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Ablation and Regeneration of Tolerance-Inducing Medullary Thymic Epithelial Cells after Cyclosporine, Cyclophosphamide, and Dexamethasone Treatment

Anne L. Fletcher,* Tamara E. Lowen,* Samy Sakkal,* Jessica J. Reiseger,* Maree V. Hamnett,* Natalie Seach,* Hamish S. Scott,† Richard L. Boyd,‡ and Ann P. Chidgey*†

Immunosuppressive drugs and cytotoxic chemotherapy agents are designed to kill or suppress autoreactive, alloaggressive, or hyperinflammatory T cells, or disseminated malignancies. However, they also cause severe immunological side effects ranging from interrupted thymopoiesis and general immunodeficiency to, paradoxically, autoimmunity. Consistent with the cross-talk between thymocytes and stromal cells, we now show that these common therapeutic agents have major effects on murine thymic epithelial cells (TEC), crucially required to rebuild immunity posttreatment. We show that the immunosuppressant cyclosporine A, which has been linked to a thymus-dependent autoimmune syndrome in some patients, causes extensive loss of autoimmune regulator (Aire)‑bearing medullary TEC (mTEC)HMedullary. Post-cyclosporine A, Aire expression was restored within 7 days. Full recovery of the mTEClow subset occurred within 10 days and was linked to a decrease in a relatively resistant MHC class IIlow mTEC subset (mTEClow), consistent with a previously described precursor-product relationship. Cyclophosphamide and dexamethasone caused more extensive ablation of thymocytes and stromal cells but again severely depleted tolerance-inducing mTEC. Together, these data show that Aire+ mTECs are highly sensitive to damage and that mTEC regeneration follows a conserved pattern regardless of the treatment regimen used. The Journal of Immunology, 2009, 183: 823–831.

Thymic stromal cells induce T cell differentiation and maturation through provision of growth factors, cytokines, chemokines, and through tightly regulated selective processes involving the presentation of MHC and self-peptides (1). Thymic epithelial cells (TEC)4 are the major component of thymic stroma, and phenotypically distinct TEC subsets fulfill different functions in selecting and shaping the developing T cell repertoire. Thymocytes and TECs exist in a dynamic codependence called cross-talk, whereby disruption of thymocyte development impacts on the thymic stroma and vice versa (1, 2).

The degree of affinity for self determines whether a thymocyte will mature and exit the thymus. Positive selection, mediated by cortical TECs (cTEC), ensures that mature T cells are self-MHC restricted, whereas negative selection occurs when dendritic cells or medullary thymic epithelial cells (mTEC) induce apoptosis in thymocytes with a high degree of self-avidity (1, 3, 4). Together, positive and negative selections strike a balance to create a broadly reactive TCR repertoire, with a low but not completely absent potential for self-reactivity (5). Fundamental to central (thymic) tolerance is the autoimmune regulator (Aire), a transcription factor regulating expression of many self-Ags by a defined subset of mTECs, deleting or functionally silencing thymocytes reactive to tissue-specific, sequestered, and late-onset self-Ags (6, 7).

Affinity for the lectin Ulex europaeus agglutinin-1 (UEA1) and expression of MHC class II can be used to divide TEC into four subsets (8). The mTECMedullary expression is the highest levels of MHC class II, CD80, UEA1, and Claudin 3/4 and has the highest capacity of any TEC subset to stimulate transgenic T cells in vitro (8–10). Importantly, thymic Aire expression is exclusive to this subset (11–13).

