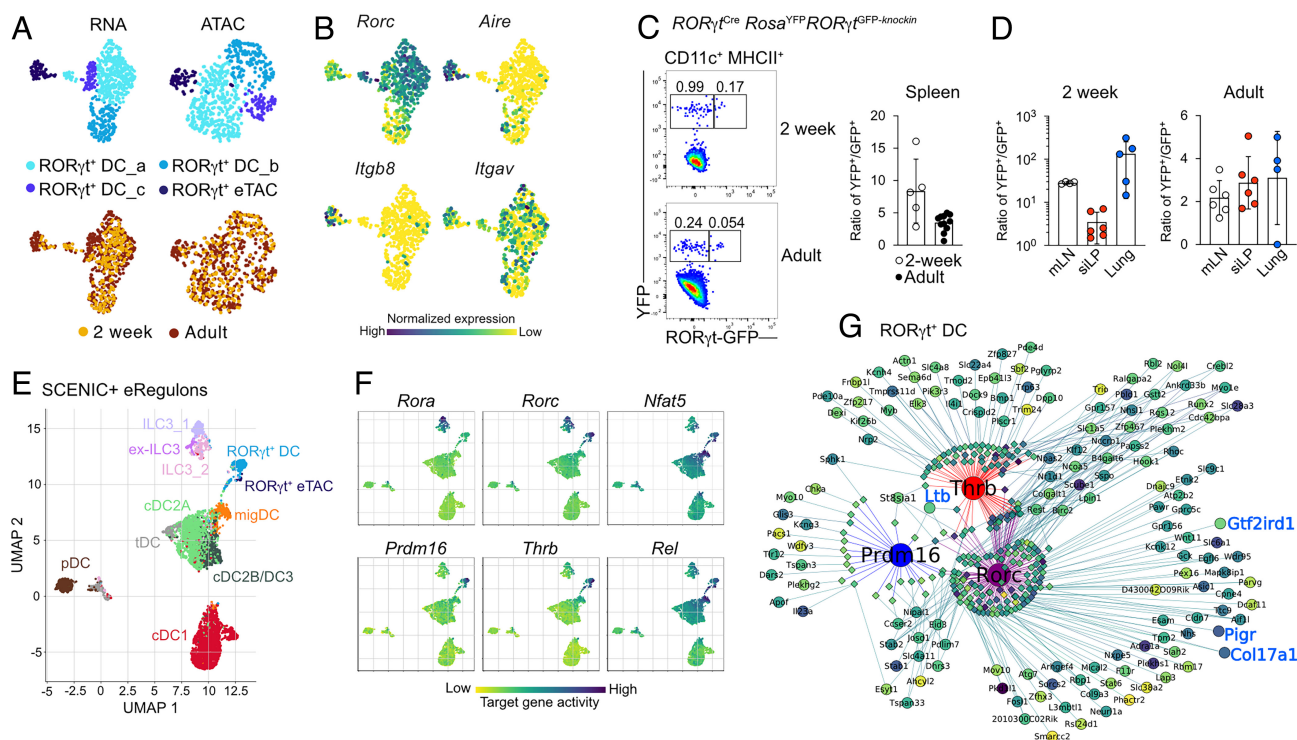


Fig. 5. Single-cell multiomic profiling reveals the unique identity of RORγt⁺ DCs. (A) RORγt⁺ DCs and RORγt⁺ eTACs from the multiome analysis in Fig. 2 A–E were reclustered. The resulting UMAPs based on RNA and ATAC profiles are shown and annotated by timepoint. (B) Expression of *Rorc*, *Aire*, *Itgb8*, and *Itgav* on the RNA-based UMAP from (A). (C and D) Spleen (C), mLN, siLP and lung (D) from 2-week-old and adult RORγt^{Cre}Rosa^{YFP}RORγt^{GFP-knockin} mice were analyzed by flow cytometry. YFP (RORγt-expression history) versus GFP (active RORγt) expression in CD11c⁺MHCII⁺ cells were plotted and the ratio of YFP⁺ to GFP⁺ cells was calculated. Each dot represents one mouse. (E) UMAP display of dimensionality reduction based on target genes and region enrichment scores generated using SCENIC+. (F) UMAP colored by target gene activity of the indicated eRegulons predicted to regulate specific cell types (SI Appendix, Fig. S9F). (G) Visualization of the gene regulatory network formed by *Prdm16*, *Thrb*, and *Rorc* in RORγt⁺ DCs. Genes that determine the identity of RORγt⁺ DCs are colored in blue.

or expression of costimulatory molecules between RORγt⁺ DCs and cDC2. Taken together, these data demonstrate that RORγt⁺ DCs can process and present antigen, and activate naïve CD4⁺ T cells in response to an inflammatory trigger.

