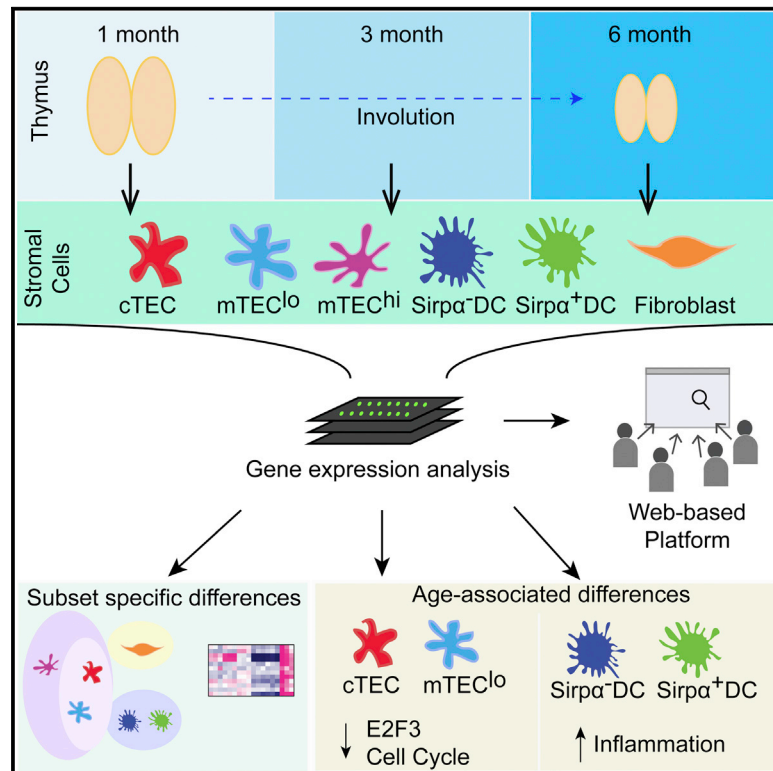# Global Transcriptional Profiling Reveals Distinct Functions of Thymic Stromal Subsets and Age-Related Changes during Thymic Involution

Graphical Abstract

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In Brief

Heterogeneous thymic stromal cells regulate proper T cell development. Age-associated thymic involution results in diminished T cell output and immunity. Ki et al. now present transcriptional profiling of six thymic stromal subsets during early thymic involution. They conclude that diminished E2F3 activity in thymic epithelial cells and an augmented inflammatory signature in dendritic cells are early hallmarks of involution. Data are provided on a web-based platform to facilitate discovery of molecular mediators of thymocyte:stromal cell crosstalk and early thymic involution.

Highlights

During involution, cell-cycle genes and E2F3 targets are down-regulated in TEC subsets

Thymic dendritic cell subsets acquire proinflammatory features during involution

Transcriptional profiling of thymic cell subsets gives insight into stromal biology

Thymic stromal and thymocyte data sets are provided in a searchable platform

Global Transcriptional Profiling Reveals Distinct Functions of Thymic Stromal Subsets and Age-Related Changes during Thymic Involution

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SUMMARY

Age-associated thymic involution results in diminished T cell output and function in aged individuals. However, molecular mediators contributing to the decline in thymic function during early thymic involution remain largely unknown. Here, we present transcriptional profiling of purified thymic stromal subsets from mice 1, 3, and 6 months of age spanning early thymic involution. The data implicate unanticipated biological functions for a subset of thymic epithelial cells. The predominant transcriptional signature of early thymic involution is decreased expression of cell-cycle-associated genes and E2F3 transcriptional targets in thymic epithelial subsets. Also, expression of proinflammatory genes increases with age in thymic dendritic cells. Many genes previously implicated in late involution are already deregulated by 3–6 months of age. We provide these thymic stromal data sets, along with thymocyte data sets, in a readily searchable web-based platform, as a resource for investigations into thymocyte:stromal interactions and mechanisms of thymic involution.

INTRODUCTION

The thymus is spatially organized into cortical and medullary regions containing heterogeneous stromal cells, including thymic epithelial cells (TECs), dendritic cells, macrophages, fibroblasts, and endothelial cells (Love and Bhandoola, 2011). As thymocytes mature, they migrate through distinct thymic microenvironments, where they undergo bidirectional crosstalk with local stromal cells, essential for the generation of a diverse and self-tolerant T cell pool (Petrie and Zúñiga-Pflücker, 2007). Signals provided by developing thymocytes are also required for differentiation and maintenance of thymic stromal cells (Nitta et al., 2011). Although some molecular signals responsible for this bidirectional signaling have been characterized, many remain to be identified.

Thymocyte:stromal cell crosstalk first occurs in the cortex where thymocyte progenitors encounter cortical TECs (cTECs) that express NOTCH1 ligands, SCF, and interleukin-7 (IL-7), which are essential for thymocyte survival, proliferation, and commitment to the T cell lineage (Anderson and Takahama, 2012; Petrie and Zúñiga-Pflücker, 2007). In addition, cTECs display self-peptide:major histocompatibility complex (MHC) complexes that promote positive selection of self-MHC-restricted thymocytes and apoptosis of autoreactive cells (McCaughtry et al., 2008). Reciprocally, unidentified signals from early thymocyte progenitors are critical for cTEC differentiation (Klug et al., 1998).

Following positive selection, thymocytes migrate into the medulla, where they interact with medullary thymic epithelial cells (mTECs). mTECs can be subdivided into mTEClo and mTEChi subsets, based on differential expression of CD80 and MHC class II. The mTEChi subset expresses the chromatin modulator AIRE, which promotes expression of tissue-restricted antigens (TRAs), genes otherwise expressed in a limited number of differentiated tissues, such as the pancreas or retina (Anderson et al., 2002; Klein et al., 2011). When medullary thymocytes engage TRAs on mTECs, they undergo apoptosis or differentiate into regulatory T cells, thus establishing central tolerance to peripheral self-antigens. Conversely, mTEClo cells must engage thymocytes entering the medulla, via tumor necrosis factor superfamily members, to drive differentiation to the mTEChi stage (Nitta et al., 2011). Thus, bidirectional signaling in the medulla between TECs and maturing thymocytes is critical for thymocyte tolerance and medullary stromal organization.

Thymic dendritic cells also play a critical role in central tolerance. Conventional thymic dendritic cells can be subdivided into Sirpα⁻CD8⁺CD11b⁻ (DC) and Sirpα⁺CD8⁻CD11b⁺ (DCS) subsets (Li et al., 2009). Thymic dendritic cells can acquire TRAs from mTECs to mediate deletion of autoreactive thymocytes (Klein et al., 2011). In addition, DCS traffic peripheral antigens into the thymus to mediate negative selection or induction of regulatory T cells (Bonasio et al., 2006; Proietto et al., 2008). Thymic dendritic cells require chemotactic signals from mTECs to accumulate in the medulla and function properly (Lei et al., 2011), underscoring the complex interplay between thymocytes

and various stromal subsets required to ensure production of a self-tolerant T cell repertoire.

The thymus involutes in an age-dependent manner, resulting in diminished TEC cellularity and turnover (Gray et al., 2006), disrupted thymic architecture, decreased thymic output, and reduced T cell function (Chinn et al., 2012; Haynes and Maue, 2009; Nikolich-Žugich et al., 2012). Both hematopoietic age-related dysfunction and degeneration of the thymic stromal compartment likely contribute to thymic involution (Berent-Maoz et al., 2012; Chinn et al., 2012). Although reduced levels of the transcription factor *Foxn1* contribute to TEC atrophy (Chen et al., 2009) and genetic manipulation of cell-cycle regulators can maintain thymic mass in aged mice (Garfin et al., 2013; Robles et al., 1996), specific molecular pathways driving degeneration of the thymic stroma early in the process of involution remain to be discerned. Furthermore, whereas manipulation of sex steroids or growth factor levels in aged individuals can transiently increase thymic size (Min et al., 2007; Sutherland et al., 2005), the resultant thymi are not functionally equivalent to young thymi and may be incapable of maintaining central tolerance (Griffith et al., 2012). Thus, identifying molecular pathways that regulate stromal changes associated with involution remains an important goal.

