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Mesenchymal Cells Regulate Retinoic Acid Receptor-Dependent Cortical Thymic Epithelial Cell Homeostasis

Katarzyna M. Sitnik,* Knut Kotarsky,* Andrea J. White,† William E. Jenkinson,† Graham Anderson,† and William W. Agace*—

The vitamin A metabolite and transcriptional modulator retinoic acid (RA) is recognized as an important regulator of epithelial cell homeostasis in several tissues. Despite the known importance of the epithelial compartment of the thymus in T cell development and selection, the potential role of RA in the regulation of thymic cortical and medullary epithelial cell homeostasis has yet to be addressed. In this study, using fetal thymus organ cultures, we demonstrate that endogenous RA signaling promotes thymic epithelial cell (TEC) cell-cycle exit and restricts TEC cellularity preferentially in the cortical TEC compartment. Combined gene expression, biochemical, and functional analyses identified mesenchymal cells as the major source of RA in the embryonic thymus. In reaggregate culture experiments, thymic mesenchyme was required for RA-dependent regulation of TEC expansion, highlighting the importance of mesenchymal-derived RA in modulating TEC turnover. The RA-generating potential of mesenchymal cells was selectively maintained within a discrete Ly51intgp38+ subset of Ly51+ mesenchyme in the adult thymus, suggesting a continual role for mesenchymal cell-derived RA in postnatal TEC homeostasis. These findings identify RA signaling as a novel mechanism by which thymic mesenchyme influences TEC development. The Journal of Immunology, 2012, 188: 4801–4809.

**The thymus is the primary site of T cell development. Anatomically, the thymus is compartmentalized into cortical and medullary regions, each containing specialized thymic epithelial cell (TEC) subsets that play a central role in supporting thymocyte differentiation and maturation. In the cortex, cortical TECs (cTECs) support the commitment and progressive differentiation of immature CD4+CD8+ double-negative thymocytes to TCR-bearing CD4+CD8+ double-positive (DP) thymocytes and their positive selection to CD4+CD8+ and CD4–CD8– single-positive (SP) thymocytes. In the medulla, medullary TECs (mTECs) are important in establishing self-tolerance by participating in the elimination of SP cells with high self-reactive TCR affinities, in the generation of regulatory T cell populations, and in postselection T cell maturation (for review, see Refs. 1, 2).

Although studies show that cTECs and mTECs derive from a common bipotent progenitor via an intermediate pool of lineage-committed cTEC and mTEC precursors (3–7), the intermediate differentiation steps that result in the generation of functionally mature TEC compartments are only partly defined. In addition to this differentiation program, TECs undergo phases of proliferation, resulting in thymus growth that is directly linked to an increase in the availability of stromal niches for T cell development. TEC development and proliferation appears to be regulated by cell-intrinsic factors, including epithelial progenitor pool size (8), as well as extrinsic signals they receive from their local environment (for review, see Refs. 9, 10). For example, thymocyte–TEC cross-talk involving double-negative and DP thymocytes is involved in later stages of cTEC development (7, 11), whereas mature mTEC development is influenced by CD4+ lymphoid tissue-inducer cells and positively selected thymocytes through provision of the TNFR superfamily ligands, receptor activator for NF-κB ligand and CD40L (12–15). In addition to thymocyte-derived signals, epithelial–mesenchymal interactions have been implicated in promoting TEC turnover. For example, neural crest-derived mesenchymal cells, which surround the thymic anlagen during thymus organogenesis and make up the majority of mesenchymal cells in the adult thymus (16, 17), have been proposed to regulate TEC progenitor proliferation through production of fibroblast growth factor (FGF)-7 and -10 (18, 19) and insulin-like growth factor (IGF)-1 and -2 (20, 21). Consistent with this, mice deficient in FGF-10 or the FGF-10/-7 receptor, FGF2-IIIb, and mice expressing soluble dominant-negative FGF2-IIIb display thymus dysgenesis (22, 23) and reduced thymic epithelial proliferation (23). Mesenchymal components are found within both the cortex and medulla (16, 24), raising the possibility that these cells may be capable of influencing both thymic compartments.

The vitamin A (retinol) metabolite retinoic acid (RA) acts as a ligand for nuclear RA receptor (RAR)/retinoic X receptor heterodimers, that function as ligand-activated transcription factors recognizing specific RA response elements in the regulatory regions of target genes (25). The generation of RA from retinol involves a two-step oxidation process via retinol. Conversion of...
retinol to retinal is mediated by alcohol dehydrogenases, whereas retinol is converted to RA by retinaldehyde dehydrogenases. Two enzyme families, the cytosolic alcohol dehydrogenases and the microsomal retinol dehydrogenases (RDHs), have been implicated in the conversion of retinol to retinal, of which RDH10 has been shown to play a nonredundant role in priming of RA synthesis during embryonic development (26). The oxidation of retinal to RA is carried out by three retinaldehyde dehydrogenases (RALDH1, RALDH2, and RALDH3; encoded by the genes Aldh1a1, Aldh1a2, and Aldh1a3, respectively), mutations in two of which (Aldh1a2 and Aldh1a3) result in early embryonic (Aldh1a2) or neonatal (Aldh1a3) lethal phenotypes (for review, see Ref. 27).