Cyclosporine A (CsA) is a common immunosuppressant used to prevent allograft rejection. It is used primarily to prevent peripheral T cell activation, but it also causes mild thymic involution (14, 15). Paradoxically, when thymi from CsA-treated mice were transplanted to athymic nude mice, recipients developed characteristic organ-specific autoimmunity (16). Reductions in dendritic cells and FoxP3+ regulatory T cells (Treg) are likely to contribute and have been examined in depth (17–19), but symptoms are also highly reminiscent of a defect in mTEC-based tolerance (7). There has been limited investigation of the thymic stroma by histology, yielding conflicting results, ranging from a broad reduction in mTECs (20, 21) to no change at all (18). Similarly, although evidence suggests thymopoiesis is grossly interrupted after treatment with the immunoablative chemotherapy agents cyclophosphamide (22) and dexamethasone (23, 24), parallel flow cytometric studies
of stromal damage and regeneration have not been thoroughly performed. Any damage to the thymic stromal microenvironment with these commonly used drugs would be expected to have a major impact on the ability of the patient to restore immune competence.

With >1.4 million cancer diagnoses expected this year in the United States alone (25), swift restoration of thymopoiesis and tolerance induction after cytoblastive or immunosuppressive therapy is of paramount clinical importance. Because TECs have a unique, critical role in restoring functional immunity and self-tolerance, regeneration of Aire and the Aire-expressing mTEC<sub>high</sub> subset is particularly important. The kinetics of mTEC recovery and resistant cell types from which TEC subsets might develop has not, however, been effectively studied.

Using sophisticated flow cytometry, we demonstrate that thymic damage following CsA, cyclophosphamide, or dexamethasone treatment includes loss of the tolerance-inducing mTEC<sub>high</sub> subset. TEC regeneration at the subpopulation level revealed characteristic developmental phenotypes and kinetics across several immunosuppressive regimens.

Materials and Methods

Animals

C57BL/6j (B6) mice ages 8–12 wk from Monash Animal Services were housed under specific pathogen-free conditions in accordance with institutional guidelines and with the approval of the Monash University Animal Ethics Committee.

Immunosuppression and cytoblastive chemotherapy

To compare thymic recovery from different treatments, timepoints are referred to as days of recovery (RD) indicating the number of days after treatment has ceased. In all experiments, the final day of treatment was RD0. Mice were given 15 mg/kg/day CsA (Novartis) i.p. for 14 or 21 days, as indicated; or 100 mg/kg/day cyclophosphamide (BD Pharmacia) i.p. in PBS for 2 days; or a single injection, at 20 mg/kg, of dexamethasone (Lyppards). Mice were humanely killed by CO2 asphyxiation at indicated time points.

Individual thymus digestion and flow cytometry

Each thymus was individually digested in collagenase D and DNAse I (Roche) as previously described (26). Supernatant fractions were pooled per thymus and filtered through a 100-μm pore size mesh. Cell counts were performed using a Z2 Coulter Counter (Beckman Coulter). Staining for plasma membrane markers was performed, using 5 × 10<sup>6</sup> cells, as previously described (26). For intracellular staining, surface-stained cells were then fixed and permeabilized using the Cytofix/Cytoperm kit (BD Biosciences) i.p. in PBS for 2 days; or a single injection, at 20 mg/kg, of dexamethasone (Lyppards). Mice were humanely killed by CO2 asphyxiation at indicated time points.