Finally, we tested whether RORγt⁺ DCs have the capacity to migrate to lymph nodes in response to inflammation. Gavage of neonatal mice with R848 induces cDC migration to mLN (64). Indeed, 24 h after R848 gavage we observed an increase in the frequency and number of cDC1, but not ILC3s in mLN, which served as positive and negative controls for migration, respectively (Fig. 6N). RORγt⁺ DCs also increased in frequency and number in mLN (Fig. 6M), suggesting that oral R848 stimulates RORγt⁺ DCs to migrate to mLN. Thus, RORγt⁺ DCs bear classical migratory features of cDCs upon inflammatory triggers, validating their affiliation with DCs.

RORγt⁺ DCs Exhibit a Proinflammatory Profile in Autoimmune Neuroinflammation. We next performed comparative gene expression analyses of RORγt⁺ DCs in three independent scRNA-seq datasets from CSF of patients with autoimmune demyelinating disease and found common genes that distinguished RORγt⁺ DCs from other cells in each dataset (Fig. 7A). These genes included *S100B* and *LTB*, which propagate neuroinflammation (65, 66), *TNFSF15* (TL1A), which promotes Th17 differentiation (67) and *CCR6*, which enables trafficking of DCs to LNs for the priming of autoimmune T cells in EAE (68). Of note, RORγt⁺ DCs also expressed the aryl hydrocarbon receptor (*AHR*) and *FFAR4* (also known as GPR120), which are reported to integrate environmental signals to modulate DC function in EAE (69, 70). We thus immunized mice with MOG to induce EAE and observed an increase in frequency and number of cDC1, RORγt⁺ DCs and MHCII⁺ ILC3s in the brain and spinal

cord of mice at peak disease compared to healthy control mice (Fig. 7 B and C and SI Appendix, Fig. S12A). Of note, in inflamed CNS, RORγt⁺ DCs became a sizable population and reached comparable numbers to cDC1 and MHCII⁺ ILC3s (Fig. 7 B and C), which influence EAE pathogenesis (27, 71).

Discussion

Here, we demonstrate the existence of RORγt-expressing DCs that are transcriptionally and developmentally distinct from other DC subtypes and entail murine Janus cells/ RORγt⁺ eTACs (29, 35), Thetis cells (30), RORγt⁺ APCs in Peyer's patches (38), as well as human R-DC-like cells (37), cDC2C (40), and ACY3⁺ DCs (39). Previous work has demonstrated that RORγt⁺ DCs—also known as Thetis and Janus cells induce peripheral T cell tolerance and Tregs, particularly at mucosal sites (29, 35, 72–74). We show that RORγt⁺ DCs, like cDCs, respond to inflammation by inducing the activation of naïve T cells and that they can migrate to lymph nodes. They further accumulate in the inflamed CNS of mice and exhibit proinflammatory features in CSF of patients with inflammatory demyelinating disease, including multiple sclerosis. Thus, RORγt⁺ DCs are bona fide immune sentinels with a versatile functional spectrum ranging from inducing T cell activation to mediating peripheral T cell tolerance (SI Appendix, Fig. S12B). This versatility makes them an attractive target for therapeutic manipulation in inflammatory diseases, cancer, and neuroinflammation.

In the spleen, RORγt⁺ DCs lack a particular tolerogenic profile and, like cDCs, respond to inflammation with the activation of naïve CD4⁺ T cells. Similarly, R-DC-like cells from human lymph nodes can stimulate allogeneic CD4⁺ T cells, although their APC potential has not been compared to that of cDCs (37). Although

