Regulatory T-cell development in the human thymus

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The thymus generates a lineage-committed subset of regulatory T-cells (Tregs), best identified by the expression of the transcription factor FOXP3. The development of thymus-derived Tregs is known to require high-avidity interaction with MHC-self peptides leading to the generation of self-reactive Tregs fundamental for the maintenance of self-tolerance. Notwithstanding their crucial role in the control of immune responses, human thymic Treg differentiation remains poorly understood. In this mini-review, we will focus on the developmental stages at which Treg lineage commitment occurs, and their spatial localization in the human thymus, reviewing the molecular requirements, including T-cell receptor and cytokine signaling, as well as the cellular interactions involved. An overview of the impact of described thymic defects on the Treg compartment will be provided, illustrating the importance of these in vivo models to investigate human Treg development.

Keywords: human thymus, regulatory T-cells, FOXP3, regulatory T-cell development, human thymic defects, primary immunodeficiency

Introduction

Regulatory T-cells (Tregs) play a major role in immune homeostasis by preventing or limiting T-cell activation, particularly in the context of auto-antigens. Expression of the transcription factor forkhead box P3 (FOXP3), considered a master regulator of Treg development and function, is essential for their role in the maintenance of dominant tolerance [reviewed in Ref. (1)]. Conditions where FOXP3 is defective or absent, such as the recessive disorder immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome, are characterized by aggressive autoimmune manifestations that are usually fatal within the first 2 years of life, unless corrected via hematopoietic stem-cell transplantation (2–4).

Regulatory T-cells develop primarily in the thymus (thymus-derived Tregs, tTregs), although they can also be differentiated in the periphery (peripherally-induced Tregs). The delineation of these two populations in the peripheral Treg compartment is difficult due to the lack of specific markers. Nevertheless, tTregs are thought to be enriched in self-reactive T-cell receptors (TCRs) and to be critical for the maintenance of self-tolerance [reviewed in Ref. (1)]. Despite extensive research on tTreg development using murine models in the past 20 years, many questions remain unanswered regarding the mechanisms involved in the establishment and maintenance of the tTreg lineage [reviewed in Ref. (5, 6)]. Additionally, human tTreg studies are constrained by the limited number of tools available. Data have been mainly generated by in vitro manipulation of human thymic tissue or in vivo models using mouse/human chimeras [reviewed in Ref. (7)].

In this regard, the information provided by genetic human diseases characterized by thymus-related disturbances, such as the DiGeorge, the Omenn, and the autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED) syndromes, have been instrumental [reviewed in...
phenotype reviewed in Ref. (8)]. Loss of corticomedullary pool of T lymphocytes with a restricted repertoire and activated causing genes and is associated with the generation of a limited mutations in severe combined immunodeficiency (SCID)-CD45RA) (12). Omenn syndrome results from hypormorphic thymic emigrants (RTEs, defined by the expression of CD31 and/or thymic DCs in their differentiation. In agreement, loss-of-function mutations in the AIRE gene (APECED syndrome) have been linked to a defective circulating Treg compartment (15–17). Not only is the frequency of naïve/ resting Tregs (defined as CD4+CD8α−FOXP3+CD45RO−CD31+) decreased in APECED patients but also their levels of FOXP3 expression, function, and repertoire are altered, further supporting an abnormal tTreg development in the absence of AIRE (16).

A recent study that involved a paired analysis of thymic and blood samples in young children (newborns to 1-year-old) showed a direct correlation between the size of the two Treg compartments, further supporting the importance of the thymus for the establishment of the peripheral Treg pool early in life (18). Of note, both human and murine RTEs are endowed with enhanced potential to convert into peripherally-induced Tregs, when compared to their more mature counterparts, implying an additional role of the thymus for the setting of the peripheral Treg compartment (19). Furthermore, patients with athymia due to complete DiGeorge or FOXN1 deficiency have been shown to recover the peripheral Treg compartment upon allogeneic thymus transplantation, irrespective of the degree of HLA mismatching (20–22).

It is vitally important to understand human tTreg development in order to devise strategies to manipulate their generation as well as their repertoire (23). This mini-review will provide an overview of the current knowledge regarding human tTreg development, as well as the fundamental questions that remain to be addressed.