Abs and conjugates

Abs and conjugates were from BD Biosciences unless indicated. We used UEA1 lectin (Vector Laboratories), anti-Ly51 (clones 6C3 and BP-1); anti-IA/IE (M5/114.15.2); anti-CD45 (30-F11), anti-Aire (5H12-2(12)), anti-CD8<sup>+</sup>, rabbit anti-bovine pan-cytokeratin (DakoCytomation), anti-rat IgG2c (Southern Biotech), anti-I-A<sub>β</sub> (Ly51), streptavidin-allophycocyanin.
they pass through a number of developmental stages (11, 27), it was surprising that by RD7, the proportion of TECs expressing Aire was significantly increased compared with untreated controls, and in fact the number of Aire\(^{+/}\)H11001 mTECs was already fully regenerated at this time point. There was a significant increase in the number of Aire\(^{+/}\)H11001 mTECs at RD10 (Fig. 2B). Because Aire\(^{+}\) mTECs develop from putative transit-amplifying (TA) Aire\(^{+}\) mTEC\(^{\mathrm{high}}\) (11, 13, 27), which in turn are purported to develop from mTEC\(^{\mathrm{low}}\) (13, 27), the ratio of these cell types was examined. Ordinarily, the ratio of Aire\(^{-}\) mTEC\(^{\mathrm{high}}\) to Aire\(^{+}\) mTEC\(^{\mathrm{high}}\) cells is 2:1 (Fig. 2C). The proportion of mTEC\(^{\mathrm{high}}\) subsets expressing Aire normalized by RD7 but increased to a 1:1 ratio at RD10, in accordance with increased numbers of Aire\(^{-}\) mTEC compared with untreated controls, suggestive of either faster maturation of Aire\(^{-}\) mTECs, or a prolonged half-life of newly generated cells. These data show that newly generated mTEC\(^{\mathrm{high}}\) cells follow a swift program of differentiation to restore Aire expression.
mTEC<sup>high</sup> cells are actively cycling during thymic regeneration

Given the intense interest in mechanisms of mTEC generation and regeneration, likely mechanisms behind the swift increase in mTEC<sup>high</sup> were addressed. Ki67 expression studies determined that the remaining mTEC<sup>high</sup> entered a state of relative quiescence by RD0 but entered cell cycle at normal proportion within 4 days after treatment ceased (Fig. 3). After RD4, and in untreated mice, the highest proportion of cells positive for Ki67 was mTEC<sup>high</sup> (Fig. 3B). Increased numbers of cTEC<sup>high</sup> were entering cell cycle at RD0 (Fig. 3A), consistent with their early increase in number (Fig. 1E), but the proportion of cTEC<sup>high</sup> expressing Ki67 was no different from that of untreated mice (Fig. 3B), suggesting that the cells were accumulating, not actively undergoing expansion.

Persistent loss of mTEC<sup>low</sup> coincides with increase in mTEC<sup>high</sup>

A large proportion of mTEC<sup>high</sup> fit the profile of TA cells, supported by a body of experimental evidence (11, 13, 27). By definition, TA cells develop from a progenitor cell, before proliferating to amplify cell numbers, and subsequently maturing into a terminally differentiated cell type (30, 31). Evidence in other systems, using adult or embryonic TECs placed into a permissive embryonic environment (11, 13, 27), suggests that mTEC<sup>low</sup> subsets represent a quiescent progenitor of the purported TA mTEC<sup>high</sup> subset. However, direct differentiation studies following adult TECs transplanted into adult thymi are not yet available.

The high level of mTEC<sup>high</sup> cell cycling seen soon after treatment withdrawal does not explain the observed numerical loss of mTEC<sup>low</sup> cells as mTEC<sup>high</sup> numbers recovered (Fig. 1C). To assess the fate of mTEC<sup>low</sup> lost during mTEC<sup>high</sup> recovery, we tracked the loss and recovery kinetics of mTEC<sup>low</sup> and mTEC<sup>high</sup> cells with a different treatment time course.

The experimental protocol was varied to address two hypotheses (depicted schematically in Fig. 4A). Hypothesis i states that the late reduction in mTEC<sup>low</sup> is intrinsically linked to the recovery of mTEC<sup>high</sup>. Here, regardless of the length of treatment, the number of mTEC<sup>low</sup> always significantly reduce during the recovery phase, as mTEC<sup>high</sup> are replenished. Hypothesis ii: The late reduction in mTEC<sup>low</sup> is independent to the recovery of mTEC<sup>high</sup>. The reduction in mTEC<sup>low</sup> should always occur at the same time point after treatment begins. B, Recovery of mTEC<sup>low</sup> (■) and mTEC<sup>high</sup> (□) subsets at time points after a lengthened (21 days) CsA treatment regimen. ■, mTEC<sup>high</sup>; □, mTEC<sup>low</sup>; †, cTEC<sup>high</sup>; ‡, cTEC<sup>low</sup>. Data are represented as means ± SD, * , p < 0.05 compared with the relevant untreated TEC subset. #, p < 0.05 compared with the relevant untreated TEC subset; †, p < 0.05 compared with the relevant untreated TEC subset.