Here, we present global transcriptional profiling of purified thymic stromal subsets from mice at 1, 3, and 6 months of age, enabling the community to query stromal subset-specific gene expression before and during early thymic involution. Griffith et al. (2009, 2012) previously reported transcriptional profiles of nonsorted thymic stroma from the cortex, medulla, and cortico-medullary junction, enabling evaluation of regionalized gene expression in young and aged thymi. The current resource provides complementary information about gene expression in specific stromal cell types during early thymic involution. Our data reveal predicted, as well as unexpected, expression of genes in different thymic stromal subsets that suggest unanticipated stromal cell functions. Interestingly, decreased expression of cell-cycle genes and downregulation of E2F3 activity are major hallmarks of early thymic involution in the TEC compartment. Upregulation of proinflammatory genes in thymic dendritic cell subsets also occurs over this time course. Analysis of genes previously implicated in late thymic involution reveals that some of these factors may contribute to early involution, whereas others are likely sequelae of the aging process. To facilitate use of this resource, we provide our thymic stromal gene expression data, along with our previous thymocyte transcriptional profiling data, as an in silico model in the readily searchable web-based platform Gene Expression Commons (GExC) (Seita et al., 2012; https://gexc.stanford.edu/model/475). Exploration of our data sets on this user-friendly interface will enhance elucidation of the molecular pathways mediating thymocyte-stromal cell crosstalk and age-associated thymic stromal changes.

RESULTS

Transcriptional Profiling of Thymic Stromal Subsets at 1, 3, and 6 Months of Age

Using fluorescence-activated cell sorting (FACS), we purified thymocyte stromal subsets from mice over the course of early thymic involution to identify subtype-specific and age-regulated transcriptional profiles. Thymic cTEC, mTEClo, mTEChi, DC, DCS, and fibroblast populations were FACS purified from C57BL/6J male mice at 1, 3, and 6 months of age as follows: cTEC (Epcam$^+$CD11c$^-$ UEA1$^-$MHCII$^+$Ly51$^+$), mTEClo (Epcam$^+$ CD11c$^-$UEA1$^+$MHCII^{lo}CD80^{lo}), mTEChi (Epcam$^+$CD11c$^-$ UEA1$^+$ MHCII^{hi}CD80^{hi}), DC (MHCII$^+$Epcam$^-$CD11c$^+$Sirpα$^-$CD80$^+$), DCS (MHCII$^+$Epcam$^-$CD11c$^+$Sirpα$^+$CD80$^+$), and fibroblasts (MHCII$^-$CD45$^-$Ter119$^-$CD31$^-$; Figure 1A). During the sort setup, we ensured that all Epcam$^+$ TECs were included in the initial MHCII$^+$ gate (Figure S1A). Purified mRNA from biological duplicates of each subset at each age was analyzed on Affymetrix Mouse 430 2.0 microarrays. Resultant data were uploaded to the GExC platform for normalization (Seita et al., 2012), and resultant normalized signal intensity values were used for all subsequent bioinformatics analyses herein.

To assess consistency between duplicate data sets and to globally compare transcriptional profiles between stromal subsets, we performed unsupervised hierarchical clustering and principal components analysis (PCA) on the 30% of genes most variable in expression across subsets (Figures 1B and 1C). Duplicate data sets clustered closely together, indicating reliability of the data. Stromal subtypes clustered together regardless of age (Figure 1B), demonstrating that each stromal subset maintains its transcriptional identity during thymic involution. Stromal subsets at 6 months of age were further from their 1- and 3-month counterparts, indicative of age-related transcriptional changes in the data. Interestingly, age-associated changes were implicated not only in TEC subsets, which have been suggested to be drivers of involution, but also in dendritic cells and fibroblasts. Overall, PCA was consistent with hierarchical clustering (Figure 1C) and showed that mTEChi were distal to other TEC subsets, likely due to expression of diverse TRAs. As expected, dendritic cell subtypes clustered together, whereas fibroblasts were distinct from other stromal subsets.

To validate our data, we queried genes previously reported to be differentially expressed in distinct stromal subsets (Figure 1D). TECs expressed cytokeratins and the transcription factor *Foxn1*, confirming their identity (Blackburn et al., 1996; Klug et al., 1998). As expected, cTEC and mTEClo expressed high levels of *Il7* (Repass et al., 2009; Ribeiro et al., 2013), whereas *Ccl25*, *Cxcl12*, *Dll4*, *Enpep/Ly51*, *Ly75/CD205*, and *Psmb11/β5t* were predominantly expressed by cTECs (Murata et al., 2007; Plotkin et al., 2003; Wurbel et al., 2000). *Aire*, *Cldn3*, and *Cldn4* were expressed specifically in mTEChi cells, as expected (Hamazaki et al., 2007). MHCII genes were expressed by dendritic cells and TECs, consistent with their roles in antigen presentation, whereas dendritic cells and mTEChi expressed high levels of *Cd80* and *Cd86*. *ItgaX* (CD11c) was expressed uniquely by dendritic cells, with *Sirpα* and *Xcr1* (Lei et al., 2011) expressed uniquely in the DCS and DC subsets, respectively. Fibroblasts expressed *Pdgfrα* and *Pdgfrβ* and the extracellular matrix (ECM) component *Col6a3*, as anticipated for mesenchymal cells (Foster et al., 2008). To further validate our data, we sorted an additional biological replicate of thymic stromal subsets from mice 1 month of age and confirmed subset-specific expression of 25 genes by quantitative RT-PCR (qRT-PCR) analysis on the Fluidigm platform (Figure S1B). Altogether, these analyses
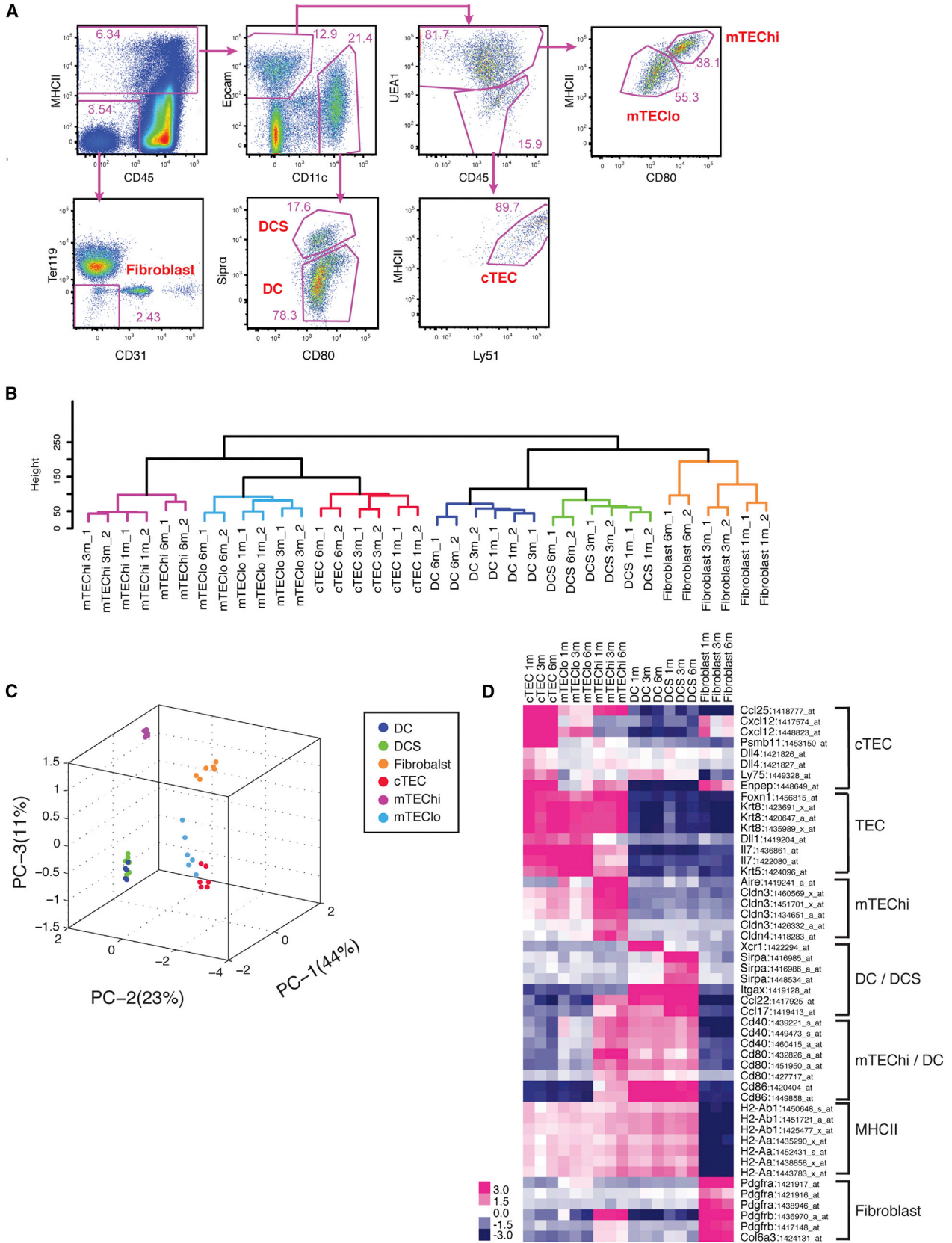
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validate the data sets and demonstrate that thymic stromal subset identity is maintained during aging.