RA is a major physiological regulator of epithelial cell homeostasis in multiple organs such as the lung (28), kidney (29), pancreas (30), gut (31), and skin (32). Regarding thymus development, mesenchymo-derived RDH10- and RALDH2-dependent RA signaling in the pharyngeal arch endoderm, a region that gives rise to TEC progenitors, is a critical prerequisite to the initial formation of the thymus (26, 33, 34). The potential ability of RA to interfere with later stages of thymus development has been suggested based on hypoplastic and ectopic thymus phenotypes generated in embryos treated with teratogenic doses of exogenous retinoids (35). Notably, there is also evidence of continual RAR signaling in the postnatal thymus (36), and the broad spectrum of immune abnormalities observed in vitamin A-deficient animals includes a marked atrophy of the thymus (37), indicating that RA signaling events may play additional roles in thymus ontogeny.

In this study, we demonstrate that mesenchymal cells represent a major source of RA in both the embryonic and adult thymus. In functional experiments involving fetal thymus organ cultures (FTOCs), we show that mesenchymal cell-derived RA plays an important role in regulating TEC numbers predominantly within the cTEC compartment, thereby highlighting a novel mechanism by which thymic mesenchyme influences the development of TEC.

Materials and Methods

Animals

C57BL/6 mice were bred and maintained at the Biomedical Centre Animal Facility (Lund University, Lund, Sweden), unless otherwise stated. BALB/c and C57BL/6 Rag−/− mice were housed in the Biomedical Services Unit at the University of Birmingham (Birmingham, U.K.). Day of vaginal plug detection was designated as day 0 of gestation. All animal procedures were approved by the Lund/Malmö animal ethics committee.

Abs and reagents

The following Abs and reagents were used: bio-Ly51/BP-1 (6C3), FITC or allophycocyanin-CD80 (16-10A1), PE-CD40 (1C10), eFluor50-CD31/PECAM-1 (390), PE-Cy5 or APC-eFluor780-Ter119 (TER119), bioluminotinin-β receptor (LTBR) (3C8), bi-platelet-derived growth factor receptor (PDGFRα) (AP54), bio-PDGRFP (AP58), Alexa 700-CD45 (30-F11), PE-Cy7-rat IgG2a,κ isotype control, PE-Cy7-Golden Syrian Hamster IgG isotype control, Alexa 647-rat IgG2a,κ isotype control, bio-rat IgG1,κ isotype control, APC-conjugated streptavidin (all from eBioscience, San Diego, CA); PE-Cy7 or APC-Cy7-epithelial cell adhesion molecule (EpCAM; G8.8), FITC-TCRβ (H57-597), Blue Pacific-1-α/4-1 (M511415.2), Alexa 700-CD452.1 (104), PE, or PE-Cy7-gp38 (8.1.1), PE-Cy7-VCAM-1 (429), Alexa 647-ICAM-1 (Y1N1/1.7.A) (all from Bio-Legend, San Diego, CA); PE-Ki67 (B56), FITC-Brdu (3D4), PE-mouse IgG1,κ isotype control, bio-rat IgG2a,κ isotype control, and PE-Cy7-conjugated streptavidin (all from BD Biosciences, Franklin Lakes, NJ). Bio-CD205/DEC205 (NDCD-145) was from Abcam (Cambridge, U.K.), and FITC-Ulex europeus agglutinin-1 (UEA-1) was from Vector Laboratories (Burlingame, CA). Propidium iodide and LIVE/DEAD Violet Fixable Dead Cell Stain Kit were from Invitrogen (Carlsbad, CA). RA, retinol, DMSO, and G418 were from Sigma-Aldrich (St. Louis, MO). LE540 was from Wako Chemicals (Osaka, Japan).

FTOC and reaggregate thymus culture

FTOCs were established as previously described (38). Briefly, thymic lobes were dissected from mouse embryos at embryonic day (E)15 gestational stage and placed at the air–medium interface on top of a 0.8-μm-isopore membrane filter (Millipore, Billerica, MA) resting on an Artiwrap gelatin sponge (Medipet, Weymouth, Dorset, U.K.). The filter and sponge were submerged in DMEM-10 (High-glucose Glutamax-supplemented DMEM, 10 mM HEPES, 100 U ml−1 penicillin, 100 μg ml−1 streptomycin, 50 μM 2-ME, 0.1 mM MEM Non-Essential Amino Acids Solution [all from Invitrogen], and 10% FCS [Sigma-Aldrich]) and incubated in 7–10% CO2 at 37˚C. Medium containing freshly prepared dilution of RA, LE540, or carrier DMSO (0.5%) was supplied to the cultures at 2- to 3-d intervals. Cell numbers were determined using a Bürkner chamber or with CountBright Absolute Counting Beads (Invitrogen).