**Commitment to the Treg Lineage: When?**

The human thymic primordium is colonized by T-cell progenitors during the 8th week of gestation, but mature T-cells are only observed in the thymus at the 12th to 13th gestational weeks (24–26). At this stage, human tTregs can already be found in the thymus (27–29). The frequency of fetal human tTregs, identified by their elevated expression of the high-affinity IL-2 receptor alpha chain (IL-2Rα/CD25), was found to be stable throughout gestation (representing 6–7% of total thymocytes) and similar to the proportion observed in infant thymuses (28).

Fetal human tTregs already express FOXP3, as assessed at the gene expression level, as well as other markers related to their suppressive phenotype, such as cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and glucocorticoid-induced TNFR-related protein (GITR) (27, 28). Moreover, these fetal human tTregs have the ability to suppress T-cell proliferation (27, 28).

One question that has been extensively addressed in mice, but due to technical limitations only in a few human studies, is when and how a thymocyte becomes committed to the Treg lineage. The discrete populations that express Treg markers, such as CD25 and FOXP3, in the human post-natal thymus mainly comprise mature CD4 single-positive (CD4SP, CD4+CD8α−) thymocytes, but also include CD8 single-positive (CD8SP, CD4−CD8β+) and double-positive (DP, CD4+CD8αβ) thymocytes, as well as cells in early pre- DP stages (27, 28, 30–36). In agreement, FOXP3+CD25+ thymocytes can be found mostly in the medullary region of the human thymus, where mature thymocytes localize, with rare cells scattered in the cortex (30, 31, 35, 37, 38). The mechanisms that allow for human tTreg commitment to occur are still ill-defined.

We and others have reported pre-DP expression of FOXP3, namely, at the triple-negative and CD4 immature single-positive stages (33, 35) (Table 1). However, the contribution of this population to the human tTreg pool remains to be addressed.

Double-positive thymocytes expressing FOXP3 and/or CD25 are clearly identified in the human thymus. They additionally express other Treg function-associated markers, such as CTLA-4, CD39, and GITR (27, 28, 30–36, and formally demonstrated by co-cultures of DP thymocytes in murine models, where Foxp3 induction, although possible at early pre-DP stages (33, 35) (Table 1). Nevertheless, the majority of human DP tTregs express high levels of CD3 and CD27, which are associated with positive selection and maturity (27, 36) (Table 1). Importantly, DP tTregs are thought to significantly contribute to the CD4SP tTreg pool in humans, as predicted by linear regression models (36), and formally demonstrated by co-cultures of DP thymocytes with either TEC (36) or mature plasmacytoid (p)DCs (38). This observation contrasts with what has been described in murine models, where Foxp3 induction, although possible at the DP stage (45–47), mostly occurs at the CD4SP stage (48). Of note, human CD4SP CD25+ thymocytes are also permissive to FOXP3 acquisition (37, 39, 49).

CD4SP tTreg represent the major population of FOXP3+ human thymocytes. They phenotypically mirror peripheral Tregs and exhibit efficient regulatory function (27, 28, 30, 36, 41, 43) (Table 1). The contribution of recirculating peripheral Tregs to this tTreg compartment is still debatable. It has long been proposed that activated T-cells may recirculate back to the thymus (50, 51), although this issue is particularly difficult to assess in humans. Recently, it was reported that a considerable proportion of human CD4SP tTregs may consist of recirculating...
express CTLA-4 (30, 36), a molecule that in mice was shown to
by TCR stimulation (55). Notably, both DP and CD4SP tTreg
expression of the role of TCR signaling in tTreg development.

Studies in mice have clearly established the requirement for
TCR signaling in Treg lineage commitment [reviewed in Ref. (5, 6)]. In humans, technical limitations preclude a direct
evaluation of the role of TCR signaling in tTreg development.

Commitment to the Treg Lineage: How?