Differentially Expressed Genes Suggest Distinct Functions of Thymic Stromal Subsets

We identified differentially expressed genes (DEGs) in each stromal subset as those genes uniquely up- or downregulated by >2-fold relative to all other stromal subsets of the same age, with an adjusted p value < 0.01 (Table S1). As expected from PCA analysis, mTEChi and fibroblast subsets had the most unique DEGs at each age (Figure 2A). We next investigated common functional characteristics of unique DEGs in each subset. The top Gene Ontology (GO) term hit for DEGs from 1-month-old fibroblasts was "cell adhesion," with "extracellular matrix" appearing as well, suggesting that, although fibroblasts were sorted based on the absence of markers in other subsets, the population is greatly enriched in mesenchymal cells. A heatmap of genes in the GO term cell adhesion category reveals ECM components and integrins that are likely relevant substrates for adhesion and migration in the thymus (Figure 2B).

The large number of DEGs and dissimilarity in overall transcriptional relatedness of mTEChi relative to other stromal subsets (Figures 1C and 2A) may have been due to expression of diverse TRAs (Derbinski et al., 2001). To address this possibility, we identified Aire-regulated genes by analyzing published expression data from Aire-deficient versus wild-type mTEChi cells (Derbinski et al., 2005). After excluding genes not shared between microarray platforms, we compared the list of Aire-regulated genes to mTEChi-upregulated DEGs. Of the 560 mTEChi-upregulated DEGs, only 150 (37%) were Aire regulated (Figure 2C), suggesting many mTEChi DEGs are Aire independent. Furthermore, comparison against a list of TRAs identified on the basis of limited tissue expression (Griffith et al., 2012) revealed overlap with only 9% of mTEChi DEGs (Figure 2D). Thus, many mTEChi DEGs may have a biological function, rather than serving as TRAs. Interestingly, the top GO hit term for 1-month mTEChi DEGs was "defense response" (p value: 8×10^{-7}), which included chemokines (*Ccl20*, *Cxcl13*, and *Cxcl3*), defensins (*Defb19*, *Defb3*, *Defb5*, *Defb6*, *Defb8*, *Defa21*, and *Defa22*), and cytokines (*Il1f6*, *Il23a*, and *Il5*; Figure 2E). Only 15 of 55 defense response genes were identified as TRAs (not shown). Thus, mTEChi cells may promote defense responses in the thymus. Consistent with a unique biological state of the mTEChi subset, Aire has been shown to regulate differentiation of mTEChi cells (Yano et al., 2008).

Because of the transcriptional relatedness of cTEC to mTEClo and DC to DCS (Figures 1B and 1C), few DEGs were identified in these subsets (Figure 2A). Although these DEGs are likely important for the unique biology of these subsets (Figure S2B; Table S1), the number of DEGs was too low to proceed with downstream bioinformatic analyses. Therefore, we identified additional DEGs in cTEC after omitting mTEClo and in DC after omitting DCS from the comparisons and vice versa (Figure S3). GO analyses of these DEGs confirmed epithelial characteristics of cTEC and mTEClo subsets (Figure 3A). The "epithelium development" and "cell adhesion" terms contained previously known as well as unreported genes in cTEC and mTEClo subsets (Figures 3B and S4), including several genes involved in Wnt signaling, such as *Wnt4* and the receptors *Fzd2* and *Fzd3* (consistent with Bredenkamp et al., 2014, Griffith et al., 2012, and Min et al., 2007). *Cldn8* was expressed in cTECs, whereas *Cldn9* and *Cldn12* were expressed in cTEC and mTEClo subsets, demonstrating TEC subset specificity of claudin family members, consistent with expression of *Cldn3* and *Cldn4* in mTEChi cells (Table S1; Hamazaki et al., 2007). The top hit from GO analyses of DC and DCS DEGs was "immune response" (Figure 3A), including Toll-like receptors, cytokines, and chemokines (Figures 3C and S5), consistent with the central role for dendritic cells in eliciting and modulating immune responses.

Thymic Involution Is Associated with Downregulation of Cell-Cycle Genes in the mTEClo Subset and Decreased Activity of E2F3 in cTEC and mTEClo Cells

Thymocyte and thymic stromal cellularity are greatest in mice around 1 month of age and subsequently decline as the thymus involutes (Gray et al., 2006). To evaluate transcriptional changes associated with early thymic involution, we compared gene expression in stromal subsets from mice at 1, 3, and 6 months of age. We identified aging-associated DEGs as those genes modulated by $\geq$2-fold, with an adjusted p value $\leq$ 0.01 in pairwise comparisons of each subset at the three ages. Using these criteria, no aging-associated DEGs were identified in fibroblasts or cTECs, with only 1 DEG in mTEChi (*Igha*). In contrast, a significant number of DEGs were identified in DC, DCS, and mTEClo subsets (Figure 4A; Table S2). Over 90% of the DEGs in DC and DCS were upregulated with age, whereas DEGs in mTEClo were both up- and downregulated with similar frequencies. We conducted K-means clustering on DEGs from each subset to identify groups of genes up- or downregulated as aging progresses from 1 through 6 months of age. Cluster 3 from mTEClo contained genes progressively and significantly downregulated with age (Figures 4B and 4C). We performed GO term analyses on each cluster to identify biological functions associated with age-related changes and found significant hits only for mTEClo cluster 3. Genes in this cluster were associated with the cell cycle (Figure 4D), implicating a progressive decrease in expression of cell-cycle regulators, such as *Aurkb*, *Cdc20*, *Cdc6*, and *Ccnb1*, in mTEClo during involution (Figure 4E).

We next utilized gene set enrichment analysis (GSEA) to identify aging-associated gene sets, without imposing an arbitrary

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Figure 1. Expression Profiling of Thymic Stromal Subsets

(A) Strategy for sorting thymic stromal subsets (gated on live events; frequencies within gates displayed).

(B and C) Hierarchical clustering (B) and PCA (C) were carried out on the top 30% of genes most variable in expression. Each symbol in the PCA represents a single array.