To test these alternatives, we extended the original treatment protocol, this time treating mice with CsA every day for 21 days rather than 14 (Fig. 4B). Our data supported hypothesis i. The number of mTEC<sup>low</sup> always further reduced between RD4–10 as mTEC<sup>high</sup> recovered, regardless of whether CsA had previously been administered for 3 wk (Fig. 4B), 2 wk (Fig. 1C), or 1 wk (data not shown). The loss of mTEC<sup>low</sup> from RD4 to RD10 did not occur
at a particular time after treatment was started; rather, it occurred at a defined time point after treatment was withdrawn.

It is possible that an as yet unknown progenitor of both mTEC<sub>low</sub> and mTEC<sub>high</sub> skews toward replenishing mTEC<sub>high</sub> in damage situations, resulting in a coincident loss of mTEC<sub>low</sub> from attrition. This would result in an experimental profile that would also fit hypothesis i. We believe that it is more likely, as suggested by other experimental systems (11, 13, 27), that residual mTEC<sub>low</sub> up-regulate MHC class II and become mTEC<sub>high</sub> cells as a developmental progression, followed by extensive proliferation and then Aire expression.

*Plet-<sup>1</sup>* TEC are proportionally resistant to CsA treatment, but *Plet-<sup>1</sup>* mTEC<sub>flow</sub> are lost during thymic regeneration

The Ab MTS24 recognizes the Plet-1 Ag (32), expressed in the emerging E10.5 thymic anlage (33–35) as well as a progenitor population of follicular keratinocytes (36). In the embryo, Plet-1 expression does not exclusively identify TEC progenitors, but does preferentially encompass the high-efficiency progenitors (34, 35, 37). We therefore examined MTS24 staining in these adult thymus models as a means of potentially enriching for progenitor TEC populations.

In the postnatal thymus, as previously reported, a large subset of TEC express Plet-1, including both MHC class II<sub>low</sub> and MHC class II<sub>high</sub> cells (35) and Fig. 5A). When assessed more specifically on gated TEC subsets, it was found that the majority of MTS24<sup>+</sup> TECs were mTEC<sub>flow</sub> (Fig. 5A).

At RD0, after 2 wk of CsA treatment, TECs expressing Plet-1 were found to be proportionally and numerically more resistant than Plet<sup>−</sup> cells (Fig. 5B). Furthermore, cTEC<sub>high</sub> and cTEC<sub>low</sub> Plet-1<sup>+</sup> cells also increased in both proportion and number following CsA treatment (Fig. 5C), showing expression of this Ag by more cells in response to thymic damage, over and above the selective sparing of Plet-1<sup>+</sup> cells. Plet-1 expression returned to normal in all subsets by RD10. The loss of Plet-1<sup>+</sup> mTEC<sub>flow</sub> cells at RD7 suggests that these cells either suddenly become susceptible to cell death 7 days after treatment was withdrawn (despite being more resistant than other cells throughout 2 wk of treatment), or that progenitor cells residing within the mTEC<sub>flow</sub> subset lose expression of Plet-1 upon differentiation to mTEC<sub>high</sub>.

