Regulatory T-cells: A potential therapeutic intervention for autoimmune diseases

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Abstract

Autoimmune diseases are associated with a breakdown in central or peripheral self-tolerance, resulting in the initiation of pathogenic immune responses against self. Regulatory T-cells (Tregs) expressing the transcription factor forkhead box P3 (FoxP3) are potent regulators of peripheral self-tolerance. Defects in the number or function of Tregs have been associated with the development of many autoimmune diseases, generating interest in the use of Tregs as cell-based immunotherapy. However, uncertainty regarding the identification, stability and function of human Tregs has prevented the application of Tregs as therapy for autoimmune diseases. Such research demonstrates that Tregs are a heterogeneous population with varying regulatory phenotypes and the potential to convert into pro-inflammatory T-helper 17 cells. Therefore, prior to using Tregs as cellular therapy for autoimmune disease, these potential drawbacks must be fully investigated. In this thesis I describe in detail the development, classification, migration, molecular characterisation and stability of Tregs. In addition to, proposing a new protocol for the isolation of highly pure FoxP3^{+} Tregs to be used as immunotherapy for autoimmune disease.

KEYWORDS: regulatory T-cells, FoxP3, Th17, plasticity, cellular therapy

Contents

Abbreviations

A2A	Adenosine A2A receptor
ADP	Adenosine diphosphate
AIRE	Autoimmune regulator 1
AMP	Adenosine monophosphate
APC	Antigen presenting cell
ATP	Adenosine triphosphate
ATx	Adult thymectomy
Bcl6	B-cell lymphoma 6 protein
CCR	C-C chemokine receptor
CD	Cluster of differentiation
CD4SP	Single-positive $CD4^+$ $CD8^-$ stage of thymocyte development
CDR	Complementarity determining region
c-Rel	Proto-oncogene c-Rel
cTEC	Cortical thymic epithelial cells
CTLA-4	Cytotoxic T-lymphocyte antigen 4
CXCR	C-X-C chemokine receptor
DC	Dendritic cells
FoxP3	Forkhead box P3
FR4	Folate receptor-4
GITR	Glucocorticoid-induced TNF-receptor
HEV	High endothelial venules
IDO	Indoleamine 2, 3-dioxygenase
IL	Interleukin
IPEX	Immunodysregulation polyendocrinopathy X-linked
IRF-4	Interferon regulatory factor 4
iTreg	Inducible/adaptive regulatory T-cells
LAG-3	Lymphocyte-activation gene 3
LAP	Latency-associated peptide
LN	Lymph nodes
MBP	Myelin basic protein
MHC	Major histocompatibility class
mTEC	Medullary thymic epithelial cells
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NOD	Non-obese diabetic
nTreg	Natural regulatory T-cells
NTx	Neonatal thymectomy
PNAd	Peripheral lymph node addressin
RA	Retinoic acid
RAG-1	Recombination activating gene-1
SCID	Severe combined immunodeficiency
SRBC	Sheep red blood cells
STAT	Signal transducer and activator of transcription
T-bet	T-box transcription factor TBX21
TCR	T-cell receptor
Teff	Effector T-cell
Tfh	Follicular helper T-cells
TGF-β	Transforming growth factor beta
Th	T-helper $CD4^+$ T-cell
Tregs	regulatory T-cells

Introduction

The ability of the immune system to discriminate between exogenous 'non-self' antigen and 'self' autoantigens, is central to our view of how the immune system elicits protective immune responses against pathogens (1,2). Failure of such cellular and molecular mechanisms is considered to give rise to a loss of self-tolerance and the generation of immune responses against autoantigens, resulting in autoimmune disease (3). Understanding of these mechanisms is therefore vital to the development of effective treatments for autoimmune disease and is an important, but challenging, area of research (4,5).

Recognition of autoantigens by the immune system is, in part, regarded as a potential consequence of T-cell receptor (TCR) development during thymic T-cell maturation (6). During this process somatic recombination of the TCR V(D)J gene segments in the thymus results in the generation of immature T-cells with varying antigen specificities, determined by the TCR complementarity determining region (CDR)(1,2). Consequently, the repertoire of immature T-cells in the thymus is considered to comprise of, 1) standard T-cells that recognise pathogenic 'non-self' antigen, and 2) autoreactive T-cells which recognise 'self' antigen and may pose threat to the maintenance of self-tolerance. Central tolerance, where T-cells expressing autoreactive TCRs are negatively selected in the thymus to undergo apoptosis or become anergic, was thought to be the main mechanism of inducing self-tolerance to autoantigens (5). However, the isolation of autoreactive T-cells in the blood of most healthy individuals, together with autoimmunity affecting only 5% of the population, suggests that other mechanisms of maintaining self-tolerance exist that are crucial to preventing autoimmune disease (4).

T-cell mediated peripheral self-tolerance was first described in the late 1960s following research which identified thymic-derived 'suppressor T-cells', shown to be critical in the prevention of autoimmune disease in mice (7-9). Later suppressor T-cells were identified in humans as $CD4^+$ $CD25^{high}$ T-cells and renamed regulatory T-cells (Tregs) (4,10,11). Subsequently, $CD4^+$ $CD25^+$ Tregs were shown to express the transcription factor forkhead box P3 (FoxP3), which was found to be critical to their development and function, leading to the characterisation of human Tregs as $FoxP3^+$ $CD25^{high}$ $CD4^+$ T-cells (8). Following these findings, research has demonstrated that patients with autoimmune disease often display reduced Treg infiltration or defective function, suggesting that modulation of Treg function or number may be a useful form of immunotherapy for autoimmune disease (4,9).

In a previous review, we proposed a protocol for isolating and expanding a population of highly purified $FoxP3^+$ Tregs from patients to use as adoptive cell therapy for the treatment of autoimmune disease (4). However, a recent review by Sakaguchi *et al.* suggests that $FoxP3^+$ T-cells are a more heterogeneous population than once thought, proposing a new delineation for human Tregs (10,12). Furthermore, current research has expressed uncertainty over the regulatory phenotype of Tregs, following concern over the stability of FoxP3 expression and evidence that Tregs can convert to pathogenic pro-inflammatory T-helper 17 like cells (4,13). In light of these findings, the aim of this thesis is to propose an improved protocol for isolating and expanding a population of highly purified $Foxp3^+$ Tregs from patients to use as immunotherapy for autoimmune disease. This will be achieved by asking two main questions; 1) how can the isolation and *ex vivo* expansion of $FoxP3^+$ Tregs from a patient be improved and tailored to a specific autoimmune disease?, and 2) could molecular interventions be used to stabilise the regulatory phenotype of isolated and *in vitro* expanded Tregs?

I. History of regulatory T-cells

Before investigating the use of Tregs as cell-based immunotherapy for autoimmune disease a literature search was performed to analyse the history behind the discovery of Tregs. By doing so recent findings regarding Tregs could be interpreted correctly and previous experiments reanalysed to support the proposed model (last chapter).

Thymic-derived suppressor T-cells

T-cell mediated self-tolerance was first suggested in the 1960s following the identification of thymic-derived suppressor T-cells using neonatal thymectomy (NTx) (7,9). This fundamental research by Nishizuka and Sakakura demonstrated that NTx of mice results in T-cell mediated 'ovarian dysgenesis' (now known as autoimmune oophoritis) if performed 3 days, but not 1 or 7 days after birth (Figure 1A, B) (7-9,14). Proposing the development of a population of thymic-derived 'suppressor T-cells' following three days of life, critical to regulating autoimmune responses (9). In 1970, Gershon and Kondon supported these findings after inoculation of mice with isolated thymic cells before injection with sheep red blood cells (SRBC), suppressed the associated immune response and specific antibody production (9,15). Moreover, isolated spleen cells from SRBC tolerant mice were shown to induce SRBC tolerance in normal mice, suggesting that thymic-derived suppressor T-cells

inhibited SRBC immune responses by inducing 'infectious tolerance' in normal T-cells (9,15-17). Alternatively, a protocol of rat adult thymectomy (ATx) with sub lethal doses of x-ray irradiation was shown to result in autoimmune thyroiditis (Figure 1 A, B) with specific autoantibody production, which was inhibited by inoculation with syngeneic T-cells from normal rats (8,18). Together these early studies suggested that a population of thymic-derived suppressor T-cells regulate immune responses against 'non-self' and 'self' antigen by inducing 'infectious tolerance' in effector T-cells (8,19).

$CD5^{high}$ $CD4^{+}$ suppressor T-cells

In the mid-1970s, research identified 'Ly antigens' as markers for T-cell subsets, resulting in the first characterisation of different T-cell subsets (20). Subsequently, the expression of Lyt-1 (CD5) and Lyt-2 (CD8), was associated with a population of $CD8^{+}$ suppressor T-cells thought to exert their suppressive function by expression of the soluble suppressor 'I-J molecule' (8,21,22). Later, research identified a population of $CD5^{+}$ $CD8^{-}$ $I\text{-}J^{-}$ suppressor T-cells expressing L3T4 (CD4), which were able to delay NTx-induced autoimmune oophoritis, generating interest in $CD4^{+}$ suppressor T-cells (8,23,24). However, splenic $CD4^{+}$ T-cells isolated from mice with NTx-induced autoimmune oophoritis were later shown to induce oocyte damage in T-cell deficient mice, by promoting autoantibody production and the activation of effector cells (8,24). Therefore, indicating the need for markers which distinguished between $CD4^{+}$ suppressor T-cells and potentially autoreactive $CD4^{+}$ T-cells. In 1985, research identified that the transfer of $CD4^{+}$ $CD5^{low}$ T-cells to T-cell deficient mice induced autoimmune thyroiditis (Figure 1C), which was not observed following the transfer of $CD4^{+}$ $CD5^{high}$ T-cells (8,22,25). These findings indicated that $CD4^{+}$ T-cells with $CD5^{high}$ expression suppressed autoimmunity, whereas $CD4^{+}$ T-cells with $CD5^{low}$ expression were responsible for autoimmune pathology. However, the lack of additional markers to further define $CD4^{+}$ suppressor T-cells, amidst uncertainty over the existence of the $CD8^{+}$ suppressor T-cell associated 'I-J molecule', led to a decline in suppressor T-cell research (9,19,26).

$CD25^{+}$ $CD4^{+}$ regulatory T-cells (Tregs)

In the mid-1990s research into suppressor T-cells was revived after analysis of peripheral $CD5^{high}$ $CD4^{+}$ T-cells by Sakaguchi *et al.* identified the IL-2 receptor α-chain (CD25) as a new marker for $CD4^{+}$ suppressor T-cells, now termed regulatory T-cells (Tregs) (4,8,27). These findings demonstrated that the transfer of $CD4^{+}$ $CD25^{low}$ T-cells to athymic mice induced multi-organ autoimmune pathology (Figure 1C), which was prevented by the co-transfer of $CD4^{+}$ $CD25^{+}$ T-cells (8,27,28). Notably, $CD25^{high}$ expression was shown to be a more reliable marker of Tregs than $CD5^{high}$ expression. Transfer of $CD4^{+}$ $CD25^{low}$ T-cells to athymic mice generated a higher incidence of autoimmune pathology, compared to the equal transfer of $CD4^{+}$ $CD5^{low}$ T-cells (8). Furthermore, $CD25^{+}$ $CD4^{+}$ T-cells were shown to appear in the spleen and periphery of normal mice after 3 days of life correlating with results from NTx, confirming that Tregs originate from the thymus (Figure 1B) (10).

In humans, *in vitro* research demonstrated that human $CD25^{+}$ $CD4^{+}$ T-cells exert a suppressor phenotype, akin to mouse $CD25^{+}$ $CD4^{+}$ Tregs (10,29-33). However, unlike in mice, the suppressor phenotype of human $CD25^{+}$ $CD4^{+}$ T-cells was found to be attributed to only $CD4^{+}$ T-cells with the highest CD25 expression (4,10,11). These findings correlated with data indicating that only 5% $CD4^{+}$ $CD8^{-}$ human thymocytes express CD25, of which were similar in function and frequency to $CD25^{high}$ $CD4^{+}$ T-cells in the peripheral blood (4,34). Therefore, indicating that $CD25^{high}$ thymocytes are the precursors to human peripheral Tregs, leading to human Tregs being characterised as $CD4^{+}$ $CD25^{high}$ T-cells (4,10,11).

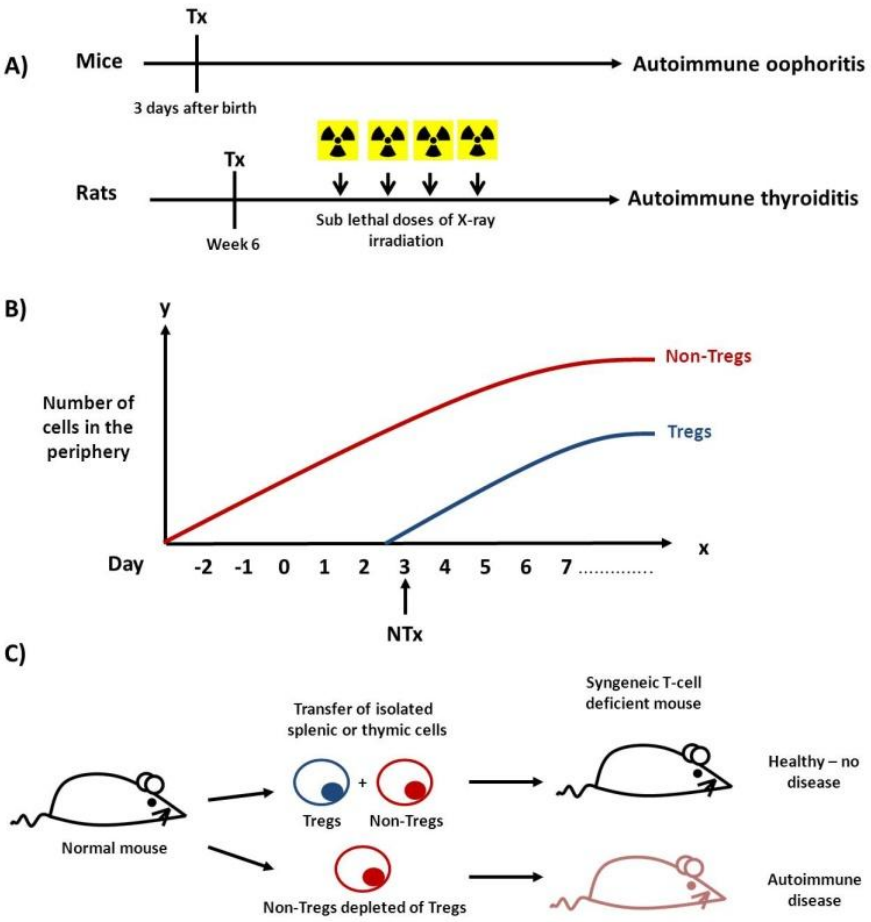

Figure 1. **A)** Schematic representation of neonatal thymectomy (mice) and adult thymectomy (rats) used to induce autoimmune disease. **B)** Thymic production of non-Tregs (including potentially autoreactive T-cells), occurs during neonatal development resulting in the presence of peripheral non-Tregs before birth (red line). However, thymic production of Tregs is delayed until 3 days after birth, resulting in the absence of Tregs in the periphery before day 3 of life (blue line). Neonatal thymectomy (mice): if performed on day 3 results in the activation of peripheral self-reactive T-cells due to the absence of Tregs, resulting in autoimmunity. Adult thymectomy (rats): peripheral Tregs are already present after 6 weeks of life. However, adult thymectomy removes the supply of Tregs, and x-ray irradiation specifically eliminates any peripheral Tregs, therefore, resulting in the activation of peripheral self-reactive T-cells and induction of autoimmunity. **C)** Transfer of a population of isolated spleen or thymic cells from adults to syngeneic T-cell deficient mice does not induce autoimmune disease as adults contain both Tregs and non-Tregs in the periphery. However, if Tregs (e.g. $CD5^{high}$ or $CD25^{high}$ $CD4^+$ T-cells) are depleted prior to transfer, recipient T-cell deficient mice develop autoimmune disease due to activation of self-reactive T-cells. Diagram and text adapted from (8).

