Regulatory T cells generated early in life play a distinct role in maintaining self-tolerance

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Aire is an important regulator of immunological tolerance, operating in a minute subset of thymic stromal cells to induce transcripts encoding peptides that guide T cell selection. Expression of Aire during a perinatal agewindow is necessary and sufficient to prevent the multiorgan autoimmunity characteristic of Aire-deficient mice. We report that Aire promotes the perinatal generation of a distinct compartment of Foxp3+CD4+ regulatory T (Treg) cells, which stably persists in adult mice. This population has a role in maintaining self-tolerance, a transcriptome, and an activation profile distinguishable from those of Tregs produced in adults. Underlying the distinct Treg populations are age-dependent, Aire-independent differences in the processing and presentation of thymic stromal-cell peptides, resulting in different T cell receptor repertoires. Our findings expand the notion of a developmentally layered immune system.

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had been labeled between 0 and 10 or 35 and 45 days after birth and analyzed their transcriptomes. Distinct sets of genes were either over- (pink) or underexpressed (green) in Tregs tagged perinatally versus the bulk Treg population of the same mice, but were not differentially transcribed in mice whose Tregs were labeled as adults (Fig. 3A and table S1). Overlaying the standard Treg signature on a volcano plot comparing the two labeled Treg populations revealed an overrepresentation of Treg 'up' genes in perinate-tagged Tregs (Fig. 3B).

Indeed, these Tregs performed better than the three comparator populations in a typical in vitro suppression assay (Fig. 3C), perhaps reflecting higher transcription of genes, such as Fgl2, Ebi3, Pdcd1, and Icos (table S1A), previously implicated in Treg effector function (13–16). The perinate-tagged Treg population was in a more activated state (Fig. 3D), which fit with its higher content of CD44hiCD62Llo cells (Fig. 3E). It was also more proliferative, as indicated by fractions of EdU-incorporating and of Ki67+ cells higher than those of the three comparator populations (Fig. 3F). Indeed, the top pathways overrepresented in perinate-tagged Tregs according to Gene-Set Enrichment Analysis (GSEA) were related to DNA replication and cell division (e.g., Fig. 3G). We confirmed the elevated expression of a number of functionally relevant genes at the protein level (Fig. 3H and fig. S9).

Lastly, we sought a molecular or cellular explanation for the distinct Treg compartments generated in perinatal and adult mice. We first used a mixed fetal-liver:bone-marrow chimera approach to rule out the possibility that T cell precursors derived from fetal liver hematopoietic stem cells, which service the developing immune system for the first few weeks after birth (17), are predisposed to yield Tregs with particular properties, assessing both reconstitution efficiencies and gene-expression profiles (fig. S10).

To facilitate comparison of the repertoires of Aire-dependent PTA transcripts in perinatal and adult MECs, we generated Adig reporter mice, which express GFP under the dictates of Aire promoter and enhancer elements (18), on either an Aire-WT or -KO background. GFP+MHC-II+ MECs were isolated from thymic stroma of <3-day-old or 5-week-old animals, and gene-expression profiling was performed. The fraction of Aire+ MHC-II+ MECs and the Aire mean fluorescence intensity (MFI) were indistinguishable in mice of the two ages (fig. S11, A and B). The repertoires of Aire-dependent MEC transcripts were also extremely similar (fig. S11C).

Next, we asked whether the similar repertoires of PTA transcripts might still yield distinct sets of MHC-presented peptides, owing to different Ag-processing and presentation machinery in mice of the two ages, which need not be Aire dependent. Transcripts encoding several molecules implicated in generating or regulating the repertoire of peptides bound to MHC-II or -I molecules were differentially expressed in perinatal MECs, we generated Adig reporter mice, which express GFP under the dictates of Aire promoter and enhancer elements (18), on either an Aire-WT or -KO background. GFP+MHC-II+ MECs were isolated from thymic stroma of <3-day-old or 5-week-old animals, and gene-expression profiling was performed. The fraction of Aire+ MHC-II+ MECs and the Aire mean fluorescence intensity (MFI) were indistinguishable in mice of the two ages (fig. S11, A and B). The repertoires of Aire-dependent MEC transcripts were also extremely similar (fig. S11C).