In further characterizing mTEC<sub>low</sub> cells in untreated mice, we noted a distinct subpopulation coexpressing Ly51, a marker commonly used to identify cortex (Fig. 5D, histogram). It has recently been shown that a minor proportion of Aire<sup>+</sup> cells (exclusively UEA1<sup>+</sup>) also express Ly51 (12); we therefore examined Ly51 expression across TEC subsets. In untreated mice, analysis of UEA1 and Ly51 expression indicates 3 cell populations (Fig. 5D, large dot plot). As expected, UEA1<sup>+</sup>Ly51<sup>−</sup> cells (population i) were primarily mTEC<sub>high</sub> and mTEC<sub>flow</sub>, where UEA1<sup>+</sup>Ly51<sup>−</sup> cells (population iii) fell within the cTEC<sub>high</sub> and cTEC<sub>low</sub> gates. However, cells coexpressing both markers (UEA1<sup>+</sup>, Ly51<sup>+</sup>; population ii) were primarily MHC class II<sub>low</sub>, falling within the mTEC<sub>flow</sub> gate (see small dot plots and bar graph, each gated from large dot plot). Any distinct function or further phenotype of this new TEC subset remains to be determined, but certainly Ly51 and UEA1 are not mutually exclusive, and the mTEC<sub>low</sub> subset is more heterogeneous than previously reported.

Conserved regeneration patterns across more severe models of thymic damage

We studied other models of thymic damage to see whether Aire<sup>+</sup> mTECs were similarly sensitive, and also to determine whether mTEC<sub>high</sub> regeneration under more severe ablative conditions

**FIGURE 5.** Plet-1<sup>+</sup> mTEC<sub>low</sub> are lost during thymic regeneration. A. Plet-1 expression (identified with MTS24 mAb) on nonhemopoietic stromal cells (dotplot; gated as CD45<sup>−</sup>) and on TEC subsets (histograms) in untreated mice. Gates were set based on staining with an appropriate isotype control. B. Proportion of each TEC subset that is MTS24<sup>+</sup> at time points post-withdrawal of CsA. C. Total number of MTS24<sup>+</sup> cells. Mice were treated daily for 14 days before RD0. Data represent responses of 8–10 mice from 2–3 experiments. D. Histogram (gated on mTEC<sub>low</sub> from untreated mice) shows a distinct subpopulation of Ly51<sup>+</sup> cells within the mTEC<sub>low</sub> subset. Numbers represent the mean ± SD for 24 mice across six experiments. Large dot plot (gated on total MHC class II<sub>TEC</sub> from untreated mice) shows three populations of cells, labeled 1, 2 and 3, identifiable using UEA1 and Ly51. Small dot plots and bar graph (respectively gated on populations i, ii, or iii from large dot plot, as indicated) show the distribution of mTEC<sub>high</sub>, mTEC<sub>low</sub>, cTEC<sub>high</sub> and cTEC<sub>low</sub> cells within populations i, ii, and iii. Data represent 16 mice across 4 experiments. ■, mTEC<sub>high</sub>; □, mTEC<sub>low</sub>; △, cTEC<sub>high</sub>; □, cTEC<sub>low</sub>; *, p < 0.05 compared with the relevant untreated TEC subset. Untr, Untreated.
followed the same pattern, proportionally increasing at the expense of mTEClow. The clinically relevant cytotoxic agent cyclophosphamide induced severe thymic involution (Fig. 6A), as previously reported (22). Similarly, the proportion (Fig. 6, B and C) of all TEC subsets was profoundly altered following treatment as epithelial involution occurred, causing a sustained reduction in TEC number across all subsets (Fig. 6D). Total TEC fell to a low of $7.2 \pm 0.4 \times 10^4$ compared with $21.4 \pm 6.8 \times 10^4$ in untreated controls (mean $\pm$ SD, $p < 0.01$). As with CsA treatment, cyclophosphamide caused the greatest numerical reduction within mTEChigh subset (Fig. 6D). This reduction again resulted in a profound loss of Aire-expressing cells (Fig. 6E). Although thymic cellularity was restored to near untreated levels by 10 days post-treatment (Fig. 6A), the proportion (Fig. 6, B and C) and number (Fig. 6D) of TEC subsets took between 14 and 28 days to return to normal.