II. Origins of regulatory T-cells: Thymic and peripheral development

Identification of FoxP3 as the master regulator of thymic Treg development and function

As previously discussed, thymectomy models in rodents established that a population of thymic-derived $CD25^+$ Tregs are crucial to the prevention of autoimmunity. However, following the identification that conventional $CD4^+$ T-cells upregulate the expression of CD25, it was not known whether $CD25^+$ Tregs represented a specific population of T-cells (5). This changed following the identification that thymic and peripheral $CD25^+$ $CD4^+$ Tregs express the unique transcription factor forkhead box P3 (FoxP3) (5,35-39). Therefore, indicating that $CD4^+$ $CD25^+$ Tregs undergo alternative development pathways compared to conventional $CD4^+$ T-cells, supporting the notion that Treg cells are a unique T-cell lineage. Subsequently, research demonstrated FoxP3-deficient *Scurfy* mice develop multi-organ autoimmune disease, and that humans with germline mutations in the *foxp3* gene develop immunodysregulation polyendocrinopathy X-linked (IPEX) autoimmune disease (5,10,13,35,37,38,40-46). Furthermore, forced expression of FoxP3 in $CD25^-$ $CD4^+$ T-cells was shown to confer the regulatory phenotype, and later studies demonstrated that absence of FoxP3 expression attenuated development of $CD25^+$ $CD4^+$ Tregs and caused the autoimmune pathology observed in *Scurfy* mice (5,35,37). These results characterised FoxP3 as the master regulator of Treg development and function, leading to Tregs being further characterised as $FoxP3^+$ $CD25^+$ $CD4^+$ T-cells (37,47,48).

Early studies of thymic Treg development

Early research of thymic Treg development proposed that thymic epithelial cells were responsible for 'imprinting' thymocytes with a stable regulatory phenotype, by presenting specific self-antigens via major histocompatibility (MHC) class-II molecules and inducing strong TCR signalling in autoreactive T-cells (5,49). This theory was supported by research demonstrating that the co-expression of haemagglutinin or ovalbumin in TCR-specific transgenic mice induced Treg development (5,14,50-53). In addition to, research showing that mice lacking the expression of TCR recombination activating gene-1 (Rag-1), were unable to produce thymic Tregs (14,34,54). Subsequently, research established that mice lacking the expression of MHC-II molecules by cortical thymic epithelial cells (cTEC) failed to develop Tregs (4,55). Suggesting that Treg development is an early cortical event dependent on autoreactive thymocytes expressing specific TCRs and encountering agonistic self-antigen presented by cTECs via MHC-II molecules.

However, the role of TCR-signalling in Treg development was opposed by research demonstrating that the co-expression of hen egg lysozyme (an enzyme involved in the innate immune response) in TCR-specific transgenic mice did not increase Treg development (14,56). In addition to, research where varying levels of transgenic antigen increased the frequency, but not the number of Tregs, suggesting that specific TCR-signalling does not induce Treg development (14,57). Furthermore, research demonstrated that the TCR-repertoires of non-Tregs and $FoxP3^+$ $CD25^+$ Tregs were significantly overlapped, and that TCRs from $FoxP3^+$ $CD25^+$ Tregs were unreactive to self-antigen (14,58). Leading to the contrasting view that thymocytes are committed to becoming Tregs prior to TCR rearrangement, proposing that TCR specificity is not required for Treg development, but is determined afterwards (14,59).

Current model of thymic Treg development – natural Tregs (nTreg)

Despite the previous controversy, current research supports the role of TCRs in thymic Treg development, but alternatively proposes that Tregs develop in the thymic medulla rather than cortex. After research demonstrated that thymic medullary epithelial cells (mTEC) select thymocytes via TCR/MHC-II interactions during the single-positive $CD4^+$ $CD8^-$ (CD4SP) stage of thymocyte development, coinciding with the induction of FoxP3 expression (5,36,60). Subsequently, research has shown that the absence of MHC-II expression by mTECs reduces $FoxP3^+$ $CD25^+$ Treg development, whereas, impaired MHC-II expression by mTECs results in preferential Treg development (4,14,61,62). Therefore, suggesting that mTECs select self-reactive CD4SP thymocytes and induce Treg differentiation based on the specificity, but also, the avidity of MHC-II/TCR interactions (Figure 2). A notion which was confirmed by recent research which indicates that $FoxP3^+$ Tregs undergo strong TCR stimulation in the thymus (14,63).

Since FoxP3 is crucial to the development and function of Tregs, research has investigated the pathways involved in the induction of FoxP3 expression during thymic Treg development. Such research has established a link between TCR-mediated CD4SP thymocyte selection and FoxP3 expression, after TCR simulation was shown to activate the NF-κB family member c-Rel in pre-Tregs and induce FoxP3 expression (14,64-66). Furthermore, TCR-independent signalling, by γ-chain cytokines such as IL-2, IL-15 or IL-7, has been shown to activate the signal transducer and activator of transcription 5 (STAT5), also inducing FoxP3 expression (14). Therefore, demonstrating FoxP3 expression is induced in CD4SP thymocytes during TCR-mediated selection, with the help of various thymic-derived cytokines.

Owing to their extensive development in the thymus, this population of Tregs are called thymic-derived or natural Tregs (nTregs). Following development in the thymus nTregs are thought to migrate to the periphery and regulate autoreactive immune responses via the recognition of self-antigens (Figure 2) (4,67).

Extrathymic Treg development – adaptive/inducible Tregs (iTregs)

Although once thought to develop solely in the thymus, there is growing support for a population of Tregs which develop in the periphery. This group of Tregs are proposed to develop in the periphery from conventional $CD25^-$ $CD4^+$ T-cells following the recognition extrathymic antigen. This group of Tregs are termed adaptive/inducible Tregs (iTregs) (Figure 2) (4,14,67). Studies of oral tolerance to myelin basic protein (MBP) were the first to propose extrathymic Treg development, identifying antigen-specific iTregs which regulate effector T-cell responses by secreting IL-10 (Tr1), or TGF-β (Th3) (Figure 2) (4,68-70). These $FoxP3^-$ iTregs are thought to develop from conventional $CD4^+$ T-cells following recognition of extrathymic antigen and stimulation with IL-2 and TGF-β (4,14). Furthermore, recent research suggests that a group of $FoxP3^+$ iTregs from conventional $CD4^+$

T-cells in the gut following the recognition of extrathymic antigen, and secretion of retinoic acid (RA) and TGF-β by lamina propria CD103^{+} dendritic cells (DC) (4,71,72).

Neonatal transfer of Tregs suggests that iTregs have a distinct TCR repertoire from nTregs, supporting their alternative development and recognition of extrathymic antigens (14,73). In addition, iTregs have been shown to be crucial to the prevention of autoimmunity in mice, after both iTregs and nTregs were needed to maintain self-tolerance in FoxP3-deficient *Scurfy* mice (14,73). However, despite these findings, the functional significance of iTregs in the maintenance of peripheral self-tolerance is currently under debate (4,5,74-77). Furthermore, due to the majority of research into iTregs being carried out in mice, we currently lack information regarding the role of human iTregs in the prevention of autoimmune disease. Therefore, this thesis will focus primarily on human nTregs as a potential therapeutic intervention for autoimmune disease.

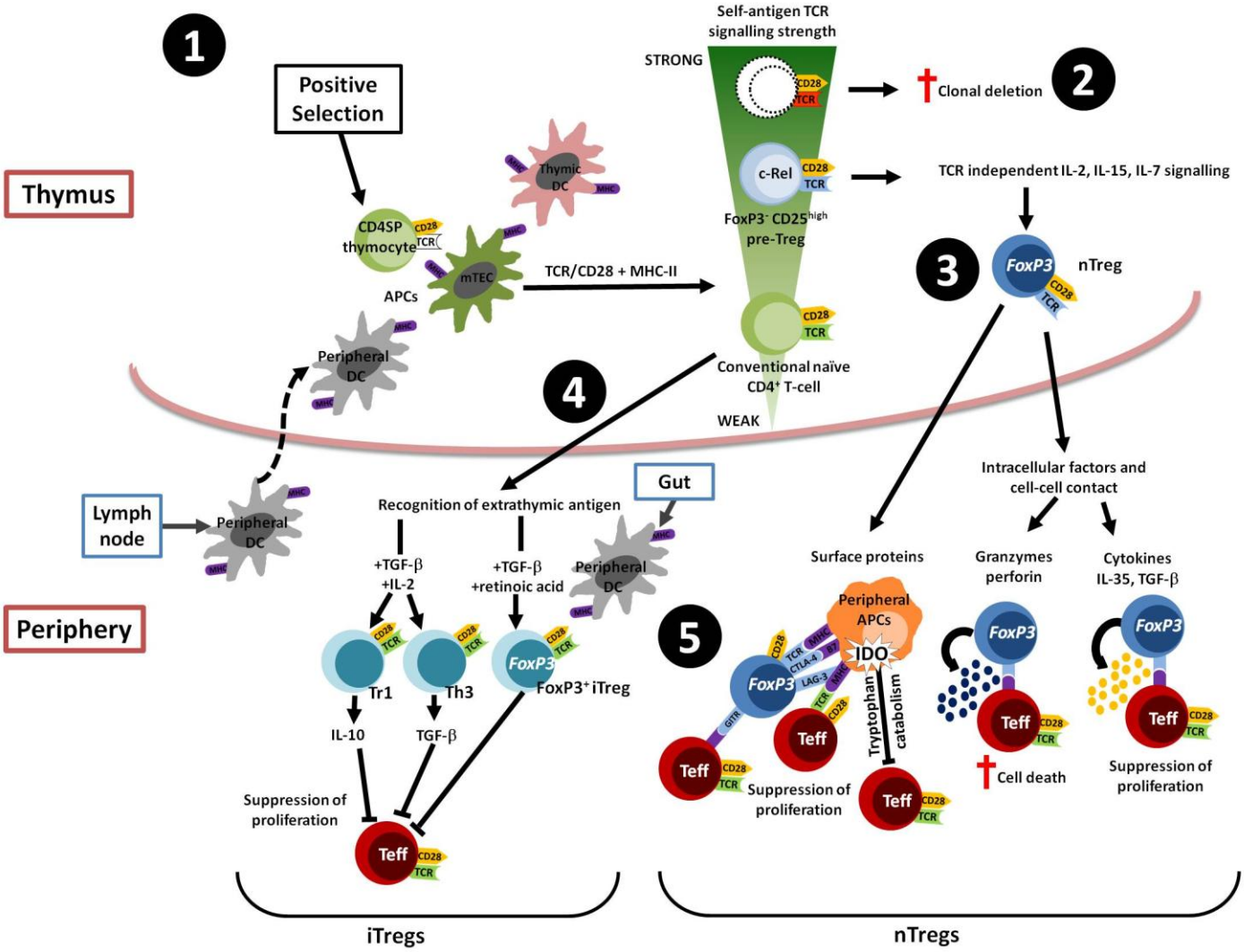

Figure 2. Overview of thymic and extrathymic Treg-cell development, and the proposed mechanisms of suppression. **1)** Following positive selection for the recognition of self MHC molecules, immature CD4SP thymocytes are presented with self-peptides by resident APCs (mTEC, thymic DC and peripheral DC). During this process three responses are hypothesised: **2)** Thymocytes expressing high affinity TCRs for self-peptides with strong activation upon MHC stimulation undergo apoptosis, resulting in the clonal deletion of highly autoreactive thymocytes and maintenance of central tolerance; **3)** Thymocytes expressing medium affinity TCRs for self-peptides with moderate activation upon MHC stimulation are thought to be FoxP3^{-} CD25^{high} Treg cells, which following stimulation with γ-chain cytokines (IL-2, IL-15 and IL-7) are induced to express FoxP3 and become nTregs cells and; **4)** Thymocytes expressing low affinity TCRs for self-peptides with weak activation upon MHC stimulation become conventional naïve CD4^{+} T-cells which upon recognition of non-self antigen in the periphery, in combination with cytokines secreted by peripheral dendritic cells, develop into iTregs cells. **5)** In the periphery, nTregs can indirectly suppress Teff cells by either reducing the ability of APCs to present antigen, or by activating indoleamine 2,3-dioxygenase (IDO) resulting in the generation of immunosuppressive metabolites. Furthermore, in conjunction with cell-to-cell contact nTregs can directly suppress Teff cells by secreting cytokines, or inducing Teff death by producing cytotoxic molecules. Following conversion from naïve CD4^{+} T-cells iTregs (e.g. T-regulatory -1, T-helper-3, and FoxP3^{+} iTregs) suppress effector T-cells by the secretion of anti-inflammatory cytokines such as IL-10 and TGF-β. Diagram and text adapted from (4,14).

III. Migration of regulatory T-cells: Targeted suppression of autoimmune responses

Like conventional naïve T-cells, naïve nTregs express L-selectin (CD62L) and the C-C chemokine receptor 7 (CCR7), and use these molecules to migrate towards secondary lymphoid tissues, such as the lymph nodes (LN) (78,79). Upon arriving in the LNs, naïve nTregs cross the high endothelial venules (HEV) guided by the expression of chemokine C-C ligand 21 (CCL21) and peripheral lymph node addressin (PNAd) by HEVs, which interact with CCR7 and CD62L expressed by naïve nTregs (78,80). During an immune response, naïve nTregs are presented with self-antigen by peripheral APCs in the LNs, resulting in the TCR-mediated maturation of naïve nTregs into antigen-specific effector nTregs (78).

In mice, it has been demonstrated that the maturation of naïve nTregs in the LNs is required for nTregs to acquire an immunoregulatory phenotype and maintain a population of effector nTregs in the periphery (10,81). However, unlike in mice, human naïve nTregs have been shown to regulate antigen-specific immune responses prior to maturation in the LNs (78,82-85). Therefore, suggesting that human nTregs do not require TCR-mediated maturation to elicit regulatory immune responses. However, research demonstrates that peripheral nTregs switch from predominantly expressing the naïve T-cell marker CD45RA in neonates, to expressing effector T-cell marker CD45RO in adulthood, indicating that TCR-mediated maturation becomes more important as we age (78,86,87). Furthermore, Tregs deficient in the LN-homing receptors show impaired migration to the LNs and reduced suppressor function in the periphery, suggesting that TCR-mediated maturation is required to effectively suppress peripheral immune responses (78,88-90).