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DO chains were expressed at a significantly lower level in perinatal than in adult MECs, independent of Aire (Fig. 4B). Perinatal MECs also had reduced amounts of intracellular DO complexes (Fig. 4C). In addition, they displayed higher intracellular amounts of DM complexes (Fig. 4E). Co-plotting intracellular amounts of the two complexes at the single-cell level revealed a subset of perinatal MECs with reduced DO and enhanced DM expression (Fig. 4F). A lower DO:DM ratio should promote more effective replacement of CLIP by other peptides. Indeed, a higher percentage of perinatal MECs displayed low amounts of or no CLIP (37.6 ± 6.4% versus 20.9 ± 2.2%), and the CLIP MFI was lower for perinatal MECs (761.7 ± 78.7%)

Fig. 2. Stability and function of perinatal- versus adult-tagged Tregs. (A) Tamoxifen was administered from 0 to 10 or 35 to 45 days of age; at various times later, splenocytes were analyzed for GFP and YFP expression by flow cytometry. Left: representative flow-cytometric dot-plots. Numbers represent percentages of CD4+3+ cells in the designated gates. Center: summary data on numbers of GFP+YFP– bulk Tregs. Right: corresponding data on GFP+YFP+ perinatal-tagged or adult-tagged Tregs from the same mice. n = 5. (B to E) Tregs (1.5 × 10^5) were transferred into Aire-KO mice on days 0.5, 3, and 7 after birth, and the recipients were monitored until 16 weeks of age. A four-way comparison as schematized in fig. S8A: GFP+YFP+ Tregs tagged from 35 to 45 days of age and isolated from a 60-day-old mouse (C), GFP+YFP– bulk Tregs from the same mouse (B), GFP+YFP+ Tregs tagged from 0 to 10 days of age and isolated from a 60-day-old mouse (E), and GFP+YFP– bulk Tregs from the same mouse (D). Data organized as in Fig. 1B. The key comparison is boxed.
versus 1019.0 ± 54%) (Fig. 4G). Thus, the repertoires of peptides presented by perinatal and adult MECs are different, the latter appearing to be more limited.

Aire-dependent PTAs can be “cross-presented” by myeloid-lineage cells in the vicinity (4, 5), primarily MHC-II⁺CD8α⁺ DCs (4). Interestingly, this cell type was present in strongly reduced amounts in thymi from perinatal mice (Fig. 4H). Because the splenic MHC-II⁺CD8α⁺ DC subset showed an even more extreme age dependence, it is unlikely that this difference is Aire dependent.

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**Fig. 3. A distinct transcriptome in perinate-tagged Tregs.** The same type of four-way comparison used in Fig. 2 was performed except that the sorted cells were analyzed for diverse phenotypic features. (A) FC/FC plots comparing perinate-tagged GFP⁺YFP⁺ cells versus bulk GFP⁺YFP⁺ cells from the same mice (x axis) and adult-tagged GFP⁺YFP⁺ cells versus bulk GFP⁺YFP⁺ cells from the same mice (y axis). Pink dots denote transcripts overrepresented in perinate-tagged GFP⁺YFP⁺ cells; green dots indicate underrepresented transcripts. (B) P-value versus FC volcano plot comparing gene expression of perinate-tagged GFP⁺YFP⁺ and adult-tagged GFP⁺YFP⁺ cells. Red and blue dots indicate up- and down-regulated Treg signature genes, respectively (31). P-values from the chi-squared test. (C) Classical in vitro suppression assay on the four sorted Treg populations. **P ≤ 0.01, ***P ≤ 0.001 (Student’s t test). (D) Same volcano plot as in (B), except that up- (red) and down- (blue) regulated activation signature genes (31) are superimposed. (E) Summary data on late activation marker (CD44⁺CD62L⁺) expression in the four Treg populations. n = 5. ***P ≤ 0.001 (Students’ t test). (F) EdU uptake (left) and Ki67 expression (right) by the four Treg populations. ***P ≤ 0.001. (G) GSEA of transcripts increased in the perinate-tagged GFP⁺YFP⁺ relative to the adult-tagged control Treg populations. NES, normalized enrichment score. FDR q-val, false discovery rate. Representative transcripts showing increased expression are shown on the right. (H) Flow cytometric confirmation of gene overexpression in perinate-tagged Tregs. For Fgl2 and PD1: Left: representative flow-cytometric histograms; red, perinate-tagged; blue, adult-tagged; black, control bulk populations; gray shading, isotype-control antibody; bar indicates marker positivity. Center: summary data for percentage of the four Treg populations expressing the marker; Right: summary data for marker MFI in the marker-positive population.
Such differences in the Ag processing and presentation machinery of MECs from perinatal and adult mice suggested that their Treg TCR repertoires might diverge. We constrained the inventory of TCRs to be examined by using an approach that had proven fruitful in the past (21, 22). BDC2.5 is a Vα1Vβ4+ T helper cell specificity directed at a pancreatic Ag presented by Aβ2 molecules; hence, generation of T_{reg} in BDC2.5/NOD mice is dependent on rearrangement of an endogenous Tera gene and thymic selection on the resulting second TCRβ complexes. The fixed Vβ4+ chain constrains the TCR repertoire, and the analysis is further delimited by sorting individual cells expressing Vα2. We sequenced 281 Vα2+ TCR CDR3 regions from splenic T_{reg} of three individual BDC2.5/NOD adults and another 232 from the corresponding population of three individual perinates. This restricted, but parallel, slice of the TCR repertoire was clearly different in the two age groups. Perinatal T_{reg} TCRs