Dexamethasone is a potent, clinically relevant glucocorticoid used to suppress immune responses. It induced a severe degree of thymic involution comparable with cyclophosphamide (Fig. 7A), and again extensively depleted total TECs ($5.8 \pm 2.0 \times 10^4$, compared with $28.9 \pm 4.6 \times 10^4$ in untreated mice; mean $\pm$ SD, $p < 0.01$). All TEC subsets were ablated, to varying degrees (Fig. 7, B–D), but again, the mTEChigh subset proved most sensitive to treatment. Although cyclophosphamide, dexamethasone, and CsA each affected mTEChigh to a comparable extent, these cells took
longer to recover after cyclophosphamide and dexamethasone treatment (compare Figs. 6D and 7D with Fig. 1E).

During recovery from both cyclophosphamide and dexamethasone, the proportion of mTEC<sub>low</sub> again steadily reduced in proportion concomitant with mTEC<sub>high</sub> recovery (Figs. 6C and 7C), as seen during mTEC<sub>high</sub> regeneration post-CsA. This pattern of mTEC regeneration held true regardless of the method used to achieve mTEC ablation or the severity of damage sustained.

Numerically, a different pattern of recovery was noted following severe ablation, where mTEC<sub>low</sub> and mTEC<sub>high</sub> both increased in number between RD7 and RD10, with mTEC<sub>high</sub> expanding proportionally faster, again befitting a TA population (Figs. 6D and 7D). The same proportional skewing of mTEC subsets, with the same kinetics of recovery, were noted regardless of the sex of the mice, suggesting similar regenerative patterns across both sexes. Aire was also severely ablated post-dexamethasone (S. Sakkal, manuscript in preparation). Unlike CsA, a significantly increased rebound in numbers and proportion of Aire<sup>+</sup> TEC beyond untreated levels was not observed in these models where damage to all TEC subsets occurred.

Discussion

The thymus undergoes acute, naturally reversible involution during pregnancy (38) or stress (39), demonstrating an ability to regenerate. Similarly, thymic damage induced by cytoablative chemotherapy or radiotherapy causes acute involution followed by eventual regeneration (22, 40, 41), at least in young animals. TEC regeneration is essential for the restoration of an immunocompetent, self-tolerant T cell pool in such conditions. This is particularly pertinent for CsA, which is commonly used to manage allograft rejection, yet can cause a T cell-mediated graft-vs-host-like autoimmune disease (17, 20, 29, 42) or organ-specific autoimmunity (16) to develop. Loss of peripheral suppressor cells is contributory (20, 21, 29, 43), but our results also clearly demonstrate a significant loss of Aire-expressing mTEC<sub>high</sub>. The mTEC<sub>high</sub> subset is also exquisitely sensitive to at least two cytoxic therapies: cyclophosphamide and dexamethasone.

The well-recognized cross-talk symbiosis between thymocytes and stromal cells makes it difficult to ascertain whether CsA directly affects mTEC number. Because TECs do not express IL-2R<sub>α</sub>, it is more likely that mTEC<sub>high</sub> are lost secondary to an effect on SP T cells, which is supported by robust evidence that SP T cells maintain the mTEC maturation program through provision of ligands for CD40 and TNF-related activation induced cytokine (44–46), but we cannot exclude a contributive role for calcineurin in mTEC maintenance.

mTEC<sub>high</sub> and Aire<sup>+</sup> mTEC recovered fully before SP thymocytes reached steady-state numbers, in principle providing appropriate tolerance-inducing mechanisms. However, SP T cells were never completely depleted. Although evidence clearly states that the pathogenic T cells arise from poorly censored, autoreactive recent thymic emigrants emerging during treatment (47), further work should aim to definitively show that loss of Aire (in addition to established effects on Tregs and dendritic cells; Refs. 17 and 20) contributes to their escape. Certainly, the reported pattern of organ-specific autoimmunity (16) in CsA-treated mice shows some hallmarks of Aire deficiency (gastritis, oophoritis, pancreatic infiltration; Ref. 7).