Expression of tissue-specific chemokine receptors by effector nTregs

Current research suggests that TCR-mediated maturation is important as it increases the expression of tissue-specific chemokine receptors (Table 1), allowing effector nTregs to migrate towards specific tissues and effectively target their suppressor functions against pathogenic cells. For example, neonatal naïve nTregs predominantly migrate to gut-associated lymphoid tissue via the expression of α4β7 integrin, where they undergo TCR-mediated maturation to extrathymic antigen (78). Following maturation neonatal effector nTregs upregulate P- and E-selectin expression, conferring the ability to migrate from the blood to tissues, allowing neonatal effector nTregs to regulate the development of the early immune system (78,87). Similarly, the analysis of naïve nTregs in adults shows that TCR-mediated maturation increases the expression of FoxP3 and induces the expression of tissue-specific chemokine receptors and integrin molecules (Table 1) (10,78,80,91). Research has identified that deficiencies in the expression of FoxP3, and certain chemokine receptors is associated with the development various autoimmune and inflammatory diseases (Table 1) (10,80,92). Therefore, TCR-mediated maturation of naïve nTregs may strengthen the regulatory phenotype of nTregs, by increasing FoxP3 expression, and target such antigen-specific nTregs to inflamed tissues, by modulating the expression of tissue-specific chemokine receptors.

Hijacking of Th-cell related migratory pathways by effector nTregs

In addition to the expression of tissue-specific chemokine receptors, effector Tregs express similar homing receptors to Th-cells (Table 1) and can be sorted into Th1, Th2, Th17 and Th22-like Treg subsets (80,92,93). This is thought to be the result of nTregs expressing various transcription factors associated with Th-cell development, allowing effector nTregs to 'hijack' Th-related migratory pathways and effectively regulate Th-specific immune responses (78,80). For example, expression of T-bet by nTregs, a transcription factor involved in interferon-γ production and Th1 pathogenesis, controls the expression of CXCR3, IL-10 and TGF-β by nTregs, and is associated with effective suppression of Th-1 induced inflammation (78,94,95). In addition, expression of IRF4, a transcription factor involved in Th2 differentiation, controls the expression of CCR8, IL-10, granzyme B and fibrinogen-like protein 2 by nTregs, and is associated with the suppression of Th2 immune responses (66,78,96). Similarly, expression of the Th17 associated cytokine STAT3, regulates CCR6, IL-10, granzyme B and perforin expression by nTregs, and is required for nTregs to suppress Th17, but not Th1 or Th2 responses (78,97). Moreover, expression of Bcl6 by nTregs, a protein involved in follicular helper T-cell (Tfh) immune responses, allows nTregs to express CXCR5 and to migrate to the germinal centres where they regulate B-cell development and antibody class switching (6). Therefore, suggesting that nTregs are able to modulate their regulatory and migratory phenotypes to suit the immune response by expressing various Th-related transcription factors, allowing them to target specific immune responses in the periphery.

Targeted peripheral immunosuppression by effector nTregs

Corresponding with the expression of various tissue- and immune-specific homing receptors, the suppressive mechanisms of nTregs require close contact with their target cells for maximum effect. Accordingly, during an immune response nTregs migrate from inflamed tissue to the LNs via the afferent lymphatics, where they can potentially interfere with interactions between APCs and conventional T-cells (Figure 2). Upon arriving at the LNs, nTregs are thought to suppress APCs via the expression of cell-surface molecules which bind to APCs. For example, nTregs express interacts with CD80/CD86 on APCs via the expression of CTLA-4, resulting in the activation of intracellular indoleamine 2,3-dioxygenase (IDO) in APCs, which suppresses effector T-cells by converting tryptophan into pro-apoptotic metabolites (4,98). Furthermore, nTregs express LAG3 which binds to MHC-II molecules on APCs modulating the activation state and maturation of APCs, resulting in the reduced activation of effector T-cells (4,99,100). In addition to suppressing the function and activation of effector T-cells in the LNs, effector nTregs may promote the development of iTregs and maturation of naïve nTregs in the LNs. Therefore, resulting in a population of effector Tregs (iTregs and nTregs), which migrate to the inflamed tissue. Here, effector nTregs are thought to use cell surface proteins CD39 and CD73 to activate A2A receptors on effector T-cells, catalysing the conversion of ATP or ADP to extracellular AMP and resulting in the deactivation of effector T-cells (4,101-103). In addition, effector nTregs can recognise potentially autoreactive T-cells via cell-to-cell contact and release intracellular factors such as granzyme-B or perforin, which induce cell death (4,104,105). Moreover, in conjunction with activated iTregs, effector nTregs may suppress effector T-cells by expressing cytokines such as IL-10, TGF-β and IL-35 (4,106). Within inflamed tissue the production of cytokines by local effector cells is thought to alter the *in situ* microenvironment and Treg stability/plasticity, allowing Tregs to convert into pathogenic T-helper 17 cells (Th17) (discussed later).

In summary, recent research demonstrates that the maturation of naïve nTregs in the LNs, upregulates the expression of tissue-specific CCRs and Th-related transcription factors, which are crucial for the effective suppression of peripheral autoimmune responses by effector nTregs. Therefore, the manipulation of CCR expression and Th-related transcription factors may be an important tool to maximize the therapeutic potential of isolated Tregs.

Table 1. The types and function of Treg homing receptors suggested by Campbell and Koch, and the similarities between other lymphocytes proposed by Ding *et al.* Adapted from (78,92).

Site of Treg migration	Receptor	Function	References	Also expressed by
Lymphoid tissues	CD62L (L-selectin)	Migration to lymph nodes	▪ Function in Treg cell migration to lymph nodes (107) ▪ Importance in the NOD–SCID model of diabetes (108)	Naïve T-cells
	CCR7	Migration to lymph nodes and the spleen	▪ CCR7$^{-/-}$ Treg cells fail to prevent colitis (109) ▪ Importance in Treg cell migration during allograft rejection (110)	Naïve T-cells
Non-lymphoid tissue (peripheral tissue)	E-selectin and P-selectin ligands	Migration to skin and inflamed tissues	▪ Expression by human Treg cells (111) ▪ Function in the cutaneous hypersensitivity response (112) ▪ Importance in cutaneous tolerance (113)	General leukocytes
	αEβ7 integrin	Epithelial localization	▪ Expression by mouse Treg cells (89) ▪ Treg cell retention during infection with *Leishmania major* (114)	General leukocytes
	α4β7 integrin	Migration to gut-associated lymphoid tissues	▪ Function in Treg cell migration to the intestine (115)	General leukocytes
	CCR2	Migration to inflamed tissues	▪ Expression by human and mouse Treg cells (116,117) ▪ Importance in Treg cell migration during allograft rejection (110)	Th17,
	CCR4	Migration to skin and other inflamed tissues	▪ Expression by human Treg cells (111) ▪ Importance in cutaneous and pulmonary tolerance(118) ▪ CCR4$^{-/-}$ Treg cells fail to prevent colitis (119)	Th2, Th17, Th22, Tr1
	CCR5	Migration to inflamed tissues	▪ Importance in Treg cell migration during allograft rejection (110) ▪ Function in migration to the inflamed intestine (120) ▪ Directing Treg cell localization during infection with L. major (121)	Th1, Tr1
	CCR6	Migration to sites of Th17 cell-mediated inflammation	▪ Expression by IL-10-producing Treg cells (122) ▪ Function in Treg cell migration during Th17 cell-mediated autoimmunity (123,124)	Th17, Th22
	CCR8	Migration to skin and sites of Th2 cell-mediated inflammation	▪ Expression by Treg cells (125)	Th2, Tr1
	CCR9	Migration to the small intestine	▪ Expression by Treg cells in the intestinal lamina propria (126)	Th17, Tr1
	CCR10	Migration to mucosal tissues and skin	▪ Expression by Treg cells in inflamed human liver (127)	Th22
	CXCR3	Migration to sites of Th1-mediated inflammation	▪ Expression by Treg cells is T-bet dependent (94) ▪ Importance in Treg cell localisation to inflamed liver (128,129)	Th1, Th17, Tr1
	CXCR5	Migration to B cell follicles and germinal centres	▪ Expression by human Treg cells (130) ▪ Inhibition of B cell responses by Treg cells (131)	Tfh
	CXCR6	Migration to the liver	▪ Expression by human Treg cells (130)	Th1, Th17
Both lymphoid and non-lymphoid tissue	CXCR4	Migration to bone marrow, Peyer's patches and tumour sites	▪ Expression by naïve phenotype Treg cells (130) ▪ Association with tumour-infiltrating Treg cells (132,133)	

IV. Molecular characterisation of regulatory T-cells

Identification of FoxP3 as the master regulatory of Treg development and function represented a major breakthrough in the characterisation of CD25^{+} Tregs, leading to human FoxP3^{+} CD25^{+} CD4^{+} T-cells being regarded as a distinct Treg lineage. However, recent research indicates that, unlike in mice, human conventional CD4^{+} non-Tregs are able to upregulate CD25 and FoxP3 expression following activation (4,10,12,37,134). These findings therefore indicate that, unlike in mice, FoxP3 is not a specific marker for identifying human Tregs. Accordingly, research has

demonstrated that human $CD4^+$ $FoxP3^+$ Tregs are highly heterogeneous in gene expression, phenotype and suppressor function, and can be classified into three groups based on expression of FoxP3, markers of Treg suppression and CD45RA (Figure 3) (10,135). Identifying, activated ($FoxP3^{high}$ $CD45RA^-$), resting ($FoxP3^{low}$ $CD45RA^+$) and non-regulatory ($FoxP3^{low}$ $CD45RA^-$) (Figure 3) (4,135). However, this model is not particularly useful to isolate Tregs as conventional naïve T-cells express CD45RA and may upregulate FoxP3 expression upon maturation. Furthermore, many markers of Treg suppression have not been fully characterised and may be expressed by other leukocytes. Therefore, there has been considerable confusion as to how to isolate a population of $FoxP3^+$ Tregs capable of regulating immune responses *in vivo*, hindering the potential use of Tregs for cell-based therapy.

Expanding on the proposed model of Treg differentiation above, a recent review by *Sakaguchi et al.* has provided a more elaborate model of Treg differentiation based on the expression of FoxP3, CDR45RA and the effector T-cell marker CDR45RO (10). This model differentiates between conventional T-cells, iTregs and nTregs, in addition to identifying, naïve, effector and terminally-differentiated Tregs (

Figure 4). Using this model it has been proposed that naïve nTregs ($CD45RA^+$ $CD45RO^-$ $FoxP3^{low}$ $CD25^{high}$) are the most suitable for isolation and use in immunotherapy, rather than effector Tregs ($CD45RA^-$ $CD45RO^+$ $FoxP3^{high}$ $CD25^{high}$). This is because research demonstrates that naïve nTregs proliferate readily *in vitro* following TCR stimulation, and are resistant to apoptosis, whereas effector Tregs have been shown to be hyporesponsive to TCR activation and prone to apoptosis *in vitro* (10,135). Furthermore, upon activation, naïve Tregs have been shown to upregulate FoxP3 expression becoming effector Tregs $CD45RO^+$ $CD25^{high}$ $FoxP3^{high}$ Treg cells with an activated Treg cell phenotype, whereas effector Tregs may lose their regulatory function (Figure 3) (78). Moreover, unlike effector nTregs, naïve nTregs express the LN-homing receptors CD62L and CCR7 (78). Therefore, transferred naïve nTregs are expected to migrate to the LNs and undergo TCR-mediated maturation, thus allowing them to target the appropriate autoimmune responses and induce infectious tolerance to self.

Since FoxP3 is an intracellular molecule we are reliant on cell surface markers for the isolation of $FoxP3^+$ Tregs. As previously discussed, CD25 is a useful marker of $CD4^+$ $FoxP3^+$ Tregs in mice. However, in humans CD25 expression is not as specific, with 30% of peripheral T-cells being $CD4^+$ $CD25^+$, and only those with $CD25^{high}$ expression (1-2%) being functionally considered as functional Tregs (10,11,136). Furthermore, isolation based on CD25 expression alone is not practical to select naïve nTregs, since FoxP3 and CD25 expression in humans is proportional, and isolation of the top 1-2% of $CD25^{high}$ Tregs may exclude $FoxP3^{low}$ $CD25^{mid}$ naïve Tregs (10,135).

Recently, research has identified that the absence of CD127 (IL-7R) is inversely correlated with FoxP3 expression and therefore may be used to isolate naïve nTregs which are $CD127^{low/-}$ (4,137,138). However, Tregs (both nTregs and iTregs) lack the expression of CD127 to a similar degree, and conventional $CD4^+$ T-cells have been shown to downregulate CD127 expression upon activation (10,139). Therefore, the lack of CD127 expression alone cannot be used to distinguish naïve nTregs from other Tregs subsets and activated conventional $CD4^+$ T-cells. CD127 together with CD25 has been used to isolate $FoxP3^+$ Tregs, however this protocol would again exclude naïve Tregs which are $CD25^{mid}$, in addition to potentially isolating unstable $CD127^{low/-}$ $FoxP3^{low}$ $CD4^+$ non-Tregs which may express pro-inflammatory cytokines (next chapter) (10,137,138). Alternatively, CD127 in conjunction with CD62L, CD45RA and CD45RO, could be used to distinguish naïve nTregs ($CD45RO^-$ $CD45RA^+$ $CD127^{low/-}$ $CD62L^+$) from conventional activated $CD4^+$ T-cells ($CD45RO^+$ $CD45RA^-$ $CD127^{low}$ $CD62L^{low}$), and conventional naïve $CD4^+$ T-cells ($CD45RO^-$ $CD45RA^+$ $CD127^+$ $CD62L^+$) (Figure 4).

In addition to CD127, research has demonstrated that nTregs express folate receptor-4 (FR4) and ectonuclease CD39, which may be used to distinguish nTregs from conventional effector $CD4^+$ T-cells (4,140,141). Furthermore, expression of latency-associated peptide (LAP) and IL-1 receptor type I (CD121a) and type II (CD121b) by activated nTregs could possibly differentiate activated nTregs from activated $CD4^+$ non-Tregs (4,142). However, many of these novel Treg cell markers have not been fully characterised and may be expressed by other Treg cell types such as iTregs (Table 2). Therefore, their ability to distinguish between different Treg and T-cell subsets is currently questionable, negating their potential use to isolate a pure population of naïve nTregs.