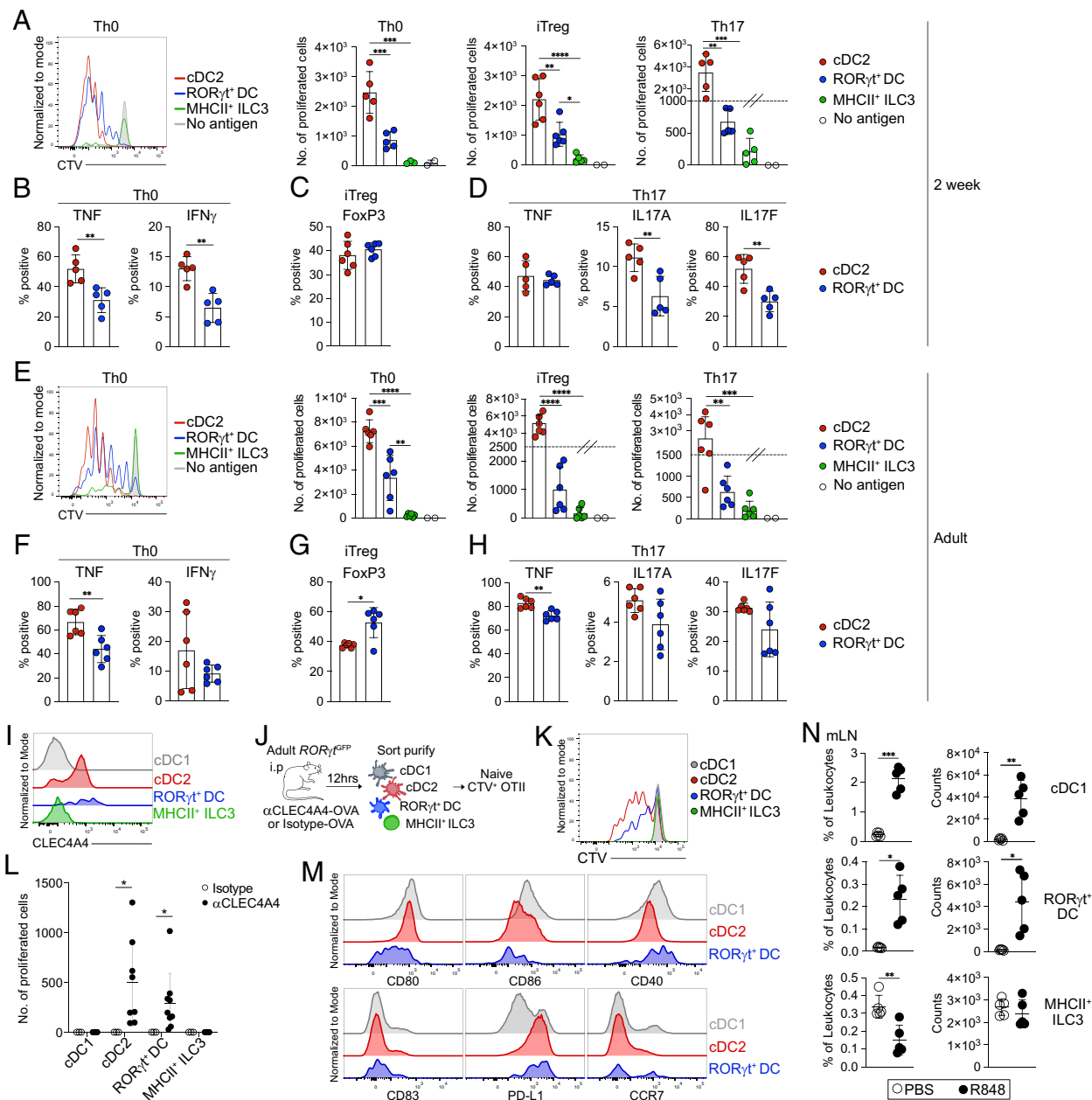


Fig. 6. RORγt⁺ DCs are bona fide dendritic cells (DCs). (A–H) 250 cDC2, MHCII⁺ ILC3, and RORγt⁺ DCs from spleens of 2-wk-old (A–D) or adult (E–H) RORγt^{GFP} mice were pulsed with OVA₃₂₃₋₃₃₉ and cocultured with 2500 CTV-labeled naïve OTII cells as indicated. 3.5 d later, proliferation (CTV dilution), cytokine production, and FOXP3 expression in proliferated T cells were quantified. (A) CTV-trace of OTII cells cocultured with cDC2, RORγt⁺ DCs, MHCII⁺ ILC3, or cDC2 without OVA₃₂₃₋₃₃₉ (gray) under nonpolarizing conditions (Th0). Right: Quantification of proliferated cells after culture with cDC2, RORγt⁺ DCs, MHCII⁺ ILC3, or cDC2 without OVA₃₂₃₋₃₃₉ (open circle) under the indicated conditions. (B–D) Proliferated OTII cells were analyzed for TNF and IFNγ production (B), FOXP3 expression (C), or TNF, IL-17A, and IL-17F production (D). (E–H) OTII T cells cocultured with cDC2, MHCII⁺ ILC3, and RORγt⁺ DCs from adult mice were analyzed as in A–D above. (I) CLE44A4 expression on the indicated populations. (J–L) Adult RORγt^{GFP} mice were injected i.p. with anti-CLE44A-OVA or isotype-OVA control antibody plus CpG-B. After 12 h 300 cDC2, cDC1, MHCII⁺ ILC3, and RORγt⁺ DCs were sorted and cocultured with 3000 naive CTV-labeled OTII cells. (J) Experimental setup. (K) CTV dilution and (L) number of proliferated OTII cells after coculture with the indicated populations (n ≥ 5). (M) Expression of the indicated markers on cDC1 (gray), cDC2 (red), and RORγt⁺ DCs (blue) 12 h after i.p. injection of CpG-B. (N) Frequency and absolute number of cDC1, MHCII⁺ ILC3, and RORγt⁺ DCs in mLN of 11-d-old RORγt^{GFP} reporter mice 24 h after oral administration of R848. Each dot represents one biological replicate pooled from two independent experiments. Horizontal bars represent mean, error bars represent SD. *P (0.0332), **P (0.0021), ***P (0.0002), ****P < 0.0001. Statistical analysis: two-tailed Welch's t test (B–D, F–H, and L) or one-way ANOVA with Tukey's multiple comparisons (A and E). Only statistically significant comparisons are indicated.