(D) Expression of known thymic stromal subset-specific genes is presented as a heatmap, using the average of biological duplicates for each subset. Probe sets with a dynamic range $\geq$3 (in GExC) were displayed if multiple probe sets were present for one gene.
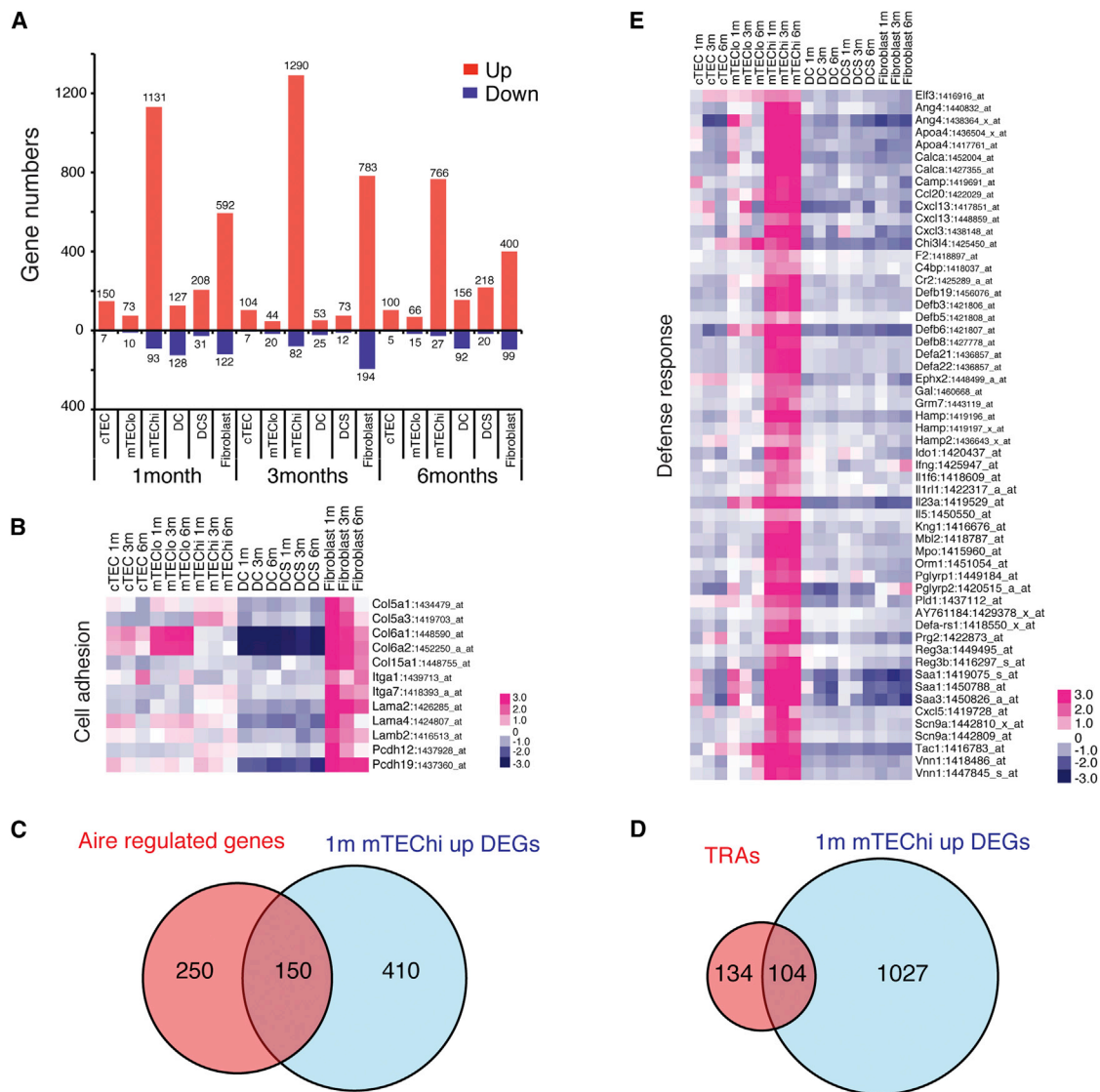
Figure 2. Mesenchymal Signature of Fibroblasts and Defense Response Function of mTEChi Revealed by DEGs

(A) The number of stromal subset-specific DEGs, identified by pairwise comparisons between stromal subsets of the same age, is displayed.

(B) The heatmap displays the relative expression of select upregulated DEGs from 1-month fibroblasts that overlap with the "cell adhesion" GO term (the heatmap of all overlapping genes is in Figure S2A).

(C) Overlap between Aire-regulated genes (Derbinski et al., 2005) and 1-month mTEChi-upregulated DEGs, shared between microarray platforms.

(D) Overlap between TRAs (Griffith et al., 2012) and 1-month mTEChi-upregulated DEGs.

(E) A heatmap displaying the relative expression of upregulated mTEChi DEGs that overlap with the "defense response" GO term hit.

fold-change cutoff. Strikingly, E2F3 target genes (Kong et al., 2007) were significantly downregulated with age in both cTEC and mTEClo subsets (Figure 4F). This decline was gradual in mTE-Clo cells from 1 to 6 months but precipitous in cTECs between 1 and 3 months (Figure 4G). E2F3 is a transcription factor critical for normal cellular proliferation (Humbert et al., 2000), and many target genes are regulators of the cell cycle, such as *Cdc6*, *Ccna2*, *Aurka*, and *Cdc7* (Figure 4H). Together, these analyses suggest that a decline in E2F3 activity results in decreased cell-cycle progression in cTEC and mTEClo cells, likely contributing to the decline in TEC cellularity early in the process of thymic involution.

DC and DCS Have an Increasingly Proinflammatory Signature with Age

Although GO term analyses did not yield significant hits for aging-associated DEGs in DC and DCS subsets, GSEA revealed an increasingly proinflammatory signature with age. Genes induced after lipopolysaccharide (LPS) simulation in human monocytes were upregulated with age in thymic DC and DCS (Figure 5A; Dower et al., 2008). Aging is associated with increased inflammation (Chung et al., 2009), and inflammatory cytokines become elevated in human thymi with age (Sempowski et al., 2000). Both previously described and unreported proinflammatory molecules,
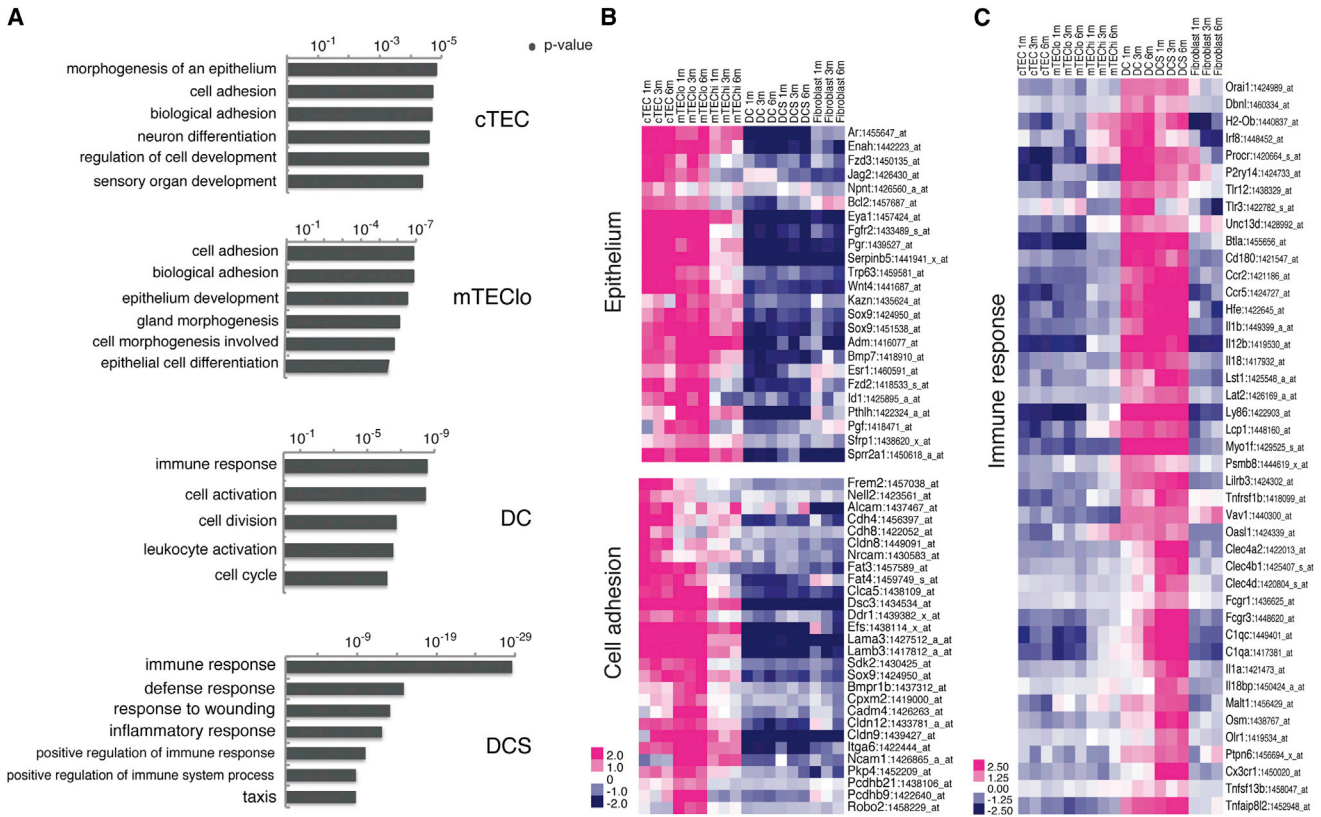
Figure 3. Epithelial Identity of cTEC/mTEClo and Immune Response Function of DC/DCS Revealed by DEGs

(A) GO term analyses of DEGs from cTEC/mTEClo confirm the epithelial identity of these subsets. GO term analysis of DEGs identified in DC/DCS reveal immune response and inflammatory signatures. See Figure S3.

(B) The heatmap displays the relative expression of select upregulated DEGs in cTEC/mTEClo that overlap with the "morphogenesis of an epithelium" and "epithelium development" (top) and "cell adhesion" (bottom) GO term hits (the heatmap of all overlapping genes is in Figure S4).