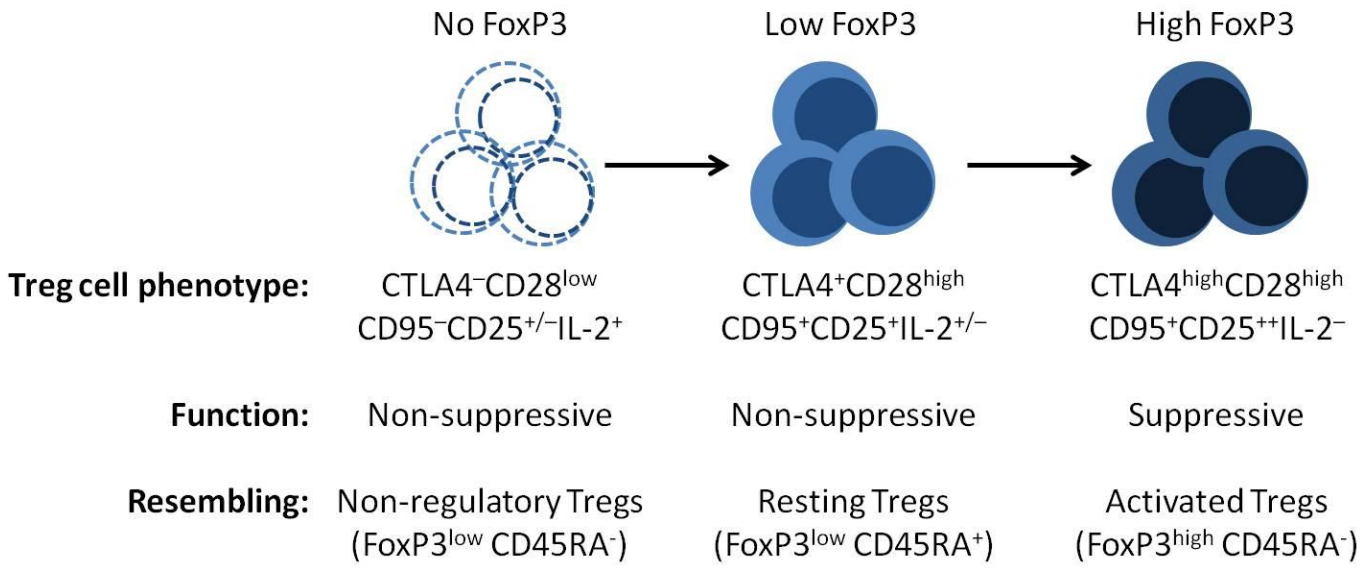

Figure 3. Varying levels of FoxP3 expression and associated regulatory molecules in human Treg cells. Absence of FoxP3 expression by Tregs results in the loss of Treg suppressive function. Low expression of FoxP3 induces the expression of various Treg-associated molecules however these cells do not elicit suppressor functions. High expression of FoxP3 is required to induce the full expression of Treg-associated molecules and elicit a suppressor functions. Diagram adapted from (10,135).

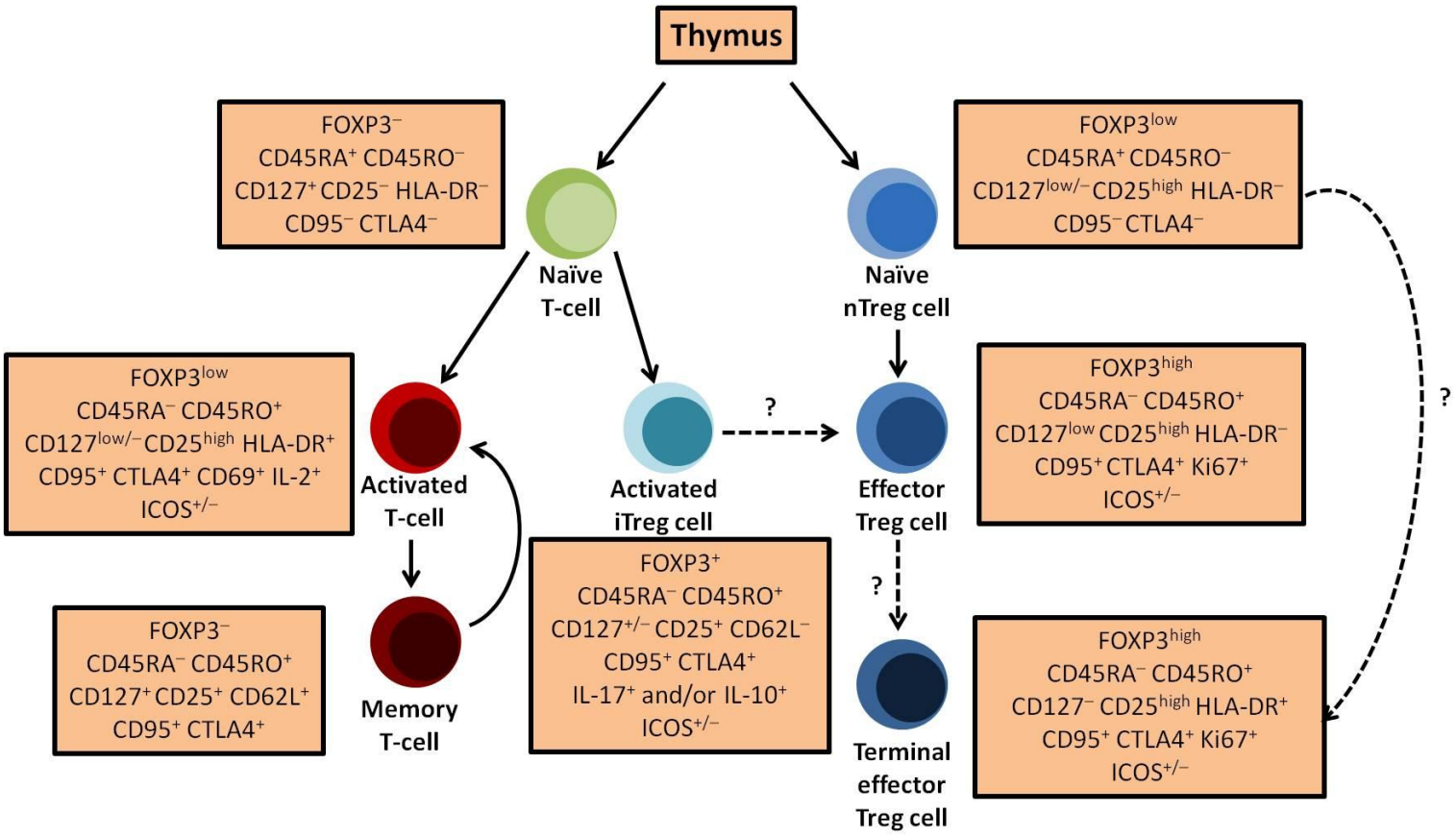

Figure 4. Proposed model of Treg cell differentiation by *Sakaguchi et al.* (10). This model uses the phenotypic markers expressed by $CD4^+$ T-cells to attempt to differentiate between conventional $CD4^+$ T-cells and the various Treg subsets. All T-cells originate from the thymus and enter the periphery expressing the naïve T-cell marker CD45RA. Following maturation in the secondary lymphoid tissues, T-cells lose the expression of CDR4RA and differentiate in to activated T-cells and natural regulatory T-cells (nTregs) expressing the effector T-cell marker CDR45RO. Activated T-cells have been shown to further differentiate into memory T-cells, which are important in maintaining long-term immunity to pathogens. Although, such memory recall responses have not been described for Tregs, effector Tregs are known to further differentiate into terminal effector Treg cells, which express unique phenotypic markers. Further contributing to the peripheral $CD45RA^-$ T-cell population are the recently identified induced/adaptive Tregs (iTregs), which develop from naïve T-cells and express a similar Treg associated molecules. Text and diagram adapted from (10).

Table 2. A comparison of Treg- associated cell marker expression between Treg subsets as shown by *Abdulahad et al.* (4)

Treg marker	Treg Subset		
	nTregs	Tr1	Th3
CD25	++	+/-	+
FoxP3 (effector/naïve)	+High/+Low	-	-
CD45RA (effector/naïve)	-/+	?	?
LAG-3	+	?	?
GITR	+	-	?
CTLA-4	+	+	+
IL-10	-	++	+/-
TGF-β	+/-	+/-	++
FR4	++	?	?
CD39	+	?	?
LAP	+	?	?
IL-1R I/II	+	?	?
Place of origin	Thymus	Periphery	Periphery
? = unknown			

V. Plasticity of regulatory T-cells: Relevance to autoimmune diseases

Recently there has been uncertainty over the stability of FoxP3$^+$ Tregs, after mouse models demonstrated that Tregs lose FoxP3 expression and differentiate into 'ex-FoxP3' Tregs, which secrete pro-inflammatory cytokines (143-147). Similarly, research has identified that human FoxP3$^+$ Tregs are capable of differentiating into IL-17 secreting Th17-like Tregs, and IFN-γ secreting Th1-like Tregs (80,143,148-151). Together these findings indicate that FoxP3$^+$ Tregs exhibit some degree of plasticity to differentiate into Th-like cells and are not a committed lineage. Therefore, leading to considerable controversy over the use of isolated Tregs for the treatment of autoimmune disease (13,152-154).

Since stable FoxP3 expression is associated with effective regulatory function, it is proposed that unstable or loss of FoxP3 expression is associated with the propensity for Tregs to convert to IL-17-secreting T cells. Research demonstrates that FoxP3 expression and stability is highly regulated by the epigenetic status of non-coding DNA sequence elements (CNS) within the *FoxP3* locus (Figure 5) (13,155). Analysis of the *FoxP3* locus indicates that nTregs, iTregs and T-helper cells exhibit different levels of methylation at CpG regions within the 'stability' associated CNS2 region, suggesting that T-cell subsets differ in the stability of FoxP3 expression (13,143). Specifically, nTregs are fully demethylated at CNS2, whereas, iTregs are partially methylated at CNS2, which suggests that FoxP3 expression by nTregs is more stable than in iTregs. Interestingly, iTregs provide researchers with a link between Tregs and Th17 cells, after *in vitro* stimulation of conventional naïve T-cells with TGF-β alone induces iTreg development, whereas the addition of IL-6 results in the Th17 development (Figure 6) (4,156). Therefore, it could be hypothesised that iTregs are less committed to the Treg cell linage and represent a population of Tregs capable of converting into Th17-like cells (80,143,157,158). Conversely, research suggests that naïve Tregs exhibit more stable FoxP3 expression and regulatory functions than effector Tregs, supporting the use of naïve Tregs in adoptive cell therapy (13,80,157,159-161).

Accordingly, the ability for Tregs to convert into IL-17-proudcing T-cells has been also associated with the TGF-β and IL-6 dependent expression of the Th-17 linage transcription factor RORγt, which binds to and inhibits FoxP3 (4,143,150,160,162-164). Furthermore, studies of Tregs *in vitro* demonstrate that pro-inflammatory cytokines IL-4, IL-6, IL-15 and IL-21, signal via Stat3 and downregulate FoxP3 expression, which may predispose Tregs to differentiating into IL-17-secreting cells *in vivo* (4,13,143,158,165-167). In contrast, anti-inflammatory cytokines such as IL-10 have been shown to stabilise FoxP3 expression and may protect Tregs from differentiating into IL-17-secreting cells *in vivo* (13,168). Moreover, research indicates that FoxP3 is able to bind to RORα and inhibit the expression of IL-17, IL-22 and CXCR3, which are associated with Th-17 differentiation (143,169). In addition to RORγt, Tregs express other Th1-, Th2- and Th17-associated molecules such as T-bet, IRF4 and Stat3, which are also regulated by FoxP3 (94,170-173). Therefore, it has been suggested that stable FoxP3 expression together with certain Th-associated molecules allows Tregs to maintain a regulatory phenotype whilst mimicking Th-cells for effective

migration and targeted suppression (see above) (78). This theory supports the identification of IL-17-secreting FoxP3$^+$ Tregs which maintain a regulatory phenotype, and suggests that IL-17-secreting FoxP3$^+$ T-cells without a regulatory phenotype represent Tregs with unstable FoxP3 expression (4,150,160,162,163). Therefore, it can be postulated that the tendency for Tregs to differentiate into Th17-secreting cells (and potentially Th1- or Th2-like Tregs) *in vivo* is determined by a delicate balance between stable FoxP3 expression and pro- inflammatory cytokines *in situ* (Figure 6).

Corresponding with the aforementioned data, IL-17 secreting and RORγt$^+$ FoxP3$^+$ T-cells have been identified in many inflammatory and autoimmune diseases, together with research identifying an increase in pro-inflammatory cytokines (Figure 3). Furthermore, recent studies indicate that Th17 cells are the main effector cells in inflammatory and autoimmune diseases (4,174,175). Therefore, it has been proposed that the reduced function or number of Tregs associated with many autoimmune diseases is due to the increased conversion of Treg to pathogenic Th17 cells (4).

Overall, these findings suggest that Tregs are an unstable lineage which lose their regulatory function and differentiate into IL-17-secreting cells following stimulation with pro-inflammatory cytokines. Given these findings the removal of unstable Tregs during the expansion of Tregs and use of epigenetic interventions to stabilise FoxP3 expression, may prove valuable for effective adoptive Treg cell therapy.

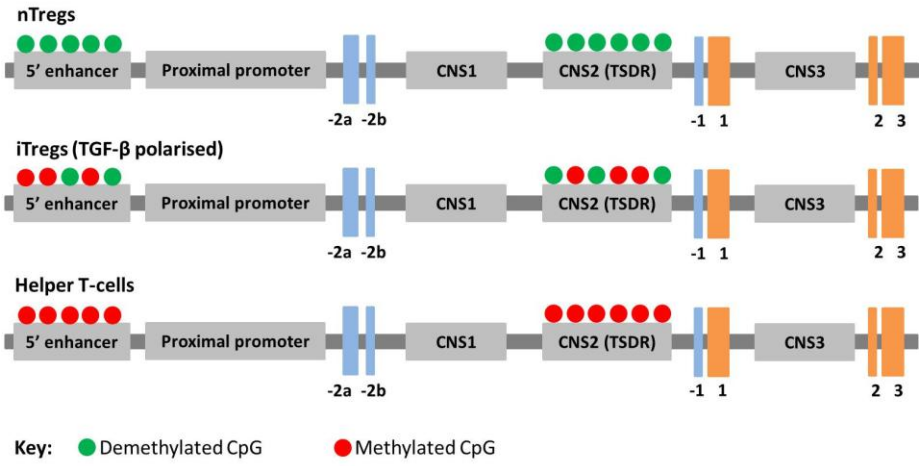

Figure 5: Comparison of the epigenetic signature at the FoxP3 locus in nTregs, iTregs and Th-cells. Transcriptional activity of the FoxP3 gene is largely determined by epigenetic status of the FoxP3 locus at various CpG islands, which lie in three non-coding DNA sequence elements (CNS), the 5' enhancer and the proximal promoter. High amount of CpG methylation is associated with closed DNA structure, whereas low amount of CpG methylation is associated with open DNA structure. The methylation status of the FoxP3 locus determines the transcription of the *FoxP3* gene in response to different stimuli such as, IL-2, TGF-β and antigen stimulation. Furthermore, different CNS regions control different aspects of FoxP3 expression, CNS1 – controls peripheral FoxP3 expression; CNS2 – controls FoxP3 stability; and CNS3 – controls the induction of FoxP3 expression during thymic and peripheral Treg development. Investigation of the methylation status of the 5' enhancer and CNS2 region suggest that 1) low methylation status in nTregs = stable and sustained FoxP3 expression, intermediate methylation status in iTregs = possibly unstable and transient FoxP3 expression, and 3) high methylation status in helper T-cells = low/no FoxP3 expression (only transiently upon activation/maturation). Diagram and text adapted from (13,143).