RORγt⁺ DCs are infrequent in steady-state tissues, we found them to increase in the inflamed mLN and in the CNS of mice with active EAE. They also accumulate in the inflamed esophagus, where they express the eosinophilic esophagitis risk gene *ATP10A* (40), and in the CSF from patients with demyelinating disease (39). In patient CSF, RORγt⁺ DCs display a proinflammatory profile and have been predicted to interact with memory CD4⁺ T cells (39). Since RORγt⁺ APCs contribute to CNS pathology in EAE (27),

the functions of RORγt⁺ DCs in neuroinflammation require further investigation. AhR cooperates with RORγt to drive IL-22 expression in Th17 cells (70). In patient CSF, RORγt⁺ DCs express *AHR*, raising the possibility that these two transcription factors drive gene expression in RORγt⁺ DCs. Since *AHR*, *FFAR4*, and *RORC* are druggable molecules, the possibility to manipulate RORγt⁺ DCs therapeutically in EAE can be explored in future studies. The prominent expression of *LTβ* in RORγt⁺ DCs suggest

that they can contribute to the spatial organization of immune cells (75). ROR γ ⁺ DCs also express the tryptophan-metabolizing enzyme IL4i1 that generates bioactive immunomodulatory metabolites (76). IL4i1-derived metabolites can for instance activate AhR or protect cells from ferroptosis (76), suggesting that ROR γ ⁺ DCs may function beyond T cell priming, for instance, by modulating metabolic communication between cells.

Taken together, the above data suggest that ROR γ ⁺ DCs exhibit tissue-specific functional diversity. Tolerogenic immune functions in ROR γ ⁺ APCs have been linked to AIRE (29, 35), the expression of which is restricted to subtypes of ROR γ ⁺ DCs (29, 30). *AIRE* shapes gene expression in tumor-associated macrophages, DCs and ILC3s (28, 49). Our data suggest that *Aire* marks a distinct functional state since AIRE⁺ROR γ ⁺ DCs express higher levels of CCR7 and MHCII than AIRE^{neg} cells and show a distinct transcriptional profile. RANK signaling induces AIRE in cTACs and ILC3s (34, 35) and RANKL or other tissue/age-specific signals may shape the transcriptional and functional attributes of ROR γ ⁺ DCs. ROR γ ⁺ DCs may receive such signals in specific tissue niches.