TEC regeneration took longer in cyclophosphamide- and dexamethasone-treated mice compared with CsA, reflecting a more severe ablation of all thymocyte and TEC subsets. Unlike recovery from CsA, all TEC subsets began to numerically recover simultaneously, reflecting an expedited program of emergency expansion and differentiation, in parallel with thymocyte subsets, to restore the thymic microenvironment. The increase in cTEC during CsA treatment was striking, and the possibility that these cells (or a subset) contribute to mTEC regeneration cannot be ruled out. However, arrested DP thymocyte emigration from cortex to medulla during treatment may be contributive, and cTEC returned to normal number before either the increase in mTEC<sub>high</sub> or decrease in mTEC<sub>low</sub>. Recent work defined cTEC differentiation in embryonic thymi by up-regulation of surface markers (48) analogous to the maturation program for mTEC proposed here and by others (11, 13, 27), but there is as yet no experimental evidence to suggest that cTEC routinely generate mTEC in adult mice.

Recent data show Aire expression as a postmitotic, end-stage differentiation step (27). Combined with our present results showing the loss of mTEC<sub>high</sub> and Aire following cytotoxic chemotheraphy and immunosuppression, the fact that Aire recovered relatively quickly raises important questions regarding the origin of mTEC, in particular the differentiation kinetics and stimuli for relevant progenitor cells. It has been suggested, using data generated in vitro, that the minimum amount of time taken for an mTEC<sub>low</sub> cell to differentiate and up-regulate Aire is 3 days (27). In these in vivo studies, the process appears to take at least 7 days, probably reflecting the additional time required for differentiation and trafficking of mature SP thymocytes and/or lymphoid tissue inducer cells (11, 27, 44–46). However, there was a statistically significant rebound in Aire<sup>+</sup> mTEC differentiation, such that more cells expressed Aire at day 10 posttreatment than at day 7 or in untreated mice, pointing to an early, prioritized restoration compared with other mTEC subsets. The significantly increased proportion of Aire<sup>+</sup> cells at day 10, even though Aire<sup>+</sup> mTEC were fully recovered at day 7, may suggest an early commitment to Aire expression, or increased half-life of newly generated cells. The return to normal number and ratio in just 4 further days suggests that these cells were not replaced after cell death to restore their homeostatic balance.

Unlike Gray et al. (27), we did not find a proportion of Aire<sup>+</sup> cells expressing low levels of MHC class II, suggesting the possibility of artifacts during TEC development in in vitro culture systems. However, our results do support the overall findings of this and similar studies (11, 27), which showed that mTEC<sub>low</sub>, when sorted and either injected into fetal thymic lobes, or re-aggregated with embryonic TEC, could differentiate into Aire<sup>+</sup> mTEC<sub>high</sub>.

As the mTEC<sub>high</sub> subset regenerated, there was consistent proportional loss of mTEC<sub>low</sub> regardless of the treatment used or degree of thymic damage sustained. Following CsA, the total number of mTEC remained relatively constant through RD0, 4, and 7, during which the number of mTEC<sub>low</sub> cells reduced in direct proportion with the increase in mTEC<sub>high</sub>. We then tested two hypotheses pertaining to the fate of the mTEC<sub>low</sub>. Hypothesis i suggested that the loss of mTEC<sub>low</sub> and recovery of mTEC<sub>high</sub> were intrinsically linked, suggesting either a precursor-product relationship, or a common progenitor of both cell types which skewed its potential post-CsA to replenish mTEC<sub>high</sub>, causing mTEC<sub>low</sub> attrition. Hypothesis ii stated that the reduction in mTEC<sub>low</sub> and recovery of mTEC<sub>high</sub> were not linked, suggesting probable attrition at the end of their natural lifespan due to nonreplacement during the treatment period. The data were clearly consistent with Hypothesis i. The reduction did not occur because of mTEC<sub>low</sub> attrition following a defined mTEC<sub>low</sub> lifespan, because the reduction in mTEC<sub>low</sub> always occurred 4–10 days after treatment ceased, even if the treatment period was extended.