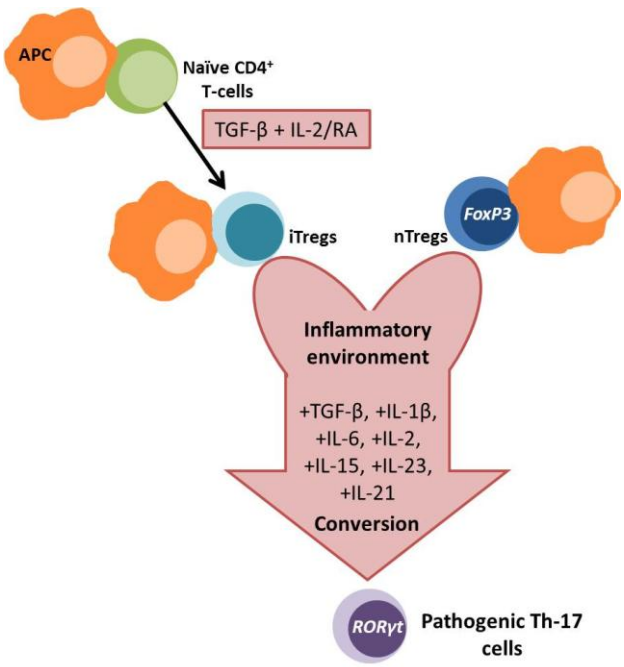

Figure 6. Schematic representation of the conversion of Tregs into IL-17 producing cells (Th17) as proposed by *Abdulahad et al.* (4). Presentation of antigen by APCs in the presence of pro-inflammatory cytokines converts unstable Tregs into Th17 cells which may contribute to autoimmune pathology.

Table 3. Table of human diseases associated with Treg to Th17 plasticity. Adapted from (13).

Disease	Identified Th17 cell type	Cytokine expression	Associated Treg defect	References
IPEX	↑frequency of IL-17 producing cells	-	-	(13,176)
Psoriasis	RORγt$^+$ IL-17$^+$ cells in skin lesions	↑IL-17, IL-22	-	(13,177,178)
Allergic rhinitis and polyposis	FoxP3$^+$ IL-17$^+$ cells in polyps	-	-	(13,179)
Crohn's disease	FoxP3$^+$ IL-17$^+$ cells in intestinal mucosa	↑TGF-β	-	(13,180,181)
Inflammatory bowel disease	↑frequency of peripheral IL-17 cells	↑IL-17A, IL-17F, IL-1β, IL-6 and IL-21	↓ number of peripheral Tregs	(182,183)
Ulcerative colitis	FoxP3$^+$ IL-17$^+$ cells in the colitic microenvironment and associated colon carcinoma ↑number IL-17 cells together with ↑RORγt mRNA expression	↑IL-5, IL-13 and Th17 cytokines	-	(13,177,181,183, 183,184)
Type 1 diabetes	↑frequency of FoxP3$^+$ IFN-γ$^+$ cells	↑IL-17 and TNF-α	FoxP3$^+$ IFN-γ$^+$ cells have reduced function CD4$^+$ CD25$^+$ T-cells - transiently lose regulatory functions in the early stages, and gradually gain stable effector functions	(13,80,148,185,18 6)
Multiple sclerosis	FoxP3$^+$ IFN-γ$^+$ cells Myelin specific Th17 cells (ex-Tregs?)	↑IL-17 (associated with disease activity)	-	(80,149,177,187, 188)
Systemic lupus erythematosus	↑frequency of IL-17 cells	↑IL-23, IL-21, IL-15 and IL-17	↓frequency of CD25^{high} FoxP3$^+$ Tregs (with reduced function), ↑frequency of CD25^{low} FoxP3$^+$ Tregs	(4,177)
Rheumatoid arthritis	↑frequency of IL-17 cells	↑IL-15 production by monocytes Highly inflammatory synovium (↑IL-21 IL-6, IL-1β and TGF-β)	Active state: ↑ number of Tregs in inflamed joint, reduced function of peripheral Tregs	(4,9,177)
Wegener's granulomatosis	↑percentage of IL-17 cells	↑IL-23, IL-15 and IL-17	↑number of Tregs (with reduced suppressor function)	(4)
Sjögren's Syndrome	↑infiltration of IL-17 cells into the glands	↑IL-17 in the glands	*Controversial* ↑number of Tregs in the glands (unknown suppressive function)	(4,9)

VI. Isolation and expansion of naïve natural regulatory T-cells for cellular therapy

Given that many autoimmune diseases display reduced Treg number or function, there has been considerable interest in the use of *ex vivo* expanded FoxP3$^+$ Tregs as a potential treatment for autoimmune disease. However, since human FoxP3$^+$ Tregs are functionally heterogeneous and may differentiate into potentially pathogenic Th-17 cells, there has been considerable confusion over which Treg cell subset should be isolated for use in adoptive Treg cell therapy. Previous research has focused on isolating CD25^{high} FoxP3^{high} effector Tregs for use in adoptive cell therapy, however effector Tregs are prone to apoptosis *in vitro* and may express an unstable regulatory phenotype. Furthermore, since effector Tregs lack the expression of LN-homing receptors and have already undergone TCR-mediated maturation, it is unsure whether these cells will effectively target specific autoimmune responses in the periphery. By utilising the new model of Treg differentiation, I propose isolating CD45RA$^+$ CD127^{low} CD62L$^+$ FoxP3^{low} naïve nTregs as an alternative to effector Tregs in adoptive cell therapy (Figure 7). As highlighted in this thesis, recent research indicates that naïve nTregs are highly proliferative *in vitro* making them ideal for isolation and expansion *in vitro*. Moreover, naïve nTregs have been suggested to display a more stable regulatory phenotype, therefore are potentially less prone to converting to pathogenic IL-17-secreting cells following transfer to the pro-inflammatory

environment present in autoimmune patients. Furthermore, naïve nTregs have not yet undergone TCR-mediated maturation and express LN-homing molecules CD62L and CCR7. Therefore, following transfer are expected to migrate to the LNs and undergo maturation, which will allow them to target peripheral autoimmune responses more effectively than effector Tregs.

Expansion of Tregs from PBMCs

There has been considerable research into expansion of Tregs from PBMCs. In contrast to our previous protocol which used anti-CD3 and anti-CD28 mABs conjugated to beads, research has looked into the use of artificial antigen presenting cells and dendritic cells (4,189-191). These novel protocols are projected to give better yields and the potential for inducing antigen-specific Tregs from naive Tregs (10,189,192). However, these novel methods of expansion may introduce contaminating cells and induce unknown effector antigen-specific Treg expansion, which could jeopardise the expansion of a naïve Treg population. Due to these concerns, our proposed protocol will continue to the use anti-CD3 and anti-CD28 mABs conjugated to beads, which although may compromise yield, is expected to ensure Treg purity. To further ensure Treg purity, PBMCs will be treated with cytokines to enhance Treg expansion and induce the differentiation of unstable Tregs into IL-17-secreting cells. This is to ensure that during the isolation of naïve nTregs, all other potentially pathogenic leukocytes are removed by positive selection.

Isolation of naïve nTregs from $CD4^+$ T-cells

Following the isolation of $CD4^+$ T-cells our previous protocol utilised the recent finding that $FoxP3^+$ Tregs are double negative for CD49d and CD127 (4,193). However, recent findings suggest that Tregs are not negative for CD49d and may express the integrin at variable levels (189). Furthermore, researchers have not yet investigated CD49d expression by naïve nTregs. Therefore, the use of CD49d to negatively isolate naïve nTregs is questionable. Instead I propose the use of CD127 and CD45RO to distinguish and negatively isolate naïve nTregs ($CD45RO^-$ $CD127^{low}$), from conventional effector T-cells ($CD45RO^+$ $CD127^{low}$), memory T-cells ($CD45RO^+$ $CD127^+$), effector Tregs ($CD45RO^+$ $CD127^{low/-}$), iTregs ($CD45RO^+$ $CD127^{+/-}$), conventional naïve $CD4^+$ T-cells ($CD45RO^-$ $CD127^+$) and IL-17 producing cells ($CD127^+$) (Figure 4) (4,10,193). Following this initial isolation I propose to eliminate any potential effector cells and increase Treg purity by culturing the cells with Rapamycin, together with IL-2 and other T-cell supporting cytokines (10,194). Furthermore, to ensure that the isolated Treg cells are naïve and able to home to the LNs, the isolated cells will undergo positive selection for CD45RA and LN-homing markers CD62L and CCR7.

Screening of isolated naïve nTregs

Given that the proposed protocol for isolating naïve Tregs is new, there will be considerable need for optimisation of the procedure and screening of the isolated cells before they can be used as therapy for autoimmune disease. Analysis of isolated naïve nTregs could be carried out using co-culture assays. However, given that the *in vitro* situation cannot replicate the *in vivo* environment, analysis of isolated naïve nTreg stability and suppressor function *in vitro* may not be relevant to the *in vivo* situation. Alternatively, isolated naïve nTregs could be transferred into immunodeficient NOD/Shi-scid $Il2rg^{-/-}$ (NOG) mice, which are a strain of mouse genetically modified to accept human cells (10). Using NOD mice, researchers will be able to assess the function of the isolated cells *in vivo,* monitoring important parameters such as migration, stability and suppressor. Following extensive screening, the isolated naïve nTregs could be used directly since they express LN-homing receptors and should be able to undergo TCR-mediated maturation in the LNs and target specific peripheral autoimmune responses.

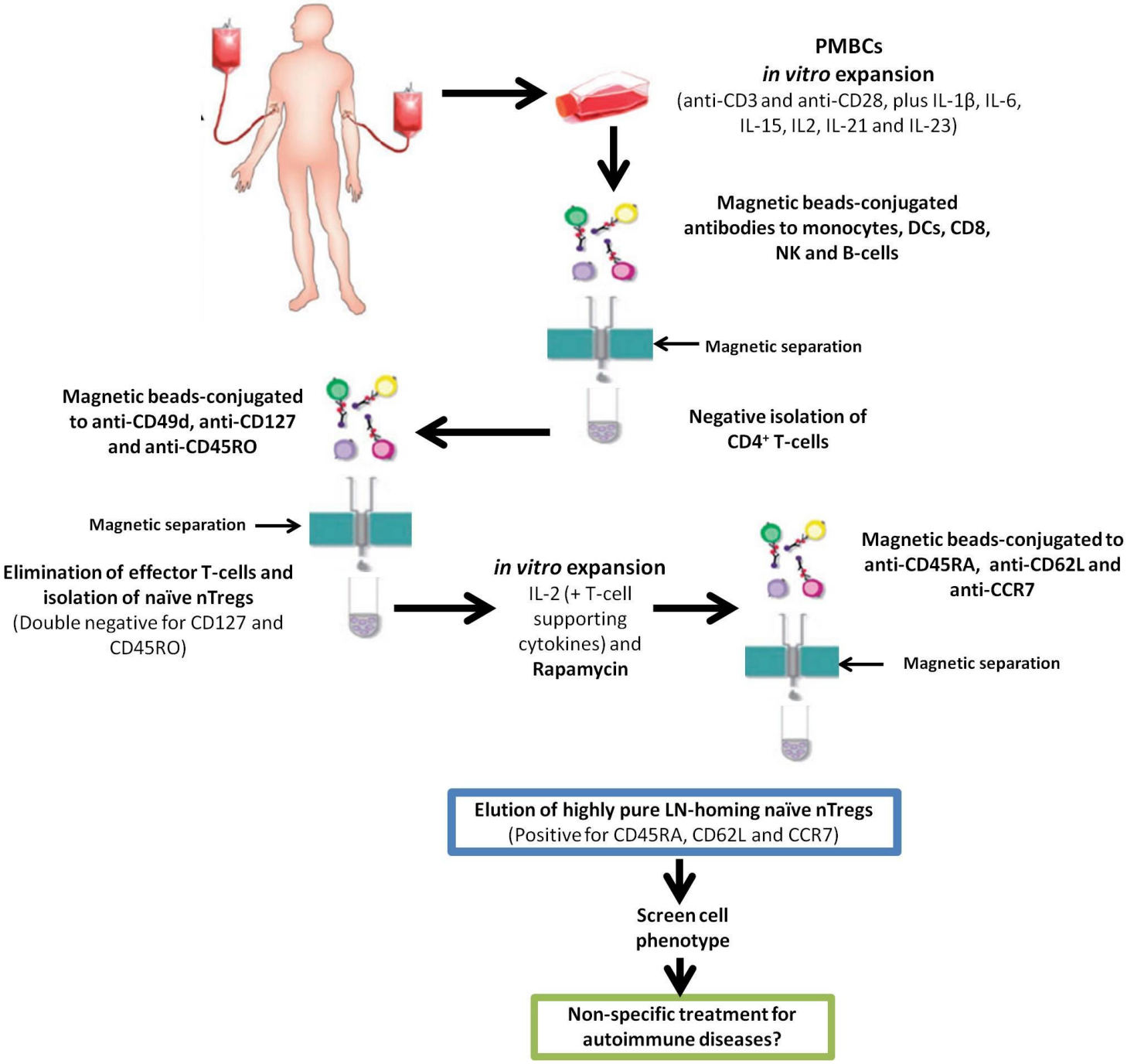

Figure 7. Proposed model for the isolation of a highly pure population of naïve nTregs for adoptive cell therapy. Similar to our previous protocol, peripheral blood will be taken from patients and the peripheral blood mononuclear cells isolated, followed by stimulation for 7 days *in vitro* by anti-CD3/anti-CD28 mABs with a cocktail cytokines including IL-1β, IL-6, IL-2, IL-15, IL-21 and IL-23. This step is vital as it results in the expansion of the FoxP3^{+} T-cells, and the differentiation of potentially pathogenic Tregs into IL-17-producing Tregs ready for elimination. Following expansion, immunomagnetic beads will be used to negatively select and isolate CD4^{+} T-cells. Next, the CD4^{+} T-cell isolate will be stained for bead-conjugated anti-CD45RO and anti-CD127. During this procedure, effector T-cells (CD45RO^{+} CD127^{low}), memory T-cells (CD45RO^{+} CD127^{+}), effector Tregs (CD45RO^{+} CD127$^{low/-}$), iTregs (CD45RO^{+} CD127$^{+/-}$), conventional naïve CD4^{+} T-cells (CD45RO^{-} CD127^{+}) and IL-17 producing cells (CD127^{+}) will be removed, and a population of naïve nTregs isolated by negative selection (double negative for CD45RO and CD127). Afterwards, the proposed naïve Treg isolate will be expanded *in vitro* using IL-2 and associated T-cell cytokines, and treated with Rapamycin to further increase Treg purity and eliminate any non-Tregs that may be present(10,194). Subsequently, to ensure that the isolated naïve Tregs are indeed naïve Tregs and are able to migrate to the LNs the isolated population will be positively selected for the expression of CD45RA, CD62L and CCR7 using immunomagnetic beads. This will result in a highly purified population of naïve nTregs (CD45RO^{-} CD45RA^{+} CD127^{low} CD62L^{+} CCR7^{+} FoxP3^{low}). Diagram adapted from: (4,10).