ROR γ -Cre lineage tracing experiments show that some cells that have previously expressed ROR γ downregulate it. Although they phenotypically still resemble those cells actively expressing ROR γ , these data indicate that ROR γ itself may not be a good lineage defining marker. CCR6, EpCAM, CLEC4A4, and CD200 are good candidates to design a combinatorial sort strategy to identify ROR γ ⁺ DCs in mice. However, ROR γ is currently the most reliable marker to identify these cells by flow cytometry in mouse tissues either by using ROR γ ^{GFP} reporter mice or intracellular staining using an anti-RORC antibody. Other genes, including *PRDM16*, may be better candidates to reliably define ROR γ ⁺ DCs across tissues and species. A gating strategy independent of ROR γ would be critical for example in neonatal lung, where cells with ROR γ ^{Cre} expression history vastly outnumber cells with active ROR γ expression. The neonatal lung is considered an environment that promotes type 2 immunity (77), while ROR γ is generally associated with type 3/Th17 mediated immunity. Factors

specifically expressed in neonatal lung may suppress ROR γ expression, which could alter the phenotype and functions of ROR γ ⁺ DCs by rendering them unresponsive to natural ligands of ROR γ .

Indeed, SCENIC+ predicted ROR γ itself to shape the transcriptional identity of ROR γ ⁺ DCs, consistent with a recent report describing a role for ROR γ in the development of Thetis cells (72). PRDM16 is a master regulator of brown fat adipogenesis that can act as transcriptional activator and repressor. Loss of PRDM16 increases $\gamma\delta$ T17 differentiation, suggesting PRDM16 antagonizes ROR γ in $\gamma\delta$ T cells (78). GTF2IRD1, a predicted target of ROR γ , can mediate the repressive action of PRDM16 (79). PRDM16 also regulates stem cell quiescence (80), raising the possibility that ROR γ ⁺ DCs serve as progenitors. Indeed, RNA velocity analyses in humans suggest that ROR γ ⁺ DCs may serve as alternative progenitors for cDC or ILC3-like cells (37). Although we found no evidence for that in steady-state neonatal spleen (10), the fact that cells with ROR γ -expression history outnumber those with active ROR γ expression would be consistent with the possibility of ROR γ ⁺ DCs serving as progenitors. However, ROR γ ⁺ DCs express costimulatory molecules and can activate naive T cells, indicating that they themselves constitute mature APCs. Consistent with previous reports (30), our observation that ROR γ ⁺ DCs arise from lymphoid bone marrow progenitors in a Rag2-common- γ -chain-independent manner suggests that these cells are a lineage distinct from myeloid cDCs and ILC3s.

Owing to their expression of CD11c, ZBTB46, and ROR γ , ROR γ ⁺ DCs need to be considered in the interpretation of studies using promoters for the above genes to drive Cre or other transgenes, such as diphtheria toxin receptor. Models to specifically manipulate this cell type are urgently needed to understand its functions in immunity. However, inducible depletion models are necessary, given the necessity of ROR γ ⁺ DCs for differentiating ROR γ ⁺FoxP3⁺ T regs, which regulate intestinal homeostasis and dampen type II immune responses (29, 35, 72, 73). In the absence of such models, the anti-CLEC4A4 based antigen-targeting