Importantly, up-regulation of MHC class II was accompanied by full acquisition of Aire expression. If the mTEC<sub>low</sub> population merely up-regulated MHC class II until mTEC<sub>high</sub> cells developed
from a separate lineage, Aire expression would lag until just before nTEC\textsuperscript{low} recovery. If, however, nTEC\textsuperscript{low} was a relatively quiescent population that sits static or turns over slowly until required to repopulate the actively cycling nTEC\textsuperscript{high} subset, the Aire ratio should recover early and quickly, as observed.

The expression of Plet-1 (identified with the MTS24 Ab) by the CsA-resistant population is interesting given that it identifies a population containing high-efficiency TEC progenitor cells in the embryo (34, 35, 37). However, although transplantation of as few as 2500 MTS24\textsuperscript{+} embryonic TECs generates a complete thymic microenvironment (35), a recent study showed that both MTS24\textsuperscript{+} and MTS24\textsuperscript{−} TEC could generate thymic grafts if much higher cell numbers (100,000 cells) were transplanted together with E12 thymic mesenchyme. These studies show that although Plet-1 is not restricted to embryonic progenitors, MTS24 can be used to enrich for these cells. After E18, MTS24\textsuperscript{+} TECs no longer reaggregate for transplant (37), but Plet-1 is still expressed by a minority of TEC throughout life (35). It has been suggested that Plet-1 expression is progressively restricted to mature, differentiated cells (37).

Conversely, we report that the nTEC\textsuperscript{low} subset, which is suggested in this study and others (11, 13, 27) to contain a subset of quiescent mTEC progenitor cells, expressed the highest levels of Plet-1. A similar finding can be inferred from an earlier study (35), which showed that most MTS24 staining in 4-wk-old (CBA × C57Bl/6)F\textsubscript{1} mice was found on MHC class II\textsuperscript{low} cells, although the enriching stromal cell isolation protocol used in that study, itself biased toward mTECs (26), results in slightly different flow cytometry profiles. We further found that MTS24\textsuperscript{+} cells were proportionally more resistant to cytotherapeutic decay, as would beft quiescent cells, and that MTS24\textsuperscript{+} nTEC\textsuperscript{low} numbers were significantly reduced during nTEC\textsuperscript{high} regeneration. A lower proportion of the nTEC\textsuperscript{high} subset was found to express Plet-1 compared with other TEC. This observation is in keeping with the hypothesis that Plet-1 is expressed by less mature epithelium, because the nTEC\textsuperscript{high} subset has been shown to encompass a large terminally differentiated Aire\textsuperscript{−} population (27).

In summary, although requisite lineage tracking studies following adult TEC subsets injected into adult thymi are yet to be performed, our data support the theory (11, 13, 27) that nTEC\textsuperscript{low} generate nTEC\textsuperscript{high}. Under this model, these data suggest that a quiescent subset of UEA1\textsuperscript{low}MHC class II\textsuperscript{low}CD80\textsuperscript{−} cells are capable of swift differentiation into the proliferating nTEC\textsuperscript{high} subset. A proportion of these cells also express cortical marker Ly51 forming a triple-low population (MHC class II\textsuperscript{low}UEA1\textsuperscript{low}Ly51\textsuperscript{−}) and showing further heterogeneity within TEC subsets. These cells now form a basis for transcriptome analysis and more detailed functional studies.

These studies document, for the first time, the regenerative kinetics of Aire\textsuperscript{−} mTEC and other TEC subsets in vivo following immunosuppression and cytotoxic chemotherapy using common clinically relevant agents. Although we show evidence herein to suggest the existence of a developmental hierarchy among mTEC subsets during thymic regeneration, a true TEC stem cell in adult mice, capable of differentiating into both cortical and medullary TECs and their subsets is yet to be unequivocally defined.

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Disclosures
R.L.B. is chief scientific officer of and A.P.C. is a consultant to Norwood Immunology, Ltd.

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