Future possibilities for autoimmune specific treatment

Since not all autoimmune disease are characterised by the same defects, recent advances in our understanding of Treg migration and stability could be used to tailor isolated naïve nTregs to specific autoimmune diseases (Figure 8). For example, autoimmune diseases with defective Treg function may benefit from isolated naïve nTregs where epigenetic editing has been used to demethylate the CNS2 element and stabilise their FoxP3 expression (195). Furthermore, autoimmune diseases with defective migration may benefit from the use of Tregs which have been genetically modified to express the appropriate tissue-specific CCRs. Moreover, autoimmune diseases with specific Th-associated pathology may benefit from isolated naïve nTregs which have modulated expression of T-bet, IRF4 or STAT3 to target their suppressive functions to specific immune responses. In addition, it may be possible to transgenically express antigen TCRs specific for certain autoimmune diseases, or inhibit pathogenic gene expression by microRNAs. However, these ideas need further development and testing before they become viable treatments for autoimmune disease. Furthermore, additional investigation of autoimmune diseases using the new model of Treg-differentiation is needed to determine the population of Tregs which are defective.

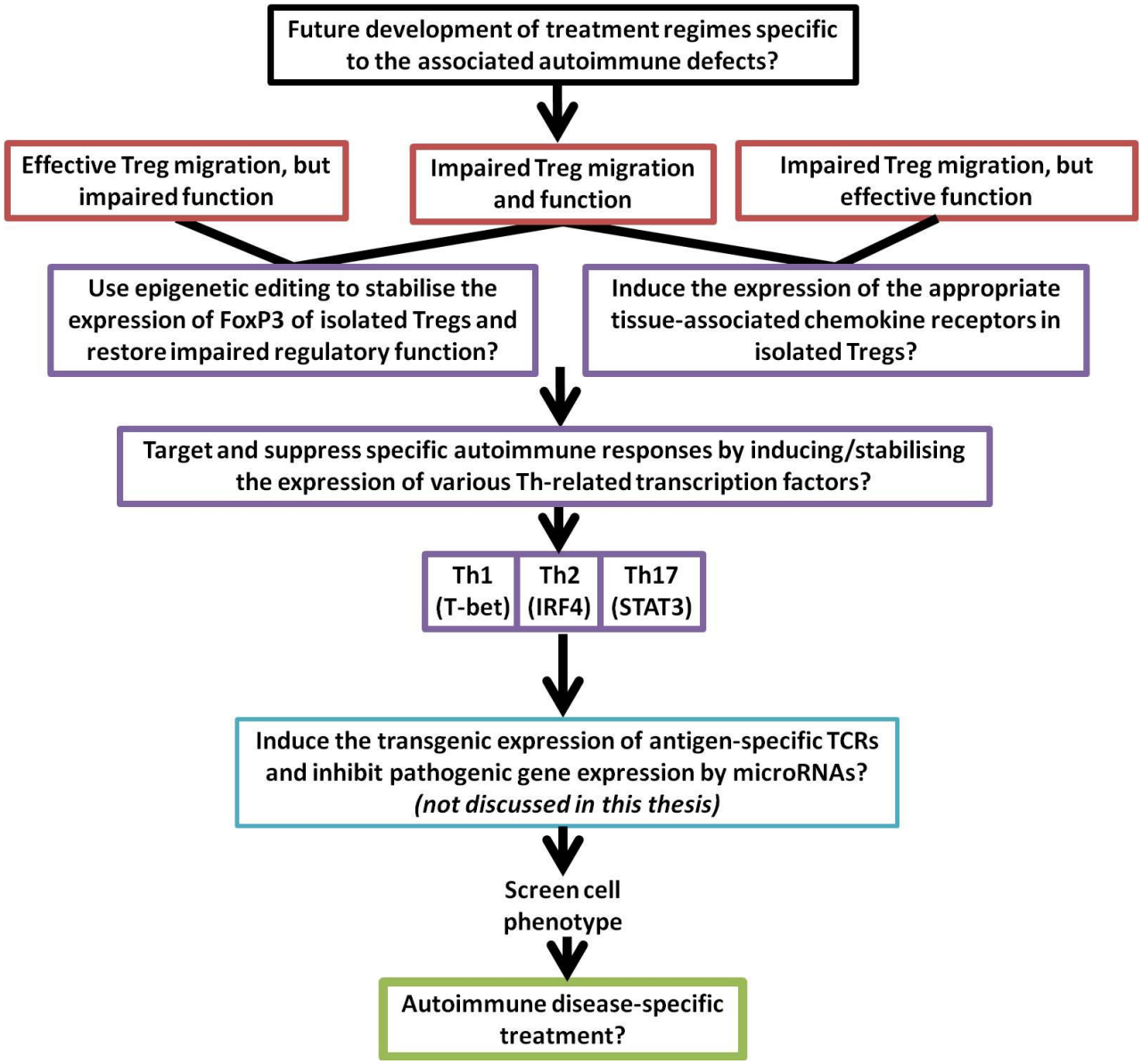

Figure 8. Potential avenues for the development of Treg-based immunotherapy for specific autoimmune diseases.

Summary

Previous research has focused on the use of effector Tregs as potential therapy for autoimmune disease. However, given recent advances in our understanding of Treg differentiation, stability and function, naïve nTregs may be a better choice for sustained regulation of the pathogenic immune responses against self. In this thesis I propose a model for isolating naïve nTregs to be used as adoptive cell therapy for autoimmune disease and highlight potential avenues for the isolation and development autoimmune-specific naïve nTregs.

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Bibliography

(1) Abbas AK, Lichtman AH, Pillai S. Cellular and molecular immunology. 7th ed. Philadelphia, Pa.: Elsevier Saunders; 2012.

(2) Murphy K, Travers P, Walport M, Janeway C. Janeway's immunobiology. 8th ed. New York: Garland Science; 2012.

(3) Piccirillo CA, d'Hennezel E, Sgouroudis E, Yurchenko E. CD4+Foxp3+ regulatory T cells in the control of autoimmunity: in vivo veritas. Curr Opin Immunol 2008 Dec;20(6):655-662.

(4) Abdulahad WH, Boots AM, Kallenberg CG. FoxP3+ CD4+ T cells in systemic autoimmune diseases: the delicate balance between true regulatory T cells and effector Th-17 cells. Rheumatology (Oxford) 2011 Apr;50(4):646-656.

(5) Hori S. Stability of regulatory T-cell lineage. Adv Immunol 2011;112:1-24.

(6) Tian L, Humblet-Baron S, Liston A. Immune tolerance: are regulatory T cell subsets needed to explain suppression of autoimmunity? Bioessays 2012 Jul;34(7):569-575.

(7) Nishizuka Y, Sakakura T. Thymus and reproduction: sex-linked dysgenesia of the gonad after neonatal thymectomy in mice. Science 1969 Nov 7;166(3906):753-755.

(8) Sakaguchi S, Wing K, Miyara M. Regulatory T cells - a brief history and perspective. Eur J Immunol 2007 Nov;37 Suppl 1:S116-23.

(9) Miyara M, Gorochov G, Ehrenstein M, Musset L, Sakaguchi S, Amoura Z. Human FoxP3+ regulatory T cells in systemic autoimmune diseases. Autoimmun Rev 2011 Oct;10(12):744-755.

(10) Sakaguchi S, Miyara M, Costantino CM, Hafler DA. FOXP3+ regulatory T cells in the human immune system. Nat Rev Immunol 2010 Jul;10(7):490-500.

(11) Baecher-Allan C, Brown JA, Freeman GJ, Hafler DA. CD4+CD25high regulatory cells in human peripheral blood. J Immunol 2001 Aug 1;167(3):1245-1253.

(12) Gavin MA, Torgerson TR, Houston E, DeRoos P, Ho WY, Stray-Pedersen A, et al. Single-cell analysis of normal and FOXP3-mutant human T cells: FOXP3 expression without regulatory T cell development. Proc Natl Acad Sci U S A 2006 Apr 25;103(17):6659-6664.

(13) da Silva Martins M, Piccirillo CA. Functional stability of Foxp3+ regulatory T cells. Trends Mol Med 2012 Aug;18(8):454-462.

(14) Lee HM, Bautista JL, Hsieh CS. Thymic and peripheral differentiation of regulatory T cells. Adv Immunol 2011;112:25-71.

(15) Gershon RK, Kondo K. Cell interactions in the induction of tolerance: the role of thymic lymphocytes. Immunology 1970 May;18(5):723-737.

(16) Andersson J, Tran DQ, Pesu M, Davidson TS, Ramsey H, O'Shea JJ, et al. CD4+ FoxP3+ regulatory T cells confer infectious tolerance in a TGF-beta-dependent manner. J Exp Med 2008 Sep 1;205(9):1975-1981.

(17) Qin S, Cobbold S, Pope H, Elliott J, Kioussis D, Davies J, et al. "Infectious" transplantation tolerance. Science 1993 February 12;259(5097):974-977.

(18) Penhale WJ, Farmer A, McKenna RP, Irvine WJ. Spontaneous thyroiditis in thymectomized and irradiated Wistar rats. Clin Exp Immunol 1973 Oct;15(2):225-236.

(19) Sakaguchi S, Fukuma K, Kuribayashi K, Masuda T. Organ-specific autoimmune diseases induced in mice by elimination of T cell subset. I. Evidence for the active participation of T cells in natural self-tolerance; deficit of a T cell subset as a possible cause of autoimmune disease. J Exp Med 1985 Jan 1;161(1):72-87.

(20) Cantor H, Boyse EA. Functional subclasses of T-lymphocytes bearing different Ly antigens. I. The generation of functionally distinct T-cell subclasses is a differentiative process independent of antigen. J Exp Med 1975 Jun 1;141(6):1376-1389.

(21) Green DR, Flood PM, Gershon RK. Immunoregulatory T-cell pathways. Annu Rev Immunol 1983;1:439-463.

(22) Shevach EM. Regulatory T cells in autoimmmunity*. Annu Rev Immunol 2000;18:423-449.

(23) Streilein JW, Niederkorn JY. Characterization of the suppressor cell(s) responsible for anterior chamber-associated immune deviation (ACAID) induced in BALB/c mice by P815 cells. J Immunol 1985 Mar;134(3):1381-1387.

(24) Sakaguchi S, Takahashi T, Nishizuka Y. Study on cellular events in post-thymectomy autoimmune oophoritis in mice. II. Requirement of Lyt-1 cells in normal female mice for the prevention of oophoritis. J Exp Med 1982 Dec 1;156(6):1577-1586.

(25) Sugihara S, Izumi Y, Yoshioka T, Yagi H, Tsujimura T, Tarutani O, et al. Autoimmune thyroiditis induced in mice depleted of particular T cell subsets. I. Requirement of Lyt-1 dull L3T4 bright normal T cells for the induction of thyroiditis. J Immunol 1988 Jul 1;141(1):105-113.

(26) Kronenberg M, Steinmetz M, Kobori J, Kraig E, Kapp JA, Pierce CW, et al. RNA transcripts for I-J polypeptides are apparently not encoded between the I-A and I-E subregions of the murine major histocompatibility complex. Proc Natl Acad Sci U S A 1983 Sep;80(18):5704-5708.

(27) Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. J Immunol 1995 Aug 1;155(3):1151-1164.

(28) Asano M, Toda M, Sakaguchi N, Sakaguchi S. Autoimmune disease as a consequence of developmental abnormality of a T cell subpopulation. J Exp Med 1996 Aug 1;184(2):387-396.

(29) Taams LS, Vukmanovic-Stejic M, Smith J, Dunne PJ, Fletcher JM, Plunkett FJ, et al. Antigen-specific T cell suppression by human CD4+CD25+ regulatory T cells. Eur J Immunol 2002 Jun;32(6):1621-1630.

(30) Levings MK, Sangregorio R, Roncarolo MG. Human cd25(+)cd4(+) t regulatory cells suppress naive and memory T cell proliferation and can be expanded in vitro without loss of function. J Exp Med 2001 Jun 4;193(11):1295-1302.

(31) Ng WF, Duggan PJ, Ponchel F, Matarese G, Lombardi G, Edwards AD, et al. Human CD4(+)CD25(+) cells: a naturally occurring population of regulatory T cells. Blood 2001 Nov 1;98(9):2736-2744.

(32) Jonuleit H, Schmitt E, Stassen M, Tuettenberg A, Knop J, Enk AH. Identification and functional characterization of human CD4(+)CD25(+) T cells with regulatory properties isolated from peripheral blood. J Exp Med 2001 Jun 4;193(11):1285-1294.

(33) Dieckmann D, Plottner H, Berchtold S, Berger T, Schuler G. Ex vivo isolation and characterization of CD4(+)CD25(+) T cells with regulatory properties from human blood. J Exp Med 2001 Jun 4;193(11):1303-1310.

(34) Itoh M, Takahashi T, Sakaguchi N, Kuniyasu Y, Shimizu J, Otsuka F, et al. Thymus and autoimmunity: production of CD25+CD4+ naturally anergic and suppressive T cells as a key function of the thymus in maintaining immunologic self-tolerance. J Immunol 1999 May 1;162(9):5317-5326.

(35) Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nat Immunol 2003 Apr;4(4):330-336.

(36) Fontenot JD, Rasmussen JP, Williams LM, Dooley JL, Farr AG, Rudensky AY. Regulatory T cell lineage specification by the forkhead transcription factor foxp3. Immunity 2005 Mar;22(3):329-341.

(37) Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. Science 2003 Feb 14;299(5609):1057-1061.

(38) Khattri R, Cox T, Yasayko SA, Ramsdell F. An essential role for Scurfin in CD4+CD25+ T regulatory cells. Nat Immunol 2003 Apr;4(4):337-342.

(39) Wan YY, Flavell RA. Identifying Foxp3-expressing suppressor T cells with a bicistronic reporter. Proc Natl Acad Sci U S A 2005 Apr 5;102(14):5126-5131.

(40) Sakaguchi S. Regulatory T cells: history and perspective. Methods Mol Biol 2011;707:3-17.

(41) Brunkow ME, Jeffery EW, Hjerrild KA, Paeper B, Clark LB, Yasayko SA, et al. Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. Nat Genet 2001 Jan;27(1):68-73.

(42) d'Hennezel E, Bin Dhuban K, Torgerson T, Piccirillo C. The immunogenetics of immune dysregulation, polyendocrinopathy, enteropathy, X linked (IPEX) syndrome. J Med Genet 2012 May;49(5):291-302.

(43) Wildin RS, Ramsdell F, Peake J, Faravelli F, Casanova JL, Buist N, et al. X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy. Nat Genet 2001 Jan;27(1):18-20.

(44) Chatila TA, Blaeser F, Ho N, Lederman HM, Voulgaropoulos C, Helms C, et al. JM2, encoding a fork

head-related protein, is mutated in X-linked autoimmunity-allergic disregulation syndrome. J Clin Invest 2000 Dec;106(12):R75-81.

(45) Bennett CL, Christie J, Ramsdell F, Brunkow ME, Ferguson PJ, Whitesell L, et al. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. Nat Genet 2001 Jan;27(1):20-21.

(46) Roncador G, Brown PJ, Maestre L, Hue S, Martinez-Torrecuadrada JL, Ling KL, et al. Analysis of FOXP3 protein expression in human CD4+CD25+ regulatory T cells at the single-cell level. Eur J Immunol 2005 Jun;35(6):1681-1691.

(47) Fontenot JD, Rudensky AY. A well adapted regulatory contrivance: regulatory T cell development and the forkhead family transcription factor Foxp3. Nat Immunol 2005 Apr;6(4):331-337.