Reaggregate thymus organ cultures (RTOCs) were performed as previously described (39). Briefly, FACS-sorted E15 mesenchymal, TEC, and thymocyte populations (for gating strategy, see Supplemental Fig. 2) were combined at a 1:0.2:4:1.4 ratio and reaggregated by depositing cells on the surface of 0.8-μm Nucleopore filters (Whatman; Maidstone, Kent, U.K.) under organ culture conditions as described above. Initial TEC numbers used to establish mesenchyme-containing and mesenchyme-depleted RTOCs were 1.3 × 105 and 1.74 × 105, respectively.

Cell isolation

Isolated embryonic thymus lobes and FTOC/RTOC cultures were digested with 0.5% trypsin-EpDA (Invitrogen) at 37˚C for 30 min and single-cell suspensions used in the aldefluor assay or directly stained with Abs for analysis and cell sorting.

To isolate stromal cells from postnatal thysems, organs were cut into tissue fragments and digested with collagenase type II (Worsham 1 μm−1, Roche Diagnostics, Basel, Switzerland) and DNase I (50 Kunitz U ml−1, Sigma-Aldrich) in R-5 medium (t-glutamine–supplemented RPMI 1640, 1 mM sodium pyruvate, 50 μg ml−1 gentamicin [all from Invitrogen], 100 U ml−1 penicillin, 100 μg ml−1 streptomycin, 10 mM HEPES, and 5% FCS) at 37˚C for 35 min using an orbital shaker (350 rpm). Enzymatic treatment was repeated for an additional 20–25 min followed by incubation with 5 mM EDTA on ice for 5–10 min. Remaining tissue fragments were mechanically dispersed by careful pipetting. Cell suspensions obtained after each digestion were pooled in ice-cold PBS containing 2% FBS and 2 mM EDTA to prevent aggregate formation. Stromal cells were enriched by immunomagnetic depletion of CD45+ cells by MACS (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s protocol and used in the aldefluor assay or directly stained with Abs for analysis and cell sorting.

Measurement of aldehyde dehydrogenase activity

Aldehyde dehydrogenase (ALDH) activity was measured using the ALDEFLUOR Kit (StemCell Technologies, Vancouver, BC, Canada) according to the manufacturer’s instructions. Briefly, cells were suspended in ALDEFLUOR assay buffer containing activated aldefluor reagent (Bodipy-aminocetaldehyde; final concentration 1.5 μM) with/without inclusion of the ALDH competitive inhibitor 4-diethylamino-benzaldehyde (DEAB; final concentration 75 μM) and incubated at 37˚C for 30–40 min. Thereafter, cells were stained with Abs and analyzed or sorted in FACSAria (BD Biosciences) using FITC channel for the detection of aldefluor signal.

Flow cytometry and cell sorting

Flow cytometry was performed according to standard procedures. Dead cells (identified as propidium iodide+ or using the LIVE/DEAD Violet Fixable Dead Cell Staining Kit [Invitrogen]) and cell aggregates (identified on forward light scatter-A versus forward light scatter-W scatter plots) were excluded from all analyses. Data acquisition was performed on FACSAria, LSRII, or LSRFortessa (BD Biosciences, Franklin Lakes, NJ) and analyzed using FlowJo software (Tree Star, Ashland, OR). Sorting was performed on an FACSaria (BD Biosciences). For analysis of BrdU incorporation, day 7 FTOCs were pulsed with BrdU (10 μM; BD Biosciences) for 5 h. Lobes were trypsinized as above and single-cell suspensions labeled with surface Abs to CD45 and EpCAM, followed by staining with FITC-conjugated anti-BrdU Ab (FITC BrdU Flow Kit; BD Biosciences) according to the manufacturer’s protocol. For analysis of Ki67 expression, surface-labeled FTOC cell suspensions were then stained with biotin-12-Wnt3 and the Foxp3 Staining Buffer Set (eBioscience) according to the manufacturer’s protocol, and stained with PE-conjugated anti-Ki67 Ab (BD Biosciences).

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RA reporter cell assay

The F9-RARE-lacZ reporter cell line (40) was maintained on gelatin-coated surfaces in DMEM/F-12 medium (Invitrogen) containing 100 U ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin, 10% FCS, and 0.8 mg mL⁻¹ G418 or in DMEM-10.

For incubation with E15 thymic populations, reporter cells (5 x 10⁴ cells/well) were plated onto gelatin-coated, round-bottom, 96-well plates in DMEM/F-12 in 2% FCS 24 h prior to the assay. FACs-sorted cell populations were suspended in DMEM/F-12 and added to the wells (5 x 10⁴ cells/well) together with 25 nM retinol. LacZ induction was measured 22 h later with the β-Glo Assay System (Promega, Madison, WI) according to the manufacturer’s protocol. Chemiluminescence was quantified in white luminometer 96-well plates with a GloMax 96 luminometer (Promega). Thymic subset-induced β-galactosidase (β-Gal) activity was calculated by subtracting the activity in cultures incubated with retinol alone.