Studies in mice have shown that human tTreg differentiation
is associated with markers of positive selection, such as CD69
and CD27 (27, 28, 34, 36, 42) (Table 1). Moreover, binding sites
for the TCR downstream targets NFAT and AP1 are present
within the human FOXP3 promoter that are directly activated
by TCR stimulation (56). Indirect evidence that enhanced TCR
signaling strength may dictate thymocyte commitment into the
Treg lineage can be further inferred by the increase in CD4SP
CD25TIMTFOXP3+ thymocyte number in humanized mice treated
with a superagonist anti-CD28 mAb (57). Moreover, human
ZAP70-deficient patients present a dramatic decrease in the
frequency and number of tTregs (58). Interestingly, CD4SP
CD25+tTregs were shown to frequently express two functional
TCRs, in association with enhanced FOXP3 expression, suggest-
ing that dual TCR expression may favor Treg lineage commit-
mance in humans (40). Overall, available evidence support that
TCR signaling strength guides thymocyte commitment into the
Treg lineage in humans, with a predicted impact on their
self-reactivity.

Several additional signaling pathways and molecular fac-
tors have been implicated in human tTreg differentiation and/or
or proliferation, namely JAK3/STAT-5, Notch, CD80/CD86,
ICOS/ICOSL, CD40/CD40L, thymic stromal lymphopoietin
(TSLP), as well as the common-gamma chain (γc) cytokines
interleukin (IL)-2 and -15 (37–39, 49, 52, 59). Watanabe et al.
demonstrated that TSLP from Hassall's bodies activate myeloid
(m)DCs, enabling them to induce Treg differentiation from
CD4SP CD25+ Foxp3+ thymocytes (37). pDCs are also capable
of driving CD4SP non-regulatory thymocytes into the human
Treg lineage, upon response to TSLP (49). Cognate interactions
between mDCs or pDCs and CD4SP non-regulatory thymo-
cytes are required, as differentiation was impaired by HLA-DR
blockade (37, 49). Activation of pDCs with anti-CD40L and
IL-3 also confers on them the capacity to differentiate post-
selection DP CD69+TCRhi thymocytes into human tTregs (38).
Additionally, mTECs were shown to promote the survival
and proliferation of human tTregs in an ICOSL-dependent
mechanism that required the presence of conventional CD4SP
cells as source of IL-2 (52). We have recently investigated the
requirement of γc cytokines in human tTreg development and
established a critical role for both IL-2 and IL-15 in their line-
age commitment, as well as in tTreg proliferation and survival
post-selection (39). This study also allowed the identification
of macrophages and B lymphocytes as main IL-15 producers
that likely represent two additional thymic antigen-presenting
cell populations involved in human tTreg differentiation (39).
In agreement, we found FOXP3+ cells in close vicinity of
both macrophages and B cells in the human thymus (39).
Accordingly, B lymphocytes were recently shown to be capable
of selecting tTreg in mice (60, 61), and the majority of human
CD4SP tTregs were shown to express CCR8, endowing them
with the capacity to migrate in response to chemokines pro-
duced by macrophages (30).

As illustrated in Figure 1, the most immature thymocyte
population that clearly expresses FOXP3, in addition to other
Treg function-associated markers, such as CD25, CTLA-4,
and CD39, and displays regulatory function is the cortical
positively selected DP population (27, 28, 36). The thymic
cellular populations and signals mediating their positive selection
and concomitant recruitment to the Treg lineage may include
cortical TECs and macrophages, as well as IL-2/IL-15, shown
by immunohistochemistry to be expressed in the human thymic

| Table 1 | Characterization of human post-natal thymic Tregs. |
|---------|---------------------------------|
| Markers | Pre-DP | DP | CD4SP | CD8SP | Reference |
|---------|--------|----|-------|-------|-----------|
| FOXP3   | +      | ++ | ++++ | ++++ | (31, 33, 35, 36, 39, 40) |
| CD25    | −      | ++ | ++++ | ++++ | (30, 33, 35, 36, 39, 41) |
| CTLA-4  | +      | ++ | ++++ | ++++ | (30, 31, 33, 36, 39, 41, 42) |
| CD127   | −/#   | +  | ++  | +    | (33–36) |
| HLA-DR  | ND    | ++ | ++  | +    | (36) |
| CD39    | ND    | ++ | ++  | +    | (36) |
| CD73    | ND    | −  | −   | −    | (36) |
| CD1103  | ND    | −  | −   | −    | (36) |
| ICOS    | ND    | ++ | ++  | +    | (34, 36, 42) |
| CD69    | ND    | ++ | ++  | +    | (34, 36, 42) |
| CD27    | ND    | ++ | ++  | +    | (36, 42) |
| Ki67    | +     | −/+| −/+ | −/+  | (36) |
| Suppressi| ND   | Yes| Yes | Yes  | (50–32, 36, 39, 41) |