(C) The heatmap displays the relative expression of select upregulated DEGs in DC/DCS that overlap with the immune response GO term hit (the heatmap of all overlapping genes is in Figure S5).

including *Il1a*, *Il1b*, *Cxcl2*, *Il6*, *Il12b*, *Il18*, and *Tnf*, were upregulated in thymic dendritic cells at 6 months of age (Figure 5B), suggesting that an increasingly inflammatory environment generated by aging dendritic cells contributes to early thymic involution.

Diminished Niche Activity, Declining TEC Homeostasis, and a Decrease in Growth Factors Are Associated with Early Thymic Involution

We analyzed pathways and genes previously implicated in late thymic involution to determine if their expression was altered in stromal subsets during the early stages of thymic degeneration. *Dll4*, *Il7*, and *KitL*, which are all required for a functional thymocyte progenitor niche, were expressed at the highest levels by cTECs. Only *Dll4* expression diminished over 1–6 months (Figure 6A), suggesting a decline in Notch signaling contributes to early thymic involution.

Several studies have implicated decreased *Foxn1* expression as a major contributor to thymic involution (Bredenkamp et al., 2014; Chinn et al., 2012). *Foxn1* was not identified as an aging-associated DEG because it did not meet the <0.01 p value criterion. However, *Foxn1* expression declined ~2-fold in both cTEC

and mTEClo subsets from 1 to 6 months of age (Figure 6B), consistent with previous findings at later stages of involution (Bredenkamp et al., 2014; Ortman et al., 2002). Two recent studies associated late thymic involution with altered expression of Wnt pathway genes (Bredenkamp et al., 2014; Griffith et al., 2012). Consistent with these studies, *Wnt3a* and *Wnt4* were downregulated in early thymic involution, specifically in cTEC and mTEClo cells, whereas *Wnt10a* was upregulated in the mTEClo subset (Figure 6B). Consistent with Griffith et al., *Wnt5b* was slightly upregulated by 6 months; however, this increase occurred in fibroblasts. Taken together, some Wnt family members and *Foxn1* are deregulated early in thymic involution, suggesting that TEC homeostasis is impaired, consistent with decreased TEC proliferation and E2F3 activity described above.

Inflammatory cytokines are upregulated in aging human thymi (Sempowski et al., 2000). Similarly, *Lif*, *Il6*, and *Osm* were upregulated in murine thymic dendritic cell subsets over 1–6 months (Figure 6C). Furthermore, *Tnf*, *Il1a*, and *Il1b* were upregulated by DCS. Interestingly, the activating receptor *Il1r1* was expressed by all TECs and fibroblasts, whereas the IL-1 antagonists *Il1rn* and *Il1r2* were expressed by mTECs, suggesting IL-1 likely

A

B

C

D

E

F

mTEClo-E2F3 targets

cTEC-E2F3 targets
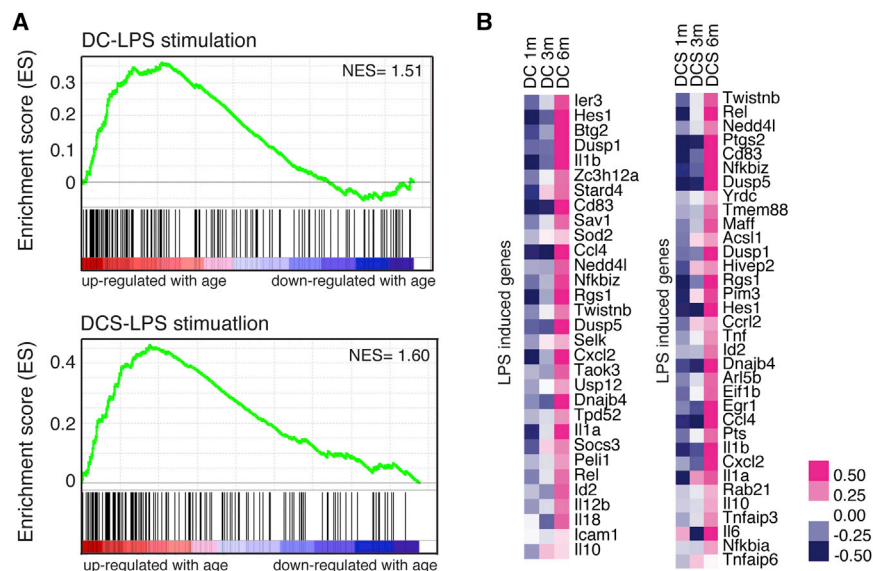
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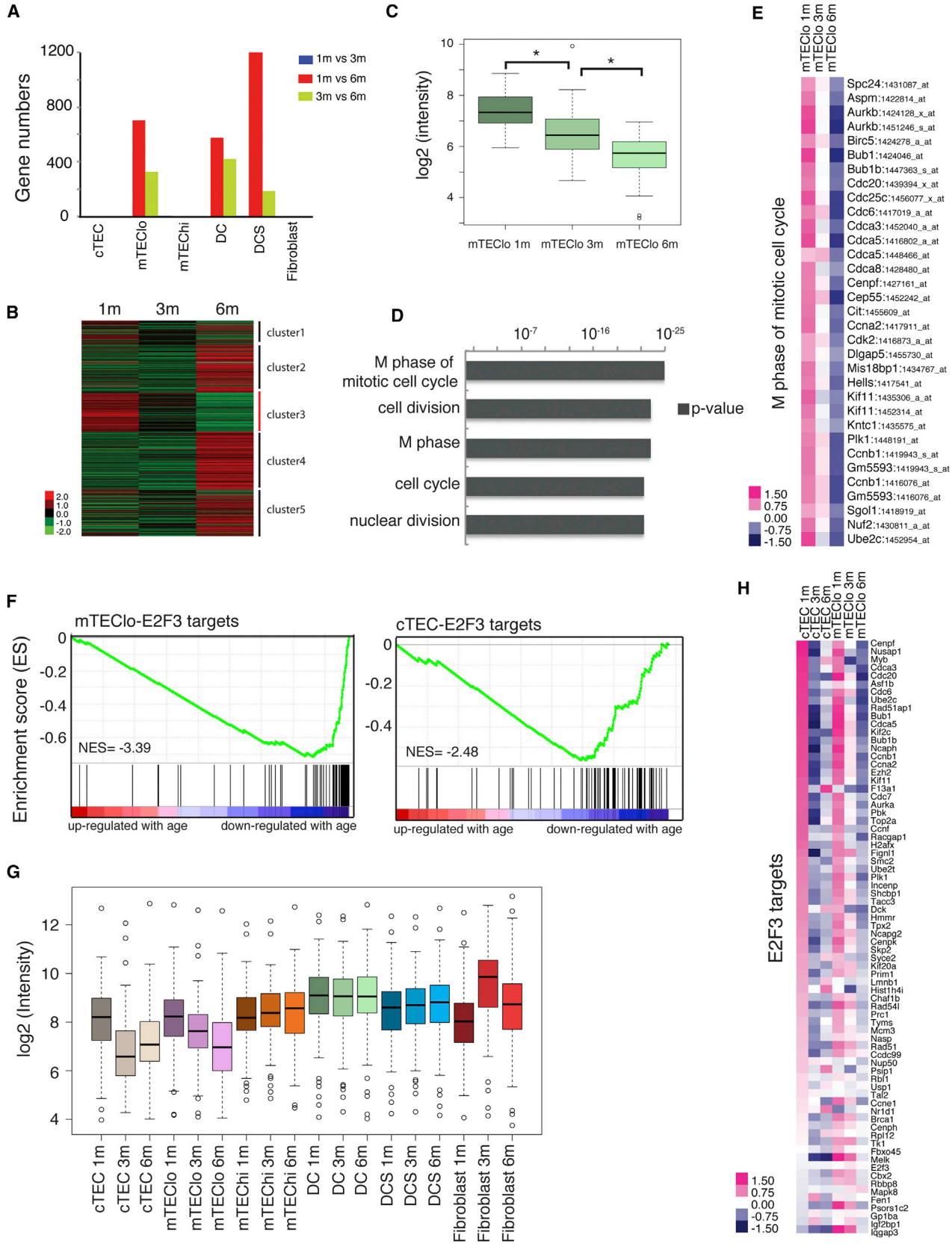
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Figure 5. Increased Expression of Proinflammatory Genes in Aging DC and DCS Subsets

(A) GSEA analysis reveals a positive correlation between LPS-stimulated genes and genes upregulated with age in DC and DCS (NOM p value = 0.054, NES = 1.51 for DC; NOM p value = 0.025, NES = 1.6 for DCS).

(B) The heatmap displays the relative expression of some LPS-induced genes that are positively correlated with aging in DC and DCS (the heatmap of all overlapping genes is in Figure S6).

impinges preferentially on cTECs and fibroblasts. We also note a trend of increased *Csf1* expression by mTEClo over 1–6 months, which can drive dendritic cell differentiation (Fancke et al., 2008).