For incubation with 2-wk-old mesenchymal populations, reporter cells (3 x 10⁵ cells/well) were plated onto gelatin-coated, flat-bottom, half-area, 96-well plates in DMEM-10 24 h prior to the assay. FACs-sorted cell populations were suspended in DMEM and added to the wells (10⁴ cells/well) together with 25 nM retinol, followed by measurement of LacZ induction 19 h later as described above.

RNA isolation and quantitative RT-PCR

Total RNA was extracted using RNeasy Micro Kit (Qiagen) and reverse transcribed with SuperScript III (Invitrogen) according to the manufacturer’s instructions. First-strand cDNA synthesis was primed with a 1:1 mixture of oligo(dT) and random oligonucleotide hexamers (both from Invitrogen). Quantitative RT-PCR (qRT-PCR) reactions (20 µl volume) were set up using Maxima SYBR Green qPCR Master Mix (Fermentas) and carried out in a Bio-Rad MyiQ Thermal Cycler (Bio-Rad) with the manufacturer’s protocol. Chemiluminescence was quantified in white luminometer 96-well plates with a GloMax 96 luminometer (Promega). Thymic subset-induced β-galactosidase (β-Gal) activity was calculated by subtracting the activity in cultures incubated with retinol alone.

Primer sequences are as follows: β-actin forward, 5’-GAGAGGGAATATCGTGGTGAACA-3’ and reverse, 5’-GTTCCTAGGATGCAGCAATGATG-3’. Gapdh forward, 5’-TGTGGCTTCGTTGATGTA-3’ and reverse, 5’- CCGTCTACACCCATCTTGATG-3’; βSt (Pab11) forward, 5’-ACTCCCGACCTCCAGAGACG-3’ and reverse, 5’-GGTTAGGCAAGAAAGCAAACGC-3’; claudin 4 forward, 5’-GACTACAGGTCCTGGGAATCTCC-3’ and reverse, 5’-GCAGTGCTCAGATAACCATCAG-3’, claudin 3 forward, 5’-ACCACTGCTCTACAAAGCCAGACG-3’ and reverse, 5’-CAGGCGGCTTGACGTAACAGCTG-3’; cactin forward, 5’-GGTGGATGCTCTGTGACTAGTC-3’ and reverse, 5’-AGGGCTGTGTCTGTTCAGTTCTC-3’; cathepsin S forward, 5’-GAAAGGGGCTGCTCACTTGTGACG-3’ and reverse, 5’-ATGTAAGCGGCGCTTACAGCTC-3’; delta-like 4 forward, 5’-AGGTGCAATTTCGTTGCAGACG-3’ and reverse, 5’-GTCCTTGTCGCTGACGTAACAGCTC-3’; Ccl21a forward, 5’-ATTGTGCAATTTCGTTGCAGACG-3’ and reverse, 5’-GTCCTTGTCGCTGACGTAACAGCTC-3’; Ccl19 forward, 5’-AGGTGCAATTTCGTTGCAGACG-3’ and reverse, 5’-GTCCTTGTCGCTGACGTAACAGCTC-3’; Fgf10 forward, 5’-TTGTGAAAGAAAGCAAGCTGAC-3’ and reverse, 5’-GA GTTCCCTTCTTGTGATCAG-3’; Igf1 forward, 5’-GGTTAGGATCTCCTCAGTTCG-3’ and reverse, 5’-ATCACTGCCGAGCAAGATCG-3’; Igf2 forward, 5’-TGCTCCTCAGGAGGGCTTCAACG-3’ and reverse, 5’-ATACTGCCGAGCAAGATCG-3’; Il7 forward, 5’-TGCTCCTCAGGAGGGCTTCAACG-3’ and reverse, 5’-ATACTGCCGAGCAAGATCG-3’; Prss16 forward, 5’-CGCGCCTGCCAAGAAGAGAC-3’ and reverse, 5’-GGTGGATGCTCTGTGACTAGTC-3’; Rank forward, 5’-GGCATTAGAGACCTTGGAGG-3’ and reverse, 5’-GTCCTTGTCGCTGACGTAACAGCTC-3’; Vegf7 forward, 5’-ATCACAGCTCCGGAAGCAACAC-3’ and reverse, 5’-GGTTAGGATCTCCTCAGTTCG-3’; Fgf10 forward, 5’-TTGTGAAAGAAAGCAAGCTGAC-3’ and reverse, 5’-GA GTTCCCTTCTTGTGATCAG-3’; Igf1 forward, 5’-GGTTAGGATCTCCTCAGTTCG-3’ and reverse, 5’-ATCACTGCCGAGCAAGATCG-3’; Igf2 forward, 5’-TGCTCCTCAGGAGGGCTTCAACG-3’ and reverse, 5’-ATACTGCCGAGCAAGATCG-3’; Il7 forward, 5’-TGCTCCTCAGGAGGGCTTCAACG-3’ and reverse, 5’-ATACTGCCGAGCAAGATCG-3’; Prss16 forward, 5’-CGCGCCTGCCAAGAAGAGAC-3’ and reverse, 5’-GGTGGATGCTCTGTGACTAGTC-3’; Rank forward, 5’-GGCATTAGAGACCTTGGAGG-3’ and reverse, 5’-GTCCTTGTCGCTGACGTAACAGCTC-3’; Adh1 forward, 5’-TAGCCAGAGACGTTC-3’; Adh1a2 forward, 5’-CAAGATCGCTGCTTGGACATG-3’ and reverse, 5’-ATACCTGCCGAGCAAGATCG-3’; Gal (β-galactosidase) activity was calculated by subtracting the activity in cultures incubated with retinol alone.