ND, not determined.
The Unique Properties of Human CD8SP tTregs

In addition to the CD4SP human tTregs, a CD8SP population with phenotypic and functional Treg characteristics is present in the human thymus. CD8SP human tTregs express several Treg-associated markers, such as FOXP3, CD25, CTLA-4, and GITR, although at lower levels than CD4SP tTregs (31, 36) (Table 1). Similarly to CD4SP tTregs, they express very low levels of the IL-7 Ra/CD127 molecule (36), and are able to suppress CD4SP CD25neg cells in a contact-dependent manner (31). A microRNA “signature” of CD8 Tregs has been recently defined in cord blood CD8 Treg (62).

Importantly, CD8SP human tTregs were found to express the αe chain (CD103) of the αeβ7 integrin (36), a marker associated with CD4 Tregs in the periphery (63). CD103 was also expressed at the DP stage but was very low within CD4SP thymocytes, supporting a precursor–product relationship between CD103+ DP and CD8SP human tTregs (36) (Table 1; Figure 1).

The role of CD103 expression on CD8SP FOXP3+ thymocytes remains to be addressed. High expression of E-cadherin, the
main ligand of CD103, on mTEC (64) may promote retention of CD8+ Tregs in the thymic medulla. Furthermore, in an in vitro cell adhesion assay, TEC-induced proliferation of CD8SP human thymocytes was inhibited by antibodies against E-cadherin or CD103, supporting a role for CD103 in CD8SP proliferation (64). Although the proliferation of CD8SP CD25+ CD103+ supporting a role for CD103 in CD8SP proliferation was not addressed in this setting, the higher levels of the active cell-cycle marker Ki67 found within CD8SP FOXP3+ as compared to CD8SP FOXP3<+ thymocytes (36) also support this hypothesis.

Despite the low frequency of CD8+ Treg in the steady state, they may be of special relevance in pathologic conditions [reviewed in Ref. (65, 66)]. CD103 has been associated with T-cell migration to mucosal sites (67, 68). In agreement, in the setting of colorectal cancer, CD8+FOXP3+CD25+ T-cells were significantly increased in peripheral blood and in colorectal cancer tissue, indicating that these cells may contribute to tumor immune escape and disease progression (69). In addition, studies in macaques have described a rapid expansion of CD8+FOXP3+CD25+ Tregs in the blood and colorectal mucosa following pathogenic SIV infection (70). An increase in the frequency of CD8+ Tregs has also been reported in HIV-1-infected patients (70).

In conclusion, a population of CD8+ Tregs is generated in the human thymus. A subset of post-selection DP FOXP3+ thymocytes expresses the tissue homing-associated molecule CD103, likely giving rise to the CD8SP FOXP3+CD103+ cells found in the medulla (Figure 1) (36). This finding supports the possibility that CD8+ Tregs egress the thymus expressing markers associated with mucosal homing, which may explain their very low frequency in the blood.

Concluding Remarks

Cumulative evidence supports the existence of different pathways of Treg commitment in the human thymus that may occur at different stages of thymocyte differentiation. Their physiological contribution and the possible implications for the tTreg repertoire diversity remain unclear.

Thymus-derived Treg development does not seem to require a dedicated antigen-presenting cell population, as studies indicate that TECs, mDC, and pDC, as well as macrophages and B cells may be involved in Treg selection. Finally, despite the proposed role of TCR signaling strength in human Treg commitment, it has become increasingly clear that γc cytokines, particularly IL-2 and IL-15, are important mediators of lineage stabilization.

The thymus and specifically tTregs represent important therapeutic targets to manipulate tolerance in many clinical settings, namely autoimmun diseases, tumor immunity, and transplantation. Furthermore, their targeting is also critical to achieve full immunological reconstitution in primary and secondary immunodeficiencies and to decrease the morbidity associated with hematopoietic stem-cell transplantation. It is therefore of utmost importance to further investigate human tTreg development, in order to take full advantage of the current development of immune-based therapies.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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