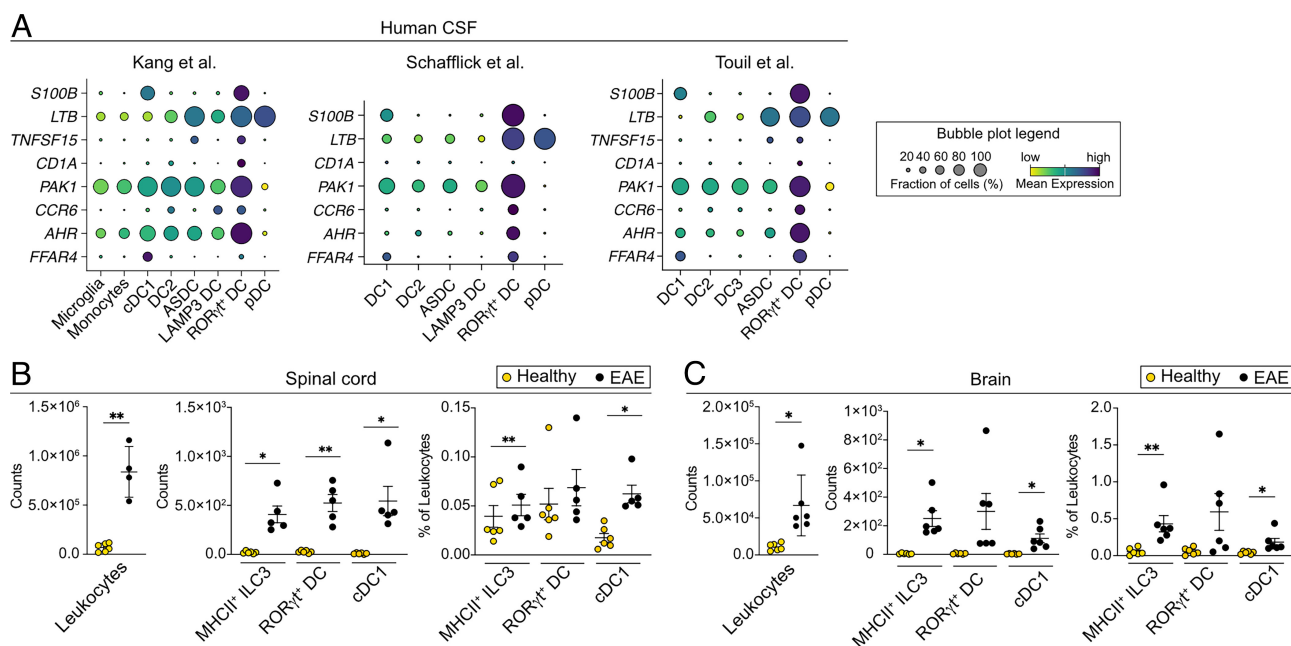


Fig. 7. ROR γ ⁺ DCs in autoimmune neuroinflammation. (A) Bubble plots depicting expression of select genes that distinguished ROR γ ⁺ DCs from other APCs in at least two of the indicated human CSF datasets. (B and C). Quantification of total leukocytes, frequency and number of MHCII⁺ ILC3, cDC1, and ROR γ ⁺ DCs in the spinal cord (B) and brain (C) of mice during peak EAE (day 16, n = 5/6) compared to healthy controls (n = 6). Each dot represents one mouse, horizontal bars represent mean, error bars represent SD. *P (0.0332), **P (0.0021), ***P (0.0002), ****P < 0.0001. Statistical analysis was performed using two-tailed Welch's t test comparing cells from healthy mice vs EAE mice for each cell type. Only statistically significant comparisons are indicated.

approach used here will prove valuable to determine the specific functions of ROR γ ⁺ DCs, especially compared to cDC2. ROR γ ⁺ DCs also express *Ly75*, the gene encoding DEC205, suggesting they are targetable by anti-DEC205, enabling functional comparison to cDC1.

ROR γ ⁺ DCs have been termed Janus cells and Thetis cells in other studies for their two-faced and shape-shifting transcriptional features and resemblance to cDCs and thymic epithelial cells. Our work shows that ROR γ ⁺ DCs are indeed multifaceted in nature and bear hallmark features of DCs including the ability to integrate signals from the environment to direct appropriate immunity. Their functional versatility ranging from inducing T cell tolerance to T cell activation highlights the potential of these cells to be targeted for therapeutic applications.

Materials and Methods

Mice. Female and male age and sex-matched mice and littermate controls were housed under specific pathogen-free conditions and used in this study. All animal procedures were performed in accordance with national and institutional guidelines for animal welfare and approved by the respective authorities. See also *SI Appendix, Material and Methods*.

Cell Isolation for Flow Cytometry. Tissues were enzymatically digested; see *SI Appendix, Material and Methods*. For SI, Peyer's patches were removed only for mice 3 wk or older. mLN from 2 to 3 one-week-old mice were pooled prior to processing; mice older than 3 wks were perfused with ice-cold PBS before lung isolation.

Flow Cytometry and Cell Sorting. Cells were incubated with CD16/32/FcBlock for 10 min at 4 °C and then for 30 min at 4 °C with antibodies against surface epitopes and Fixable Viability Dye eFluor 780. To preserve GFP signal after intranuclear staining, cells were prefixed with 2% paraformaldehyde at RT for 15 min. Intracellular cytokine and transcription factor staining was performed using Intracellular Fixation and Permeabilization Buffer Set and FOXP3 Transcription Factor Staining Set, respectively (both Thermo Fisher Scientific). Anti-RORC staining was performed for 1 h at RT.