RNA isolation and quantitative RT-PCR

Statistical analysis

Statistical analyses were performed using unpaired two-tailed Student t test except for Fig. 1E and Supplemental Fig. 1, in which one-way ANOVA with Bonferroni’s multiple comparison test were used: *p < 0.05, **p < 0.01, ***p < 0.001.

Results

RA signaling regulates TEC proliferation in FTOC

To investigate the potential role of RA signaling during thymus development, we studied the effects of the pan-RAR antagonist LE540 (41) on embryonic thymus growth in FTOC. To this aim, E15 thymus lobes were cultured in the presence/absence of LE540, and TEC, mesenchymal, and thymocyte numbers were assessed 7 to 8 d later by flow cytometry. RA signaling regulates TEC proliferation in FTOC. E15 thymus lobes from C57BL/6 (A–C) or Rag-1⁻/⁻ and BALB/c control mice (D) were cultured in the presence/absence of the pan-RAR antagonist LE540 (8 µM) for 7 to 8 d. Size of thymic lobes (A) and number of cells/lobe (B) under indicated conditions. Results are the mean (SD) of three biological replicates/condition using pooled cells from two lobes/replicate and are from one representative experiment of three performed. TEC proliferation was assessed by 5 h BrdU incorporation (C) or analysis of Ki67-staining (C, D). Representative TEC FACs plots (left panels) and percent of Ki67- and BrdU-labeled TECs (right panels). Results are the mean (SD) of four biological replicates/condition using pooled cells from one lobe/replicate (Experiment [Exp] 1) and of four biological replicates/condition using pooled cells from two lobes/replicate (Exp 2). (E) E15 thymuses were cultured with LE540 (8 µM) or RA at the indicated doses. TEC numbers/lobe (upper panel) and TEC proliferation by Ki67 labeling (lower panel) was assessed after 5-d culture. Results are the mean (SD) of three biological replicates/condition using pooled cells from two lobes/replicate and are from one representative experiment of two performed. *p < 0.05, **p < 0.01, ***p < 0.001. Mes, Mesenchymal cells.

Figure 1. Retinoic acid signaling regulates TEC proliferation in FTOC.
FTOC may be due to enhanced TEC expansion, Ki67 staining and short-term BrdU incorporation studies were performed. LE540 induced a dose-dependent increase in TEC numbers and TEC proliferation rate compared with control FTOC (Fig. 1C, Supplemental Fig. 1), suggesting that RA signaling restricts TEC cellularity by inhibiting TEC proliferation. TEC proliferation was also enhanced in LE540-treated Rag-1<sup>−/−</sup> FTOC, indicating that this effect was independent of DP and SP thymocytes, although changes in total TEC number were less dramatic (Fig. 1D). Consistent with these findings addition of exogenous RA to FTOC led to a dose-dependent reduction in TEC numbers and TEC proliferation rates (Fig. 1E), without affecting cell viability (data not shown). Together, these results identify RA signaling as a novel pathway that negatively controls TEC expansion and organ size during embryonic thymus development in vitro.

RA signaling acts preferentially to regulate cTEC homeostasis

To determine which TEC populations were affected by inhibiting RA signaling in FTOC, the phenotype of TEC in 7-d FTOC was assessed by flow cytometry (Fig. 2A). LE540 treatment increased the total number of CD80<sup>+</sup> TEC (Fig. 2A) that are thought to comprise mature and immature cTEC and mTEC progenitors (14), but did not affect CD80<sup>+</sup>/UEAh<sup>+</sup> mature mTEC numbers (42). To further define the CD80<sup>+</sup> cells that expanded in the absence of RA signaling, CD80<sup>+</sup> TEC were sorted from LE540-treated and control FTOC and assessed for expression of a panel of genes differentially expressed in cTEC and mTEC lineages by QRT-PCR (Fig. 2B). CD80<sup>+</sup> TEC that were isolated from LE540-treated cultures expressed significantly higher levels of genes associated with the cTEC lineage including βSt, Dil4, and Pprs16 (43–46) and lower levels of the mTEC-associated genes such as cathepsin S, claudin 3, claudin 4, and Ccl21a (4, 7, 47, 48), indicating that RA signaling preferentially regulates cTEC differentiation/proliferation. Consistent with these findings, the proportion and total number of CD80<sup>+</sup> cells expressing high levels of the cTEC maturation marker CD205 (DEC205) increased significantly in LE540-treated cultures (Fig. 2C). Together, these results suggest that RA primarily regulates cTEC homeostasis in FTOC.