Sex steroids and growth factors can modulate thymic size (Lynch et al., 2009). For example, castration transiently increases thymic size and output (Griffith et al., 2012; Sutherland et al., 2005). Androgen receptor, *Ar*, is expressed predominantly on cTEC but is not upregulated over 1–6 months (Figure 6D). Administration of growth hormone and KGF also induce growth of involuted thymi (Lynch et al., 2009). Expression of the growth hormone receptor *Ghr* was not altered over 1–6 months in TECs (Figure 6D); however, it was decreased with age in fibroblasts, which expressed the highest levels of *Ghr* at 1 month. The expression of *Fgfr2/Kgfr* on cTEC and mTEClo cells did not decline over 1–6 months. However, expression of the ligand *Fgf7/Kgf* diminished in both mTEChi cells and fibroblasts between 3 and 6 months (Figure 6D). Taken together, our data suggest that reduced growth factor signaling during early involution could affect both TECs and fibroblasts.

A Searchable Web-Based Platform Containing Thymocyte and Thymic Stromal Cell Gene Expression Data

To make our thymic stromal expression data easily accessible, we have uploaded the data sets to the web-based platform

GExC, where we have generated an online model containing both thymic stromal microarray data sets and our previously published thymocyte data sets (Seita et al., 2012; https://gexc.stanford.edu/model/475). Users can readily query expression of genes of interest in thymocyte or thymic stromal subsets (Figure 7A). One of the most-powerful features of this platform is that data are normalized against a common reference of ~12,000 diverse data sets; thus, gene expression values can be compared against the full dynamic range of expression values for each probe set (Seita et al., 2012). In addition to normalized signal intensity values, "gene expression activity values" are provided on GExC. After using a step function to determine the threshold of expression for each probe set, each intensity value above and below this cutoff was assigned a percentile rank, reflecting the distribution of values in the common reference, as seen in the histograms (Figures 7C and 7D). For example, if a 30% gene expression activity value is obtained, the subset queried expresses the gene at a level above the first 30% of the common reference data sets over the threshold and below the remaining 70%. This enables users to determine how robustly a gene is expressed relative to the range of expression values across diverse tissues. A second key advantage is the ability to readily search for expression patterns of interest, such as genes uniquely expressed in cTEC (Figure 7B). Finally, the combination of thymic stromal data with our previously published thymocyte data (Seita et al., 2012) will enable users to search for partner genes, such as receptors and ligands, that may contribute to thymocyte:stromal cell crosstalk. For example, the chemokine receptor *Cxcr4* is expressed in cortical double-negative and double-positive thymocytes, as well as in thymic dendritic cells and fibroblasts,

Figure 4. Cell-Cycle Genes and E2F3 Target Genes Are Significantly Downregulated in mTEClo and cTEC Subsets in Early Thymic Involution

(A) The number of up- or downregulated DEGs identified from pairwise comparisons of stromal subsets at different ages is shown.

(B) The heatmap displays K-means clustering of all age-associated DEGs identified in the mTEClo subset.

(C) The boxplot displays expression levels of mTEClo DEGs in cluster 3 from (B), which are progressively downregulated with age (p value < 2.2×10^{-16}).

(D) The top five GO term hits from cluster 3 of mTEClo DEGs reveal downregulation of cell-cycle-associated genes during aging.

(E) The heatmap displays the relative expression of downregulated age-associated DEGs in mTEClo that overlap with the "M phase of mitotic cell cycle" GO term.

(F) GSEA reveals expression of E2F3 target genes is downregulated with age in mTEClo and cTEC subsets (cTEC nominal [NOM] p value < 10^{-3}, normalized enrichment score [NES] = −2.48; mTEClo NOM p value < 10^{-3}, NES = −3.39).

(G) The box-and-whisker plot shows expression levels of E2F3 target genes in stromal subsets at 1, 3, and 6 months of age. Decreased expression of E2F3 target genes is apparent in aging cTEC and mTEClo subsets. Whiskers represent data within 1.5 of the interquartile range of the first and third quartiles.

(H) Relative expression of E2F3 target genes in cTEC and mTEClo subsets at 1, 3, and 6 months of age.
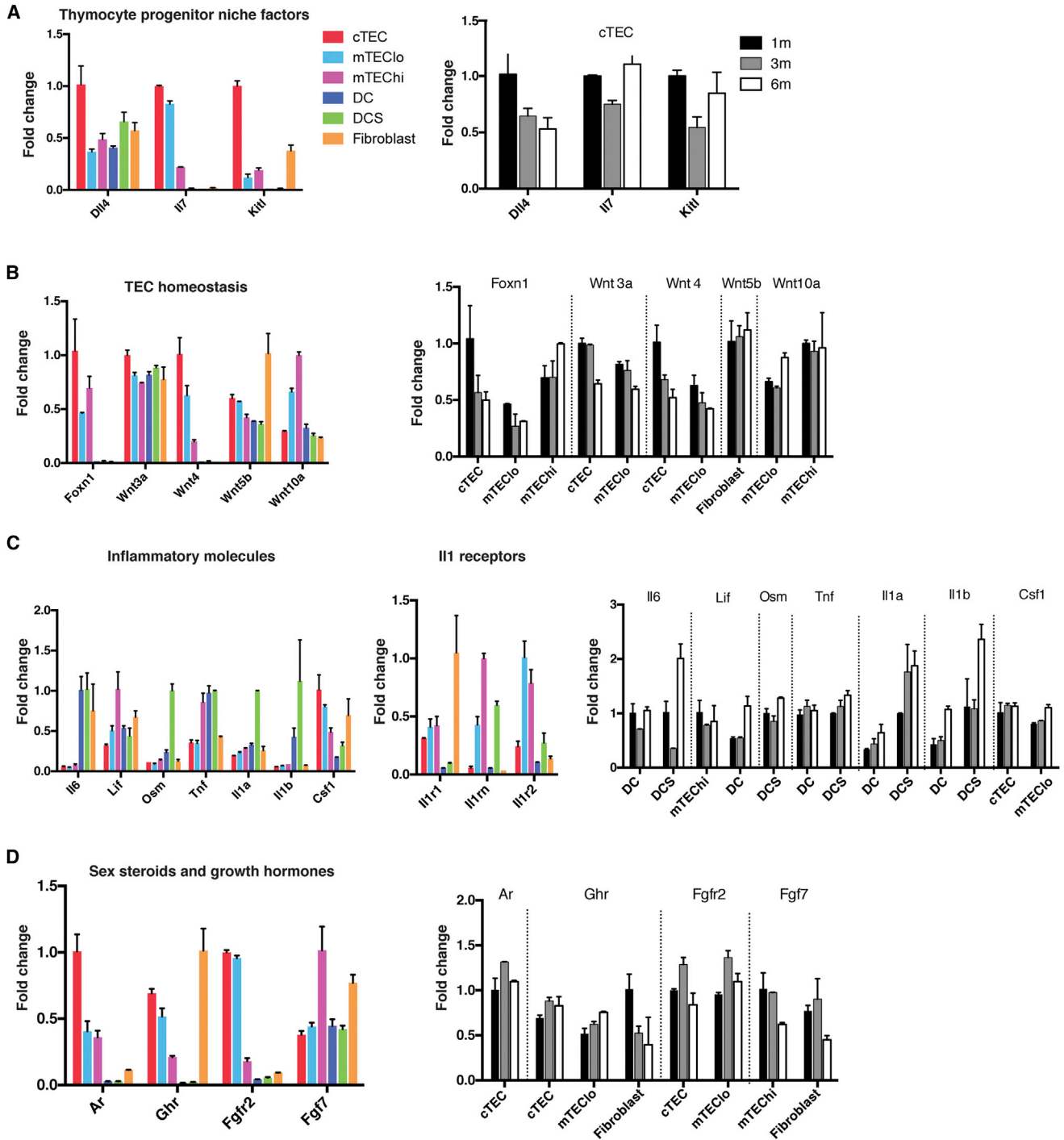
Figure 6. A Subset of Genes Implicated in Late Thymic Involution Are Deregulated in Thymic Stroma by 3–6 Months