(48) Hori S, Sakaguchi S. Foxp3: a critical regulator of the development and function of regulatory T cells. Microbes Infect 2004 Jul;6(8):745-751.

(49) Modigliani Y, Bandeira A, Coutinho A. A model for developmentally acquired thymus-dependent tolerance to central and peripheral antigens. Immunol Rev 1996 Feb;149:155-120.

(50) Jordan MS, Boesteanu A, Reed AJ, Petrone AL, Holenbeck AE, Lerman MA, et al. Thymic selection of CD4+CD25+ regulatory T cells induced by an agonist self-peptide. Nat Immunol 2001 Apr;2(4):301-306.

(51) Kawahata K, Misaki Y, Yamauchi M, Tsunekawa S, Setoguchi K, Miyazaki J, et al. Generation of CD4(+)CD25(+) regulatory T cells from autoreactive T cells simultaneously with their negative selection in the thymus and from nonautoreactive T cells by endogenous TCR expression. J Immunol 2002 May 1;168(9):4399-4405.

(52) Lohr J, Knoechel B, Jiang S, Sharpe AH, Abbas AK. The inhibitory function of B7 costimulators in T cell responses to foreign and self-antigens. Nat Immunol 2003 Jul;4(7):664-669.

(53) Apostolou I, Sarukhan A, Klein L, von Boehmer H. Origin of regulatory T cells with known specificity for antigen. Nat Immunol 2002 Aug;3(8):756-763.

(54) Olivares-Villagomez D, Wensky AK, Wang Y, Lafaille JJ. Repertoire requirements of CD4+ T cells that prevent spontaneous autoimmune encephalomyelitis. J Immunol 2000 May 15;164(10):5499-5507.

(55) Bensinger SJ, Bandeira A, Jordan MS, Caton AJ, Laufer TM. Major histocompatibility complex class II-positive cortical epithelium mediates the selection of CD4(+)25(+) immunoregulatory T cells. J Exp Med 2001 Aug 20;194(4):427-438.

(56) Liston A, Lesage S, Wilson J, Peltonen L, Goodnow CC. Aire regulates negative selection of organ-specific T cells. Nat Immunol 2003 Apr;4(4):350-354.

(57) van Santen HM, Benoist C, Mathis D. Number of T reg cells that differentiate does not increase upon encounter of agonist ligand on thymic epithelial cells. J Exp Med 2004 Nov 15;200(10):1221-1230.

(58) Pacholczyk R, Kern J, Singh N, Iwashima M, Kraj P, Ignatowicz L. Nonself-antigens are the cognate specificities of Foxp3+ regulatory T cells. Immunity 2007 Sep;27(3):493-504.

(59) Pennington DJ, Silva-Santos B, Silberzahn T, Escorcio-Correia M, Woodward MJ, Roberts SJ, et al. Early events in the thymus affect the balance of effector and regulatory T cells. Nature 2006 Dec 21;444(7122):1073-1077.

(60) Fontenot JD, Dooley JL, Farr AG, Rudensky AY. Developmental regulation of Foxp3 expression during ontogeny. J Exp Med 2005 Oct 3;202(7):901-906.

(61) Aschenbrenner K, D'Cruz LM, Vollmann EH, Hinterberger M, Emmerich J, Swee LK, et al. Selection of Foxp3+ regulatory T cells specific for self antigen expressed and presented by Aire+ medullary thymic epithelial cells. Nat Immunol 2007 Apr;8(4):351-358.

(62) Hinterberger M, Aichinger M, Prazeres da Costa O, Voehringer D, Hoffmann R, Klein L. Autonomous role of medullary thymic epithelial cells in central CD4(+) T cell tolerance. Nat Immunol 2010 Jun;11(6):512-519.

(63) Moran AE, Holzapfel KL, Xing Y, Cunningham NR, Maltzman JS, Punt J, et al. T cell receptor signal strength in Treg and iNKT cell development demonstrated by a novel fluorescent reporter mouse. J Exp Med 2011 Jun 6;208(6):1279-1289.

(64) Isomura I, Palmer S, Grumont RJ, Bunting K, Hoyne G, Wilkinson N, et al. c-Rel is required for the development of thymic Foxp3+ CD4 regulatory T cells. J Exp Med 2009 Dec 21;206(13):3001-3014.

(65) Ruan Q, Kameswaran V, Tone Y, Li L, Liou HC, Greene MI, et al. Development of Foxp3(+) regulatory t cells is driven by the c-Rel enhanceosome. Immunity 2009 Dec 18;31(6):932-940.

(66) Zheng Y, Josefowicz S, Chaudhry A, Peng XP, Forbush K, Rudensky AY. Role of conserved non-coding DNA elements

in the Foxp3 gene in regulatory T-cell fate. Nature 2010 Feb 11;463(7282):808-812.

(67) Horwitz DA, Zheng SG, Gray JD. Natural and TGF-beta-induced Foxp3(+)CD4(+) CD25(+) regulatory T cells are not mirror images of each other. Trends Immunol 2008 Sep;29(9):429-435.

(68) Chen Y, Kuchroo VK, Inobe J, Hafler DA, Weiner HL. Regulatory T cell clones induced by oral tolerance: suppression of autoimmune encephalomyelitis. Science 1994 Aug 26;265(5176):1237-1240.

(69) Sundstedt A, O'Neill EJ, Nicolson KS, Wraith DC. Role for IL-10 in suppression mediated by peptide-induced regulatory T cells in vivo. J Immunol 2003 Feb 1;170(3):1240-1248.

(70) Groux H, O'Garra A, Bigler M, Rouleau M, Antonenko S, de Vries JE, et al. A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. Nature 1997 Oct 16;389(6652):737-742.

(71) Belkaid Y, Oldenhove G. Tuning microenvironments: induction of regulatory T cells by dendritic cells. Immunity 2008 Sep 19;29(3):362-371.

(72) Coombes JL, Siddiqui KR, Arancibia-Carcamo CV, Hall J, Sun CM, Belkaid Y, et al. A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. J Exp Med 2007 Aug 6;204(8):1757-1764.

(73) Haribhai D, Williams JB, Jia S, Nickerson D, Schmitt EG, Edwards B, et al. A requisite role for induced regulatory T cells in tolerance based on expanding antigen receptor diversity. Immunity 2011 Jul 22;35(1):109-122.

(74) Floess S, Freyer J, Siewert C, Baron U, Olek S, Polansky J, et al. Epigenetic control of the foxp3 locus in regulatory T cells. PLoS Biol 2007 Feb;5(2):e38.

(75) Selvaraj RK, Geiger TL. A kinetic and dynamic analysis of Foxp3 induced in T cells by TGF-beta. J Immunol 2007 Jun 15;178(12):7667-7677.

(76) Nishio J, Feuerer M, Wong J, Mathis D, Benoist C. Anti-CD3 therapy permits regulatory T cells to surmount T cell receptor-specified peripheral niche constraints. J Exp Med 2010 Aug 30;207(9):1879-1889.

(77) Polansky JK, Kretschmer K, Freyer J, Floess S, Garbe A, Baron U, et al. DNA methylation controls Foxp3 gene expression. Eur J Immunol 2008 Jun;38(6):1654-1663.

(78) Ding Y, Xu J, Bromberg JS. Regulatory T cell migration during an immune response. Trends Immunol 2012 Apr;33(4):174-180.

(79) Forster R, Davalos-Misslitz AC, Rot A. CCR7 and its ligands: balancing immunity and tolerance. Nat Rev Immunol 2008 May;8(5):362-371.

(80) MacDonald KG, Orban PC, Levings MK. T regulatory cell therapy in transplantation: stability, localization and functional specialization. Curr Opin Organ Transplant 2012 Aug;17(4):343-348.

(81) Fisson S, Darrasse-Jeze G, Litvinova E, Septier F, Klatzmann D, Liblau R, et al. Continuous activation of autoreactive CD4+ CD25+ regulatory T cells in the steady state. J Exp Med 2003 Sep 1;198(5):737-746.

(82) Wing K, Larsson P, Sandstrom K, Lundin SB, Suri-Payer E, Rudin A. CD4+ CD25+ FOXP3+ regulatory T cells from human thymus and cord blood suppress antigen-specific T cell responses. Immunology 2005 Aug;115(4):516-525.

(83) Seddiki N, Santner-Nanan B, Tangye SG, Alexander SI, Solomon M, Lee S, et al. Persistence of naive CD45RA+ regulatory T cells in adult life. Blood 2006 Apr 1;107(7):2830-2838.

(84) Valmori D, Merlo A, Souleimanian NE, Hesdorffer CS, Ayyoub M. A peripheral circulating compartment of natural naive CD4 Tregs. J Clin Invest 2005 Jul;115(7):1953-1962.

(85) Fritzsching B, Oberle N, Pauly E, Geffers R, Buer J, Poschl J, et al. Naive regulatory T cells: a novel subpopulation defined by resistance toward CD95L-mediated cell death. Blood 2006 Nov 15;108(10):3371-3378.

(86) Booth NJ, McQuaid AJ, Sobande T, Kissane S, Agius E, Jackson SE, et al. Different proliferative potential and migratory characteristics of human CD4+ regulatory T cells that express either CD45RA or CD45RO. J Immunol 2010 Apr 15;184(8):4317-4326.

(87) Grindebacke H, Stenstad H, Quiding-Jarbrink M, Waldenstrom J, Adlerberth I, Wold AE, et al. Dynamic development of homing receptor expression and memory cell differentiation of infant CD4+CD25high regulatory T cells. J Immunol 2009 Oct 1;183(7):4360-4370.

(88) Ermann J, Hoffmann P, Edinger M, Dutt S, Blankenberg FG, Higgins JP, et al. Only the CD62L+ subpopulation of CD4+CD25+ regulatory T cells protects from lethal acute GVHD. Blood 2005 Mar 1;105(5):2220-2226.

(89) Huehn J, Siegmund K, Lehmann JC, Siewert C, Haubold U, Feuerer M, et al. Developmental stage, phenotype, and

migration distinguish naive- and effector/memory-like CD4+ regulatory T cells. J Exp Med 2004 Feb 2;199(3):303-313.

(90) Kocks JR, Davalos-Misslitz AC, Hintzen G, Ohl L, Forster R. Regulatory T cells interfere with the development of bronchus-associated lymphoid tissue. J Exp Med 2007 Apr 16;204(4):723-734.

(91) Wei S, Kryczek I, Zou W. Regulatory T-cell compartmentalization and trafficking. Blood 2006 Jul 15;108(2):426-431.

(92) Campbell DJ, Koch MA. Phenotypical and functional specialization of FOXP3+ regulatory T cells. Nat Rev Immunol 2011 Feb;11(2):119-130.

(93) Duhen T, Duhen R, Lanzavecchia A, Sallusto F, Campbell DJ. Functionally distinct subsets of human FOXP3+ Treg cells that phenotypically mirror effector Th cells. Blood 2012 May 10;119(19):4430-4440.

(94) Koch MA, Tucker-Heard G, Perdue NR, Killebrew JR, Urdahl KB, Campbell DJ. The transcription factor T-bet controls regulatory T cell homeostasis and function during type 1 inflammation. Nat Immunol 2009 Jun;10(6):595-602.

(95) Szabo SJ, Kim ST, Costa GL, Zhang X, Fathman CG, Glimcher LH. A novel transcription factor, T-bet, directs Th1 lineage commitment. Cell 2000 Mar 17;100(6):655-669.

(96) Zheng Y, Chaudhry A, Kas A, deRoos P, Kim JM, Chu TT, et al. Regulatory T-cell suppressor program co-opts transcription factor IRF4 to control T(H)2 responses. Nature 2009 Mar 19;458(7236):351-356.

(97) Chaudhry A, Samstein RM, Treuting P, Liang Y, Pils MC, Heinrich JM, et al. Interleukin-10 signaling in regulatory T cells is required for suppression of Th17 cell-mediated inflammation. Immunity 2011 Apr 22;34(4):566-578.

(98) Fallarino F, Grohmann U, You S, McGrath BC, Cavener DR, Vacca C, et al. The combined effects of tryptophan starvation and tryptophan catabolites down-regulate T cell receptor zeta-chain and induce a regulatory phenotype in naive T cells. J Immunol 2006 Jun 1;176(11):6752-6761.

(99) Huang CT, Workman CJ, Flies D, Pan X, Marson AL, Zhou G, et al. Role of LAG-3 in regulatory T cells. Immunity 2004 Oct;21(4):503-513.

(100) Liang B, Workman C, Lee J, Chew C, Dale BM, Colonna L, et al. Regulatory T cells inhibit dendritic cells by lymphocyte activation gene-3 engagement of MHC class II. J Immunol 2008 May 1;180(9):5916-5926.

(101) Deaglio S, Dwyer KM, Gao W, Friedman D, Usheva A, Erat A, et al. Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. J Exp Med 2007 Jun 11;204(6):1257-1265.

(102) Borsellino G, Kleinewietfeld M, Di Mitri D, Sternjak A, Diamantini A, Giometto R, et al. Expression of ectonucleotidase CD39 by Foxp3+ Treg cells: hydrolysis of extracellular ATP and immune suppression. Blood 2007 Aug 15;110(4):1225-1232.

(103) Kobie JJ, Shah PR, Yang L, Rebhahn JA, Fowell DJ, Mosmann TR. T regulatory and primed uncommitted CD4 T cells express CD73, which suppresses effector CD4 T cells by converting 5'-adenosine monophosphate to adenosine. J Immunol 2006 Nov 15;177(10):6780-6786.

(104) Grossman WJ, Verbsky JW, Barchet W, Colonna M, Atkinson JP, Ley TJ. Human T regulatory cells can use the perforin pathway to cause autologous target cell death. Immunity 2004 Oct;21(4):589-601.

(105) Gondek DC, Lu LF, Quezada SA, Sakaguchi S, Noelle RJ. Cutting edge: contact-mediated suppression by CD4+CD25+ regulatory cells involves a granzyme B-dependent, perforin-independent mechanism. J Immunol 2005 Feb 15;174(4):1783-1786.

(106) Nakamura K, Kitani A, Fuss I, Pedersen A, Harada N, Nawata H, et al. TGF-beta 1 plays an important role in the mechanism of CD4+CD25+ regulatory T cell activity in both humans and mice. J Immunol 2004 Jan 15;172(2):834-842.

(107) Venturi GM, Conway RM, Steeber DA, Tedder TF. CD25+CD4+ regulatory T cell migration requires L-selectin expression: L-selectin transcriptional regulation balances constitutive receptor turnover. J Immunol 2007 Jan 1;178(1):291-300.

(108) Szanya V, Ermann J, Taylor C, Holness C, Fathman CG. The subpopulation of CD4+CD25+ splenocytes that delays adoptive transfer of diabetes expresses L-selectin and high levels of CCR7. J Immunol 2002 Sep 1;169(5):2461-2465.

(109) Schneider MA, Meingassner JG, Lipp M, Moore HD, Rot A. CCR7 is required for the in vivo function of CD4+ CD25+ regulatory T cells. J Exp Med 2007 Apr 16;204(4):735-745.

(110) Zhang N, Schroppel B, Lal G, Jakubzick C, Mao X, Chen D, et al. Regulatory T cells sequentially migrate from inflamed tissues to draining lymph nodes to suppress the alloimmune response. Immunity 2009 Mar 20;30(3):458-469.