Sorting: splenocytes were depleted of T cells (CD3e), B cells (CD19), Neutrophils (Ly6G), and Erythroid cells (Ter119) using magnetic beads. Cells were sorted on a BD FACSAria Fusion. Data were acquired on an LSR Fortessa (BD Biosciences) and analyzed using FlowJo V10.8.1 (Tree Star). For antibodies and further details see *SI Appendix, Material and Methods*.

In Vitro T Cell Proliferation. 250 APCs were pulsed with 10 μ g/mL OVA₃₂₃₋₃₃₉ (Invivogen) for 3 h, washed, and cocultured with 2500 naïve CTV-labeled OTII T cells. 5 ng/mL TGF- β , 10 μ g/mL anti-IL-4, and 10 μ g/mL anti-IFN γ were added for Treg polarization, and 5 ng/mL TGF- β , 20 ng/mL IL-6, 10 μ g/mL anti-IL-4, and 10 μ g/mL anti-IFN γ for Th17 polarizing condition. 3.5 d later cells were restimulated with 10 ng/mL phorbol 12-myristate 13-acetate (PMA) (Calbiochem) and 1 μ g/mL Ionomycin (Sigma-Aldrich) for a total of 5 h. Brefeldin A (BioLegend) was added during the last 3 h. See also *SI Appendix, Material and Methods*.

In Vivo Targeting. Adult ROR γ ^{GFP} mice were injected intraperitoneally (i.p.) with 10 μ g anti-CLEC4A4-OVA or 10 μ g OVA-coupled-isotype matched control antibody plus 0.2 μ g/g body weight CpG-B ODN 1826 (InvivoGen). 12 h later 300 APCs were cocultured with 3000 naïve CTV-labeled OTII cells as above.

R848 Treatment. Ten-day-old ROR γ ^{GFP} mice were orally gavaged with 2 μ g R848 (Invivogen) in 50 μ L PBS or 50 μ L PBS as control and analyzed 24 h later.

Single-Cell Multiomics and Transcriptomics. MHCII⁺ ILC3, CD11c⁺ MHCII⁺ DC, and ROR γ ⁺ DCs were sorted from 2-wk-old (P13, $n = 2$) and adult (8-wk-old, $n = 3$) ROR γ ^{GFP} *Clec9a^{cre/wt} Rosa26^{Tom/wt}* mice. EDTA-free buffer was used for cell isolation. For each timepoint, cells were pooled at a ratio of 1:1:10 (ROR γ ⁺ DCs: MHCII⁺ ILC3: CD11c⁺ MHCII⁺ DC) and nuclei isolated following the 10X Genomics Low Cell Input Nuclei Isolation protocol. 20,000 cells were lysed for 3 min on ice, resuspended in diluted Nuclei Buffer and lysis efficiency and nucleus quality assessed by trypan blue staining. 8,400 nuclei for the adult

timepoint and 10,400 nuclei for the 2-wk timepoint were loaded for transposition. RNA and ATACseq libraries were prepared with the 10X Genomics Chromium Next GEM Single Cell Multiome ATAC + Gene Expression Kit and sequenced as recommended. Details of the analyses and all other transcriptomics are provided in *SI Appendix, Material and Methods*.

EAE. 12-wk-old male C57BL/6 mice were immunized subcutaneously with 250 μ L of a 1:1 volume emulsion of recombinant MOG₁₋₁₂₅ (400 μ g) and Complete Freund's Adjuvant (Sigma). Pertussis toxin was injected i.p. on Day 0 and Day 2. Animals were scored as described in *SI Appendix, Material and Methods* and analyzed at peak disease three to five days after disease onset with mice scoring 2.5 to 3. Cell isolation from brain and spinal cord as described in *SI Appendix, Material and Methods*.

Statistics. Statistical significance was calculated in Prism 10 software (GraphPad). For pairwise comparisons two-tailed t test with Welch's correction was used. For multiple comparisons, one-way ANOVA with Tukey's test was performed. The Wilcoxon nonparametric ranked sum test was used to calculate differences in AUC scores in R. A P -value < 0.05 was considered significant.

Data, Materials, and Software Availability. Single-cell RNA sequencing and Multiome sequencing data have been deposited in BioStudies ([S-BST1322](https://www.ebi.ac.uk/biostudies/studies/S-BST1322)) (81). All study data are included in the article and/or [supporting information](#).

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