Graphs display the relative expression levels of genes involved in (A) thymocyte progenitor niche activity, (B) TEC homeostasis, (C) inflammation, and (D) sex steroid and growth hormone signaling in the thymus. For all graphs, expression values are normalized to the subset expressing the highest level of each gene at 1 month of age. The color graphs on the left display expression levels of genes of interest for each stromal subset at 1 month. The grayscale graphs on the right display expression levels of the same genes from 1 to 6 months for the indicated thymic stromal subsets. Graphs depict the mean plus or minus range of expression values.
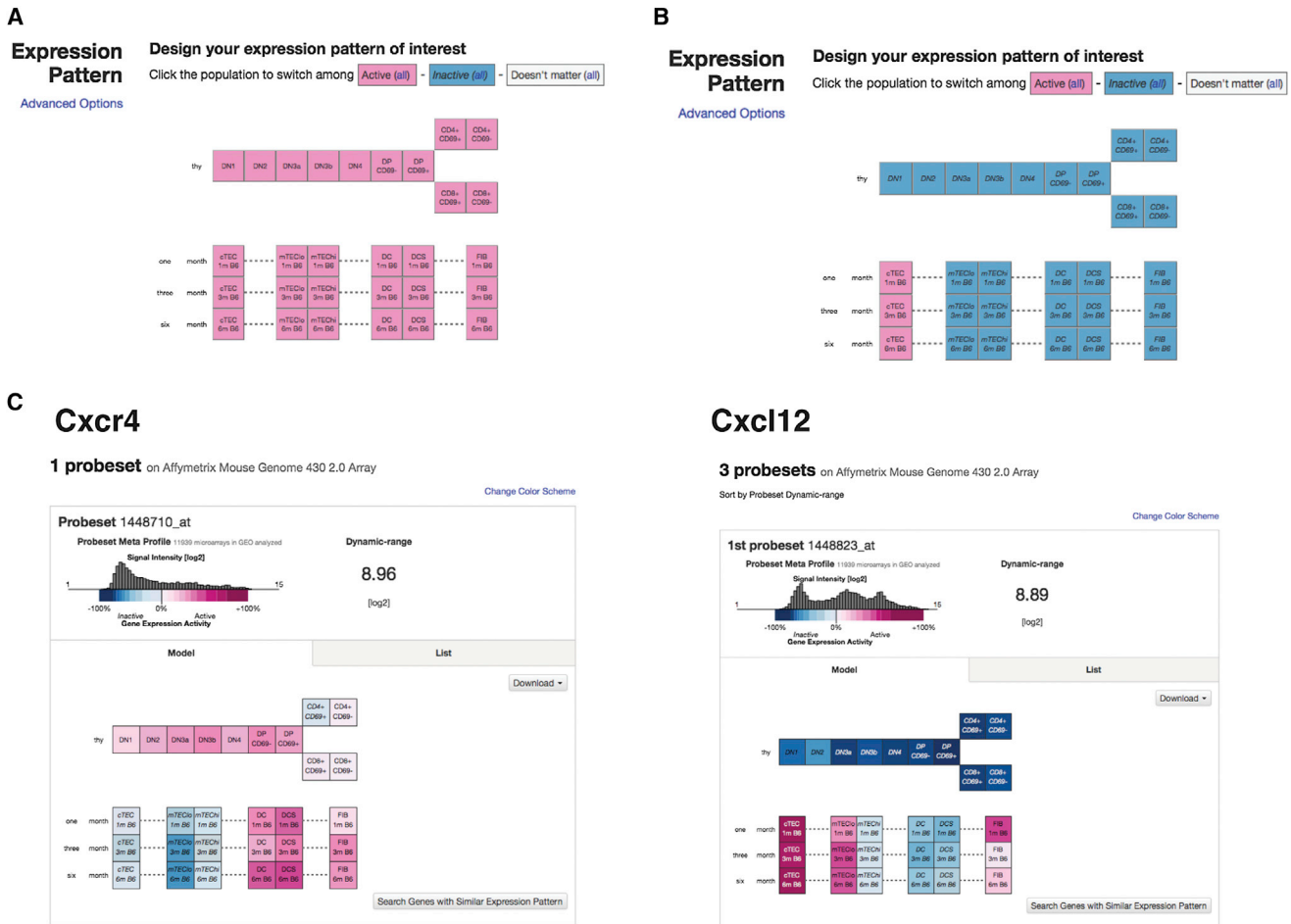
Figure 7. GExC Platform for Exploring Expression Data from Thymocytes and Thymic Stromal Subsets

(A) The "complete thymocyte:stromal interaction" model in GExC (https://gexc.stanford.edu/model/475). Eleven thymocyte subsets (at 1 month of age) and six thymic stromal subsets (at 1, 3, or 6 months of age) are represented as boxes, with colors representing the average expression level of data from biological replicates (n = 3 and n = 2 replicates for thymocyte and stromal subsets, respectively).

(B) In this example of the pattern search feature on GExC, high expression in cTECs relative to all other subsets will be queried.

(C) The database is useful for querying ligand-receptor pairs involved in thymocyte:stromal cell crosstalk. For example, the chemokine *Cxcr4* is expressed by cortical thymocytes, as well as dendritic cells, whereas the ligand *Cxcl12* is expressed at high levels by cTEC. The dynamic range of each probe set is displayed as a histogram, enabling users to ascertain expression levels relative to the range of values in publically available biological space (>12,000 diverse data sets are represented).

whereas its ligand *Cxcl12* is highly expressed by cTECs (Figure 7C). This in silico model should greatly facilitate discovery of molecular mediators of thymocyte:stromal cell crosstalk, thymic stromal function, and altered gene expression in stromal cells during early thymic involution.

DISCUSSION

We present global gene expression data from six thymic stromal subsets over the course of early thymic involution (1, 3, and 6 months of age) on a web-based platform as a resource for the scientific community (https://gexc.stanford.edu/model/475). Unsupervised clustering reveals the data sets are highly consistent (Figure 1), and qRT-PCR analyses on independently sorted samples validate the microarray expression data (Figure S1B). Nonetheless, there are several potential caveats to consider when querying the GExC database. First, thymic fibroblasts were sorted based on the absence of markers on hematopoietic cells, erythrocytes, and endothelial cells, enhancing the possibility for contamination with other cell types. Nevertheless, strong transcription of adhesion molecules, ECM components, and platelet-derived growth factor receptors reveals this population is highly enriched in mesenchymal cells. Second, the sorted populations may be heterogeneous. The transcriptional profile of rare TEC progenitors included in cTEC or mTEClo subsets would be entangled in the larger population. As additional TEC subsets are identified, it will be important to compare their transcriptional profiles against the parental TEC population. Third, when viewing gene expression activity values in GExC, it is important to remember that gene expression activity values are scaled

relative to the large common reference. This can cause a visual flattening of expression changes; for example, because *Foxn1* is uniquely expressed in TECs, the ∼2-fold decrease in expression over 1–6 months in cTEC and mTEClo cells (Figure 6B) is not as visually striking as the high expression in TECs relative to all other cell types. Also, gene expression activity values can be saturated (seen as 100%), as in the case of *Psmb11/β5t* in cTEC, obscuring the 20- to 50-fold increase in expression relative to other TEC subsets. In both cases above, it is important to compare the normalized signal intensity values, provided for download or on the "list" tab of GExC, to assess relative changes in expression with age or between subsets, respectively.

Surprisingly, our analyses revealed a significant association of mTEChi DEGs with the defense response GO term. mTEChi is the most distal thymic stromal population by PCA analysis (Figure 1C), and its distinct transcriptional profile could result from expression of diverse TRAs. TRA expression is largely driven by Aire (Anderson et al., 2002), and 38% of genes uniquely expressed in mTEChi overlapped with Aire-regulated genes (Figure 2C). However, not all Aire-regulated genes are TRAs (Derbinski et al., 2005), and the remaining 62% of mTEChi DEGs may serve a function beyond elimination of autoreactive thymocytes. The GO term category defense response includes genes such as defensins and cytokines (Figure 2E), which are critical for early innate immune responses against pathogens. Some of these genes (*Defb8* and *Defb3*) are expressed at high levels in mTEChi relative to canonical TRAs (*Crp* and *Gad67*; not shown), which tend to be expressed at low levels (Derbinski et al., 2001, 2008). Therefore, we speculate that these defense response genes may serve a functional role in mTEChi biology, perhaps to protect the thymus against infection.

Thymic involution results in diminished T cell output, leading to decreased immune function with age (Chinn et al., 2012; Lynch et al., 2009). Both CD4+ and CD8+ T cells generated in aged mice have impaired effector functions (Haynes and Maue, 2009; Nikolich-Žugich et al., 2012). These deficiencies can be attributed in part to the impact of an aged thymic environment, as fully functional T cells were generated when bone marrow precursors from aged mice were transferred into young, but not old, recipients (Eaton et al., 2008). Therefore, it is important to understand the cellular and molecular drivers of thymic involution, with the ultimate goal of restoring functional T cells to aged individuals or patients following cytoablative therapies or infections.