**Fig. 4. Age-dependent, Aire-independent differences in the processing and presentation of MEC-generated peptides.** (A) Microarray-based quantification of transcripts encoding a set of proteins involved in processing and presentation of MHCII-bound peptides. (B) Microarray-based quantification of DOα and DOβ in MECII from Aire-WT or -KO adults or perinates. (C) Intracellular expression of DOβ protein. Left: representative flow-cytometric histograms. Red, perinate; blue, adult; gray shading, negative control staining. Right: summary MFI data. (D and E) Same as in (B) and (C) except that DMa and DMb were examined. (F) Coordinate intracellular staining of DOβ and DMab. (G) Surface expression of Ab:CLIP complexes on MECII. Left: representative flow-cytometric histograms. Red, perinate; blue, adult; gray shading, negative control staining. Center: summary data for percentage of MECII expressing little or no CLIP. Right: summary data for MFI. (H) Flow cytometric quantification of MHCII/CD8α DCs in perinatal versus adult thymus (left) and spleen (right). Summary data for representation in the CD11c+ (left) and CD45+ (right) compartments. (I) High-frequency Vα2+ TCRs from 5-week-old (upper) and 4-day-old (lower) BDC2.5/NOD females. These sequences correspond to those in table S2. Bars represent frequency of each sequence. (A to H) *P < 0.05, **P < 0.01, ***P < 0.001 (Student’s t test). n = 3 to 6.
were less clonally expanded (fig. S12A), had shorter CDR3 stretches (fig. S12B) and, as expected (23), had fewer Tera N-region additions (fig. S12C). To permit a more statistically robust assessment, we focused on repeat sequences. There were many more repeated sequences in the adult mice, and very low values were obtained for both the Morisita-Horn index (0.069 on a scale from 0 to 1) and the Chao abundance-based Jaccard index (0.058 on a scale from 0 to 1), indicating that the two repertoires were very different (table S2 and Fig. 4).

Thus, our data highlight Aire’s ability to promote the generation of a distinct compartment of Foxp3+CD4+ T_{reg} as the explanation for its importance during the perinatal age window. Given the age-dependent differences in Ag processing machinery and presenting cells that we documented, juvenile and older mice are likely to have distinct repertoires of both Aire-dependent and Aire-independent T_{reg}, selected primarily on Ag:MHC complexes encountered on MECs. These findings add to, rather than negate, Aire’s role in clonal deletion of self-reactive thymocytes, established in multiple experimental contexts (4, 5, 24, 25).

There are notable similarities in the autoimmune diseases provoked by constitutive genetic ablation of Aire, thymectomy at 3 days of age, and perinatal depletion of Foxp3-expressing cells—in particular, the pattern of target tissues on different genetic backgrounds (26, 27) (Fig. 1). Our studies yield a unifying explanation for these phenocopies: The perinatally generated, Aire-dependent T_{reg} compartment is particularly proficient at protecting a defined set of tissues from autoimmune attack, and there may be little overlap with the tissues guarded by adult T_{reg}. This notion is consistent with the observations that mice that underwent a thymectomy 3 days after birth exhibited multi-organ autoimmune disease but do not have a numerically diminished T_{reg} compartment when they get older (28, 29), and that mice constitutively devoid of T_{reg} or inducibly depleted of them as adults show a very different spectrum of pathologies (9–11). Such a dichotomy could also explain why the autoimmune disease characteristic of both APECED patients and Aire-KO mice is restricted to such a limited set of tissues. An important implication of this dichotomy is that therapies based on transfer of T_{reg} isolated from adult donors may not be able to impact a particular subset of autoimmune diseases. Thus, our findings extend the notion of a “layered” immune system (30).

REFERENCES AND NOTES

8. Materials and methods are available as supplementary materials on Science Online.

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SUPPLEMENTARY MATERIALS

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Materials and Methods
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