Mesenchymal cells are the major source of RA in the embryonic thymus

The impact of the RAR antagonist on the expansion and phenotype of TEC in FTOC suggested ongoing vitamin A metabolism and RA production in the cultured organs. To determine which populations in the embryonic thymus are capable of generating RA, TEC, thymocytes, and mesenchymal cells were sorted from E15 thymuses (Supplemental Fig. 2) and analyzed for expression of RALDH1–3 (Aldh1a1, -2, and -3), which catalyze the last rate-limiting step during RA synthesis (49) by real-time PCR (Fig. 3A). TEC were sorted into CD205<sup>+</sup>/CD40<sup>−</sup> cTEC and CD205<sup>−</sup>/CD40<sup>+</sup> mTEC, which represent the major TEC compartments at this stage of thymus ontogeny (7). Importantly, Aldh1a2, the presence of which most strongly correlates with RA generation in vivo (27), was highly and selectively expressed by CD45<sup>+</sup> EpCAM<sup>−</sup> mesenchymal cells, whereas Aldh1a1 and -3 were mainly produced by cTEC and at much lower levels (note the different y-axis scales of the graphs in Fig. 3A). To further evaluate the RA-generating potential of thymic cell populations, ALDH activity in mesenchyme, TEC, and thymocytes was assessed by flow cytometry using the fluorescent ALDH substrate Bodipy-aminocetaldehyde (commercially called aldefluor) (50). Consistent with their expression of Aldh1a2, mesenchymal cells, but not TEC or thymocytes, showed prominent aldefluor staining (Fig. 3B, 3C). This staining was specifically reflecting ALDH-mediated enzymatic activity because it was blocked by the specific ALDH inhibitor DEAB (51) (Fig. 3B, 3C). To directly assess the RA-generating capacity of mesenchymal cells, TEC, and thymocytes, each population was sorted from E15 thymuses and incubated together with the RA-reporter cell line F9-RARE- lacZ (40), which express β-Gal under control of an RA-sensitive promoter. Consistent with the data above, mesenchymal cells but not TEC or thymocytes induced RA-dependent β-Gal activity in the reporter cells (Fig. 3D). Together, these data demonstrate that mesenchymal cells are a major source of RA in the embryonic thymus.

Mesenchymal cells regulate RAR-dependent TEC expansion in RTOCs

To determine whether mesenchymal cells mediated the RA-dependent regulation of TEC proliferation in FTOCs, TEC and thymocytes were sorted from E15 thymuses and used to establish reaggregate cultures in the presence or absence of E15 mesenchymal cells.
Mesenchymal cells continue to generate RA in the adult thymus

To assess whether adult mesenchymal cells are capable of metabolizing vitamin A and thus could potentially modulate TEC growth in the adult thymus, ALDH activity in adult thymic mesenchyme was assessed by flow cytometry using the aldefluor assay (for gating strategy, see Fig. 5A). Thymic endothelial cells, defined as CD31^+CD45^−EpCAM^−^ cells, did not possess ALDH activity (Fig. 5B). Two major mesenchymal subsets were identified based on expression of Ly51 and gp38 (podoplanin): Ly51^+gp38^− and Ly51^+gp38^+ cells (Fig. 5B), both of which expressed the mesenchymal marker PDGFRβ (data not shown). The minor CD31^+Ly51^− subset of CD45^−EpCAM^−^ cells was devoid of mesenchymal marker expression (i.e., PDGFRα, PDGFRβ, and LTβR; data not shown) and was not included in the analysis. Of the two Ly51 mesenchymal populations, only the Ly51^+gp38^− subset contained cells that displayed high ALDH activity (Fig. 5B, 5C), indicating that RA—generating capacity persists among Ly51^+gp38^− mesenchymal cells in the adult thymus. Next, FACS-purified gp38^− and gp38^+PDGFRβ^− mesenchymal populations were assessed for expression of RALDHs by QRT-PCR. To obtain sufficient number of cells for analysis, sorting was performed on thymuses from 2-wk-old mice. Consistent with the data above, gp38^− but not gp38^+ mesenchymal cells expressed Aldh1a1, -2, and -3 (Fig. 5D), and Aldh1a2 was present at similar levels to that observed in embryonic mesenchyme (Fig. 3A). In accordance with the hypothesis that Ly51^+gp38^− mesenchymal subset contains cells that are able to produce RA in the postnatal thymus, FACS-sorted 2-wk-old gp38^−, but not gp38^+PDGFRβ^− mesenchymal
fraction induced RAR-dependent β-Gal activity in the RA reporter cell line F9-RARE-lacZ (Fig. 5E). Together, these data demonstrate that RA-producing mesenchymal cells persist in the adult thymus, suggesting continual involvement of mesenchymal cell-derived RA in postnatal TEC homeostasis.