Unbiased analyses of aging-associated transcriptional changes during early thymic involution revealed that cell-cycle-related genes and E2F3 transcriptional targets were significantly downregulated in the mTEClo and cTEC subsets (Figure 4). This is consistent with the reduced proportion and number of cycling TECs with age (Gray et al., 2006) and the finding that overexpression of cyclinD1 or inactivation of Rb family members prevents thymic involution (Garfin et al., 2013; Robles et al., 1996). Our data add the perspective that E2F3 target genes, many of which are required for cell-cycle progression, are downregulated in both cTEC and mTEClo subsets by 6 months of age, suggesting that diminished cell-cycle progression in TECs is an early hallmark of thymic involution. A recent study revealed that *Foxn1* is an E2F3

target gene (Garfin et al., 2013). Thus, diminished E2F3 activity could directly reduce *Foxn1* levels, consistent with the reduction in *Foxn1* expression in TECs by 6 months of age (Figure 6B). *Foxn1* is a critical regulator of thymic involution, as diminished *Foxn1* expression results in premature involution (Chen et al., 2009), whereas enforced re-expression in aged TECs increases TEC cellularity and function and thymocyte output (Bredenkamp et al., 2014). However, maintenance of *Foxn1* expression in TECs was insufficient to prevent thymic involution (Zook et al., 2011), suggesting modulation of other E2F3 targets and pathways contribute to involution. *Foxn1* has also been implicated in regulating genes associated with cell cycle, such as *Ccnd1* (Bredenkamp et al., 2014); thus, there is likely a complex feedback mechanism driving both diminished cell-cycle progression and *Foxn1* levels. Expression of *E2f3* itself is not significantly altered in cTEC and mTEClo from 1 to 6 months (not shown), suggesting that E2F3 activity is modulated over the course of thymic involution. Identifying initial drivers of reduced E2F3 function will likely reveal critical regulators of thymic involution.

Diminished expression of Wnt pathway genes has also been implicated in age-associated TEC degeneration (Bredenkamp et al., 2014; Griffith et al., 2012). In agreement with these studies, we find that *Wnt3a* is diminished ∼1.5-fold in cTEC and mTEClo at 6 months, whereas *Wnt4* is decreased ∼2-fold in cTEC (Figure 6B). As *Wnt4* can induce *Foxn1* expression (Balciunaite et al., 2002) and *Wnt3a* is implicated in epithelial proliferation (Liu et al., 2010), diminished expression could contribute to impaired TEC homeostasis. Furthermore, the subtle increase in expression of *Wnt5b* by fibroblasts, which may drive adipogenesis (van Tienen et al., 2009), suggests thymic mesenchymal cells could promote the age-associated increase in adipose tissue early in involution. We did not find evidence for altered expression of other *Wnt* family members (not shown), suggesting that deregulation of some *Wnts* (Bredenkamp et al., 2014; Griffith et al., 2012) may occur late in the involution process.

Reduced potency of the thymocyte progenitor niche may also impair thymopoiesis during involution. *IL-7*, *Dll4*, and *Kitl*, expressed by TECs, are all critical for promoting survival and differentiation of early thymocyte progenitors (Anderson and Takahama, 2012). Of these niche factors, only expression of *Dll4* was diminished in cTECs by 6 months of age (Figure 6A), suggesting the thymic niche may have a reduced capacity to stimulate Notch signaling, thus impairing thymopoiesis in early involution. IL-7 levels are reduced in late thymic involution (Ortman et al., 2002); however, the lack of transcriptional downregulation over 6 months suggests that diminished IL-7 may be due to reduced cTEC numbers in the involuted thymus.

Signaling via sex steroids and growth hormones has also been implicated in thymic involution (Lynch et al., 2009). Our data do not reveal altered expression levels of *Ar* in TECs over 1–6 months (Figure 6D) or in estrogen or progesterone receptors (not shown). Taken together with the findings that sex steroid ablation induces only transient and partial regeneration of the thymus (Griffith et al., 2012) and that sex steroids diminish with age, whereas the thymus continues to involute (Chinn et al., 2012), our data do not suggest that sex steroids play a significant role in early thymic involution. However, it is possible that altered

sensitivity to signaling by sex steroid hormones in aging TECs could promote involution. Our data suggest that altered growth factor signaling could contribute to early thymic involution because *Ghr* and *Fgf7/Kgf* expression was decreased in fibroblasts by 6 months (Figure 6D). Because both TECs and fibroblasts express *Ghr*, thymic rebound induced by growth hormone (Chen et al., 2003) may be due to signaling in both stromal subsets. Reduced expression of *Fgf7/Kgf* by aging fibroblasts may diminish signaling through Fgfr2 on cTEC and mTEClo cells, contributing to TEC atrophy. Consistent with this notion, treatment with KGF causes transient thymic regeneration (Min et al., 2007).

Unbiased analyses of our data revealed an increasingly proinflammatory signature of DC and DCS with age (Figure 5). A previous study demonstrated that expression of *Osm*, *Lif*, *Scf*, and *Il6* increased in aged human thymi, and administration of these cytokines promoted thymic atrophy in mice (Sempowski et al., 2000). Our analyses add further insight that *Osm*, *Lif*, and *Il6* are upregulated by thymic dendritic cells early in the course of involution, though we found no evidence for increased *Scf*, either due to species or temporal differences. We also identified additional genes associated with inflammation that are upregulated by thymic dendritic cells by 6 months of age (Figure 5). *Il1a* and *Il1b* are strikingly upregulated by thymic dendritic cells, whereas activating, but not inhibitory, Il-1 receptors are expressed on cTEC and fibroblasts (Figure 6C). IL-1 administration promotes thymic atrophy (Morrissey et al., 1988), and *Nlrp3*-deficient mice, which cannot generate active IL-1, preferentially maintain the cTEC compartment with age (Youm et al., 2012). Altogether, these data suggest that IL-1 produced by thymic dendritic cells is likely a potent mediator of early thymic involution, signaling to both cTEC and thymic fibroblasts. Interestingly, *Csf1*, which can promote dendritic cell differentiation, is upregulated in mTEClo at 6 months (Figure 6C). Thus, a feedback loop may exist in which aging TECs promote differentiation of dendritic cells, whose inflammatory cytokines in turn promote TEC degeneration. Further elucidation of the mechanisms driving DCs to adopt a proinflammatory signature and causing TECs to reduce E2F3 activity and cycling will be key to understanding the etiology of age-associated thymic involution.

EXPERIMENTAL PROCEDURES

Mice
C57BL/6J mice were housed at the UT Austin animal facility, under conditions approved by the Institutional Animal Care and Use Committee.

FACS Isolation of Thymic Stromal Subsets and Transcriptional Profiling
Thymi from 1-, 3-, and 6-month-old male C57Bl/6J mice were enzymatically digested and FACS purified by double sorting to >95% purity on a FACSAria (BD Biosciences). Stromal subsets were collected directly in TRIzol (Life Technologies), and RNA was purified, amplified, and hybridized on Mouse Genome 430 2.0 arrays (Affymetrix). Data were uploaded to GExC (Seita et al., 2012) for robust multiarray analysis normalization (McCall et al., 2010). Gene expression data from thymic stromal subsets and previous thymocyte subsets (GSE34723) are available in GExC (https://gexc.stanford.edu/model/475) and the NCBI Gene Expression Omnibus (GEO; GSE56928). See the Supplemental Experimental Procedures.

Bioinformatics Analyses
Identification of DEGs was performed in R using "limma" and "affy" packages, and the Molecular Signature Database was queried for GSEA. See the Supplemental Experimental Procedures.

RT-PCR on the Fluidigm Platform
FACS-purified stromal subsets were subject to high-throughput qPCR on the Fluidigm platform, using manufacturer's reagents and procedures (Fluidigm). See the Supplemental Experimental Procedures.

ACCESSION NUMBERS

The NCBI GEO accession number for gene expression data from thymic stromal subsets reported in this paper is GSE56928.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.08.070.

AUTHOR CONTRIBUTIONS

S.K. and D.P. performed bioinformatics analyses with advice from V.R.I. and L.I.R.E. and wrote the manuscript with L.I.R.E. S.K. and H.C. generated Fluidigm data with guidance from J.K. H.J.S. and L.I.R.E. generated primary data. J.S. provided advice on GExC.

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