**Comparative analysis of aldefluor+ and aldefluor− Ly51intgp38+ thymic mesenchymal cells.** Finally, we determined whether Ly51int gp38+ mesenchymal cells that displayed ALDH activity (aldefluor+ cells) and Ly51intgp38− mesenchymal cells that did not have ALDH activity (aldefluor− cells) differed in their expression of known mesenchymal markers and mesenchyme-derived soluble mediators. Aldefluor+ and aldefluor− Ly51int gp38+ mesenchymal cells expressed similar levels of PDGFRβ and LTβR, whereas aldefluor−Ly51int gp38− mesenchymal cells expressed higher levels of PDGFRα and lower levels of PDGFRβ and lower levels of ICAM-1 and VCAM-1 (Fig. 6A). Gene-expression analysis demonstrated that aldefluor− and aldefluor+ Ly51int gp38− mesenchymal subsets expressed similar levels of FGF-10, IGFR-1, stem cell factor, and IL-7 mRNA, whereas aldefluor− mesenchyme expressed significantly more FGF-7 and IGF-2 and significantly less CCL19 and CCL21a transcripts than aldefluor+ mesenchyme (Fig. 6B).

**Discussion**

The establishment and maintenance of organized cortical and medullary epithelial microenvironments play an essential role in the development and selection of a self-tolerant T cell pool. The control of TEC growth for optimal T cell development is a non–cell-autonomous process that has been linked to numerous factors including IGFs and FGFs, with the latter implicated as both positive and negative regulators (18, 19, 52). In this study, we identify mesenchymal cell-derived RA as a novel negative regulator of TEC proliferation in FTOCs that acts preferentially on the cortical TEC compartment. We also demonstrate that a subset of mesenchymal cells maintains RA-generating activity in the adult thymus, suggesting continual involvement of mesenchymal cell-derived RA in postnatal TEC homeostasis.

**FIGURE 5.** Mesenchymal cells continue to generate RA in the adult thymus. (A–C) ALDH activity of mesenchymal and endothelial cells in 7–10-wk-old adult thymus was assessed by the aldefluor assay. (A and B, upper panel) Gating strategy used to identify CD31+ endothelial and Ly51intgp38− and Ly51intgp38+ mesenchymal populations in adult thymus digests. (B) Representative histograms of aldefluor signal (lower panel) in the absence (black line) or presence (gray filled) of DEAB. (C) Median fluorescence intensity of aldefluor signal in ALDH+ Ly51int gp38− and Ly51intgp38+ mesenchymal populations. Results are the mean (SD) from three biological replicates using pooled cells from two to three thymuses/replicate and are from one representative experiment of two performed. (D) gp38+ and gp38− PDGFRβ+ mesenchymal populations were sorted from 2-wk-old thymuses and expression of Aldh1a1, -2, and -3 was determined by real-time PCR. Results are the mean (SD) of three biological replicates using cells sorted from eight mice/replicate. (E) FACS-sorted gp38+ and gp38− PDGFRβ+ mesenchymal populations were sorted from 2-wk-old thymuses and incubated with the RA-reporter cell line F9-RARE-lacZ in the presence/absence of 1 μM LE540, and β-Gal activity was measured in cell lysates 19 h later. Results are mean (SD) from three biological replicates using cells sorted from seven to eight mice per replicate. ***p < 0.001. n.a., Not analyzed.

**FIGURE 6.** Comparative analysis of aldefluor+ and aldefluor− Ly51int gp38+ thymic mesenchymal cells. (A) Phenotypic characterization of aldefluor− (ALDH−) and aldefluor+ (ALDH+) Ly51intgp38+ mesenchyme in 7–10-wk-old thymus. Representative FACS plots from three to five biological replicates using pooled cells from two to eight mice/replicate from two (PDGFRβ, LTβR) or three (PDGFRα, ICAM-1, and VCAM-1) independent experiments. (B) Aldefluor− and aldefluor+ Ly51intgp38+ mesenchymal populations were sorted from 7- to 8-wk-old thymuses for analysis of gene expression by real-time PCR. Results are the mean (SD) of 3 biological replicates using cells sorted from 10 mice/replicate. *p < 0.05, **p < 0.01.
thymus, suggesting a potential role for mesenchymal-derived RA in regulating TEC homeostasis in the adult. These results add RA to the list of mesenchymal cell-derived signals that regulate TEC homeostasis and highlight a role for thymic mesenchyme as both positive and negative regulators of TEC growth.

Notably, RAR antagonism had a dramatic effect on TEC composition in day 7 FTOC. The RAR antagonist appeared not to affect CD80+ TEC numbers but significantly enhanced the number and proportion of CD80+ cells expressing high levels of the cTEC marker CD205. Furthermore, CD80+ TECs, which contain both immature mTEC and cTEC (14), showed increased levels of mTEC and decreased levels of mTEC-specific gene transcripts in RAR antagonist-treated cultures. Although the precise stages in cTEC development that are influenced by RA are unclear, fully mature MHC class IIhigh mature cTEC are largely absent from the fetal stages of thymus development analyzed in this study (7), suggesting that RA signaling may act preferentially to limit the size of the cTEC compartment by promoting cell-cycle exit of committed cTEC progenitors. We cannot, however, rule out that in addition to inhibiting cell proliferation, RA signaling may also affect fate decision events in common cTEC/mTEC progenitors.

Our observation that the RAR antagonist promoted TEC expansion in Rag-1−/− FTOC as well as preliminary FTOC experiments using CD3εTCg26 thymuses, which have a developmental block in the earliest T cell precursors, indicate that thymocytes are not required for RA-dependent regulation of TEC proliferation. One possibility is that RA functions directly on TECs to regulate their proliferation, as previously observed in primary and transformed epithelial cell lines from the skin (53), intestine (54), cervix (55), and mammary gland (56, 57). Indeed, RA has been shown to downregulate FGF binding protein production (58) and inhibit bone morphogenic protein signaling (59, 60), a pathway central to FGF-7–induced TEC proliferation (19). In addition, RA can influence the expression of IGF binding proteins (61) and has been shown to inhibit expression of deltanp63, a P63 transcription factor family member that is required for maintaining the proliferative potential of TEC precursors (62, 63) in nasopharyngeal epithelial cells (64). An alternative possibility is that RA indirectly regulates TEC proliferation by modulating growth-promoting signaling pathways. For example, RA regulates FGF-7 expression (65) and inhibits FGF-10 transcription (66). Notably, we have found that mesenchymal expression of FGF-7 and FGF-10 is not altered in RAR antagonist-treated day 7 FTOC (K.M. Sitnik, unpublished observations), indicating that transcriptional regulation of these factors is not involved in RA regulation of TEC growth. The exact mechanism(s) by which RA regulates TEC homeostasis awaits further studies.

Our observation that a subset of mesenchymal cells maintains the ability to generate RA after birth suggests that RA signals may help to regulate TEC homeostasis in the adult. Consistent with this possibility, endogenous RA signaling in the postnatal thymus has been demonstrated using RA-reporter mice (36, 67). Based on the presence of Aldh1a1 expression in TECs and the detection of dexamethasone-induced Aldh1a1 and Aldh1a2 in thymic macrophages (36, 67), these studies proposed that TECs and macrophages contribute to the generation of RA in the steady-state and glucocorticoid-stimulated thymus, respectively. By flow cytometry, we identified two major mesenchymal cell populations in the adult thymus based on expression of Ly51 and gp38: Ly51highgp38+ and Ly51lowgp38− cells. Although Ly51+ mesenchymal cells are neural crest derived (16, 24), to our knowledge, such division within the Ly51+ mesenchyme population has not previously been described. Functionally Ly51+gp38− and Ly51lowgp38− cells appear distinct from one another, as only the former subset expressed Aldh1a1, and -2, and -3 and contained within it a population of cells with high ALDH activity. Further comparative analysis of the aldelfluor+ and aldelfluor− populations within the Ly51+gp38− mesenchymal subset demonstrated that both populations expressed similar levels of FGF-10 and IGF-1, whereas the former subset expressed significantly higher levels of FGF-7 and IGF-2. Thus, it appears that the same subset of mesenchymal cells can produce both positive and negative regulators of TEC proliferation. The interplay between these pathways for the maintenance of TEC homeostasis remains to be determined. Our data also demonstrate that aldelfluorLy51+gp38− cells express significantly lower levels of CCL19 and CCL21 and express lower levels of ICAM-1 and VCAM-1 compared with aldelfluor−Ly51lowgp38+ cells. Upregulation of VCAM-1, ICAM-1, CCL19, and CCL21 is known to characterize the LTβR-dependent differentiation of mesenchyme-derived stromal organizer cell compartment in developing lymph nodes (68, 69), raising the interesting possibility that aldelfluor+ and aldelfluor− subsets of Ly51intgp38− mesenchyme represent progressive developmental stages with a lower and higher maturation status, respectively.

In summary, we have identified mesenchymal cell-derived RA as a novel regulator of TEC homeostasis and identify a subset of RA-producing mesenchymal cells in the adult thymus. Future studies using genetic deletion of the RA-generating and signaling pathways in mesenchymal cells and TEC, respectively, should help further delineate the role of this pathway in the in vivo setting.

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