(111) Hirahara K, Liu L, Clark RA, Yamanaka K, Fuhlbrigge RC, Kupper TS. The majority of human peripheral blood CD4+CD25highFoxp3+ regulatory T cells bear functional

skin-homing receptors. J Immunol 2006 Oct 1;177(7):4488-4494.

(112) Siegmund K, Feuerer M, Siewert C, Ghani S, Haubold U, Dankof A, et al. Migration matters: regulatory T-cell compartmentalization determines suppressive activity in vivo. Blood 2005 Nov 1;106(9):3097-3104.

(113) Dudda JC, Perdue N, Bachtanian E, Campbell DJ. Foxp3+ regulatory T cells maintain immune homeostasis in the skin. J Exp Med 2008 Jul 7;205(7):1559-1565.

(114) Suffia I, Reckling SK, Salay G, Belkaid Y. A role for CD103 in the retention of CD4+CD25+ Treg and control of Leishmania major infection. J Immunol 2005 May 1;174(9):5444-5455.

(115) Denning TL, Kim G, Kronenberg M. Cutting edge: CD4+CD25+ regulatory T cells impaired for intestinal homing can prevent colitis. J Immunol 2005 Jun 15;174(12):7487-7491.

(116) Lee JH, Kang SG, Kim CH. FoxP3+ T cells undergo conventional first switch to lymphoid tissue homing receptors in thymus but accelerated second switch to nonlymphoid tissue homing receptors in secondary lymphoid tissues. J Immunol 2007 Jan 1;178(1):301-311.

(117) Lim HW, Lee J, Hillsamer P, Kim CH. Human Th17 cells share major trafficking receptors with both polarized effector T cells and FOXP3+ regulatory T cells. J Immunol 2008 Jan 1;180(1):122-129.

(118) Sather BD, Treuting P, Perdue N, Miazgowicz M, Fontenot JD, Rudensky AY, et al. Altering the distribution of Foxp3(+) regulatory T cells results in tissue-specific inflammatory disease. J Exp Med 2007 Jun 11;204(6):1335-1347.

(119) Yuan Q, Bromley SK, Means TK, Jones KJ, Hayashi F, Bhan AK, et al. CCR4-dependent regulatory T cell function in inflammatory bowel disease. J Exp Med 2007 Jun 11;204(6):1327-1334.

(120) Kang SG, Piniecki RJ, Hogenesch H, Lim HW, Wiebke E, Braun SE, et al. Identification of a chemokine network that recruits FoxP3(+) regulatory T cells into chronically inflamed intestine. Gastroenterology 2007 Mar;132(3):966-981.

(121) Yurchenko E, Tritt M, Hay V, Shevach EM, Belkaid Y, Piccirillo CA. CCR5-dependent homing of naturally occurring CD4+ regulatory T cells to sites of Leishmania major infection favors pathogen persistence. J Exp Med 2006 Oct 30;203(11):2451-2460.

(122) Kleinewietfeld M, Puentes F, Borsellino G, Battistini L, Rotzschke O, Falk K. CCR6 expression defines regulatory effector/memory-like cells within the CD25(+)CD4+ T-cell subset. Blood 2005 Apr 1;105(7):2877-2886.

(123) Hirota K, Yoshitomi H, Hashimoto M, Maeda S, Teradaira S, Sugimoto N, et al. Preferential recruitment of CCR6-expressing Th17 cells to inflamed joints via CCL20 in rheumatoid arthritis and its animal model. J Exp Med 2007 Nov 26;204(12):2803-2812.

(124) Yamazaki T, Yang XO, Chung Y, Fukunaga A, Nurieva R, Pappu B, et al. CCR6 regulates the migration of inflammatory and regulatory T cells. J Immunol 2008 Dec 15;181(12):8391-8401.

(125) Soler D, Chapman TR, Poisson LR, Wang L, Cote-Sierra J, Ryan M, et al. CCR8 expression identifies CD4 memory T cells enriched for FOXP3+ regulatory and Th2 effector lymphocytes. J Immunol 2006 Nov 15;177(10):6940-6951.

(126) Guo Z, Jang MH, Otani K, Bai Z, Umemoto E, Matsumoto M, et al. CD4+CD25+ regulatory T cells in the small intestinal lamina propria show an effector/memory phenotype. Int Immunol 2008 Mar;20(3):307-315.

(127) Eksteen B, Miles A, Curbishley SM, Tselepis C, Grant AJ, Walker LS, et al. Epithelial inflammation is associated with CCL28 production and the recruitment of regulatory T cells expressing CCR10. J Immunol 2006 Jul 1;177(1):593-603.

(128) Santodomingo-Garzon T, Han J, Le T, Yang Y, Swain MG. Natural killer T cells regulate the homing of chemokine CXC receptor 3-positive regulatory T cells to the liver in mice. Hepatology 2009 Apr;49(4):1267-1276.

(129) Oo YH, Weston CJ, Lalor PF, Curbishley SM, Withers DR, Reynolds GM, et al. Distinct roles for CCR4 and CXCR3 in the recruitment and positioning of regulatory T cells in the inflamed human liver. J Immunol 2010 Mar 15;184(6):2886-2898.

(130) Lim HW, Broxmeyer HE, Kim CH. Regulation of trafficking receptor expression in human forkhead box P3+ regulatory T cells. J Immunol 2006 Jul 15;177(2):840-851.

(131) Lim HW, Hillsamer P, Kim CH. Regulatory T cells can migrate to follicles upon T cell activation and suppress GC-Th cells and GC-Th cell-driven B cell responses. J Clin Invest 2004 Dec;114(11):1640-1649.

(132) Grauer OM, Nierkens S, Bennink E, Toonen LW, Boon L, Wesseling P, et al. CD4+FoxP3+ regulatory T cells gradually accumulate in gliomas during tumor growth and efficiently suppress antiglioma immune responses in vivo. Int J Cancer 2007 Jul 1;121(1):95-105.

(133) Wald O, Izhar U, Amir G, Avniel S, Bar-Shavit Y, Wald H, et al. CD4+CXCR4highCD69+ T cells accumulate in lung adenocarcinoma. J Immunol 2006 Nov 15;177(10):6983-6990.

(134) Lahl K, Loddenkemper C, Drouin C, Freyer J, Arnason J, Eberl G, et al. Selective depletion of Foxp3+ regulatory T cells induces a scurfy-like disease. J Exp Med 2007 Jan 22;204(1):57-63.

(135) Miyara M, Yoshioka Y, Kitoh A, Shima T, Wing K, Niwa A, et al. Functional delineation and differentiation dynamics of human CD4+ T cells expressing the FoxP3 transcription factor. Immunity 2009 Jun 19;30(6):899-911.

(136) Allan SE, Crome SQ, Crellin NK, Passerini L, Steiner TS, Bacchetta R, et al. Activation-induced FOXP3 in human T effector cells does not suppress proliferation or cytokine production. Int Immunol 2007 Apr;19(4):345-354.

(137) Liu W, Putnam AL, Xu-Yu Z, Szot GL, Lee MR, Zhu S, et al. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. J Exp Med 2006 Jul 10;203(7):1701-1711.

(138) Seddiki N, Santner-Nanan B, Martinson J, Zaunders J, Sasson S, Landay A, et al. Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. J Exp Med 2006 Jul 10;203(7):1693-1700.

(139) Mazzucchelli R, Durum SK. Interleukin-7 receptor expression: intelligent design. Nat Rev Immunol 2007 Feb;7(2):144-154.

(140) Yamaguchi T, Hirota K, Nagahama K, Ohkawa K, Takahashi T, Nomura T, et al. Control of immune responses by antigen-specific regulatory T cells expressing the folate receptor. Immunity 2007 Jul;27(1):145-159.

(141) Mandapathil M, Lang S, Gorelik E, Whiteside TL. Isolation of functional human regulatory T cells (Treg) from the peripheral blood based on the CD39 expression. J Immunol Methods 2009 Jul 31;346(1-2):55-63.

(142) Tran DQ, Andersson J, Hardwick D, Bebris L, Illei GG, Shevach EM. Selective expression of latency-associated peptide (LAP) and IL-1 receptor type I/II (CD121a/CD121b) on activated human FOXP3+ regulatory T cells allows for their purification from expansion cultures. Blood 2009 May 21;113(21):5125-5133.

(143) Gao Y, Lin F, Su J, Gao Z, Li Y, Yang J, et al. Molecular mechanisms underlying the regulation and functional plasticity of FOXP3(+) regulatory T cells. Genes Immun 2012 Jan;13(1):1-13.

(144) Cobb BS, Hertweck A, Smith J, O'Connor E, Graf D, Cook T, et al. A role for Dicer in immune regulation. J Exp Med 2006 Oct 30;203(11):2519-2527.

(145) Chong MM, Rasmussen JP, Rudensky AY, Littman DR. The RNAseIII enzyme Drosha is critical in T cells for preventing lethal inflammatory disease. J Exp Med 2008 Sep 1;205(9):2005-2017.

(146) Liston A, Lu LF, O'Carroll D, Tarakhovsky A, Rudensky AY. Dicer-dependent microRNA pathway safeguards regulatory T cell function. J Exp Med 2008 Sep 1;205(9):1993-2004.

(147) Zhou X, Jeker LT, Fife BT, Zhu S, Anderson MS, McManus MT, et al. Selective miRNA disruption in T reg cells leads to uncontrolled autoimmunity. J Exp Med 2008 Sep 1;205(9):1983-1991.

(148) McClymont SA, Putnam AL, Lee MR, Esensten JH, Liu W, Hulme MA, et al. Plasticity of human regulatory T cells in healthy subjects and patients with type 1 diabetes. J Immunol 2011 Apr 1;186(7):3918-3926.

(149) Dominguez-Villar M, Baecher-Allan CM, Hafler DA. Identification of T helper type 1-like, Foxp3+ regulatory T cells in human autoimmune disease. Nat Med 2011 Jun;17(6):673-675.

(150) Beriou G, Costantino CM, Ashley CW, Yang L, Kuchroo VK, Baecher-Allan C, et al. IL-17-producing human peripheral regulatory T cells retain suppressive function. Blood 2009 Apr 30;113(18):4240-4249.

(151) Marwaha AK, Crome SQ, Panagiotopoulos C, Berg KB, Qin H, Ouyang Q, et al. Cutting edge: Increased IL-17-secreting T cells in children with new-onset type 1 diabetes. J Immunol 2010 Oct 1;185(7):3814-3818.

(152) Zhou X, Bailey-Bucktrout S, Jeker LT, Bluestone JA. Plasticity of CD4(+) FoxP3(+) T cells. Curr Opin Immunol 2009 Jun;21(3):281-285.

(153) Hori S. Developmental plasticity of Foxp3+ regulatory T cells. Curr Opin Immunol 2010 Oct;22(5):575-582.

(154) Bailey-Bucktrout SL, Bluestone JA. Regulatory T cells: stability revisited. Trends Immunol 2011 Jul;32(7):301-306.

(155) Lal G, Bromberg JS. Epigenetic mechanisms of regulation of Foxp3 expression. Blood 2009 Oct 29;114(18):3727-3735.

(156) Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, Oukka M, et al. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. Nature 2006 May 11;441(7090):235-238.

(157) Hippen KL, Merkel SC, Schirm DK, Nelson C, Tennis NC, Riley JL, et al. Generation and large-scale expansion of human inducible regulatory T cells that suppress graft-versus-host disease. Am J Transplant 2011 Jun;11(6):1148-1157.

(158) Komatsu N, Mariotti-Ferrandiz ME, Wang Y, Malissen B, Waldmann H, Hori S. Heterogeneity of natural Foxp3+ T cells: a committed regulatory T-cell lineage and an uncommitted minor population retaining plasticity. Proc Natl Acad Sci U S A 2009 Feb 10;106(6):1903-1908.

(159) Hoffmann P, Boeld TJ, Eder R, Huehn J, Floess S, Wieczorek G, et al. Loss of FOXP3 expression in natural human CD4+CD25+ regulatory T cells upon repetitive in vitro stimulation. Eur J Immunol 2009 Apr;39(4):1088-1097.

(160) Voo KS, Wang YH, Santori FR, Boggiano C, Wang YH, Arima K, et al. Identification of IL-17-producing FOXP3+ regulatory T cells in humans. Proc Natl Acad Sci U S A 2009 Mar 24;106(12):4793-4798.

(161) d'Hennezel E, Yurchenko E, Sgouroudis E, Hay V, Piccirillo CA. Single-cell analysis of the human T regulatory population uncovers functional heterogeneity and instability within FOXP3+ cells. J Immunol 2011 Jun 15;186(12):6788-6797.

(162) Koenen HJ, Smeets RL, Vink PM, van Rijssen E, Boots AM, Joosten I. Human CD25highFoxp3pos regulatory T cells differentiate into IL-17-producing cells. Blood 2008 Sep 15;112(6):2340-2352.

(163) Ayyoub M, Deknuydt F, Raimbaud I, Dousset C, Leveque L, Bioley G, et al. Human memory FOXP3+ Tregs secrete IL-17 ex vivo and constitutively express the T(H)17 lineage-specific transcription factor RORgamma t. Proc Natl Acad Sci U S A 2009 May 26;106(21):8635-8640.

(164) Burgler S, Mantel PY, Bassin C, Ouaked N, Akdis CA, Schmidt-Weber CB. RORC2 is involved in T cell polarization through interaction with the FOXP3 promoter. J Immunol 2010 Jun 1;184(11):6161-6169.

(165) Tsuji M, Komatsu N, Kawamoto S, Suzuki K, Kanagawa O, Honjo T, et al. Preferential generation of follicular B helper T cells from Foxp3+ T cells in gut Peyer's patches. Science 2009 Mar 13;323(5920):1488-1492.

(166) Yang XO, Nurieva R, Martinez GJ, Kang HS, Chung Y, Pappu BP, et al. Molecular antagonism and plasticity of regulatory and inflammatory T cell programs. Immunity 2008 Jul 18;29(1):44-56.

(167) Ferretti S, Bonneau O, Dubois GR, Jones CE, Trifilieff A. IL-17, produced by lymphocytes and neutrophils, is necessary for lipopolysaccharide-induced airway neutrophilia: IL-15 as a possible trigger. J Immunol 2003 Feb 15;170(4):2106-2112.

(168) Murai M, Turovskaya O, Kim G, Madan R, Karp CL, Cheroutre H, et al. Interleukin 10 acts on regulatory T cells to maintain expression of the transcription factor Foxp3 and suppressive function in mice with colitis. Nat Immunol 2009 Nov;10(11):1178-1184.

(169) Du J, Huang C, Zhou B, Ziegler SF. Isoform-specific inhibition of ROR alpha-mediated transcriptional activation by human FOXP3. J Immunol 2008 Apr 1;180(7):4785-4792.

(170) Pallandre JR, Brillard E, Crehange G, Radlovic A, Remy-Martin JP, Saas P, et al. Role of STAT3 in CD4+CD25+FOXP3+ regulatory lymphocyte generation: implications in graft-versus-host disease and antitumor immunity. J Immunol 2007 Dec 1;179(11):7593-7604.

(171) Chaudhry A, Rudra D, Treuting P, Samstein RM, Liang Y, Kas A, et al. CD4+ regulatory T cells control TH17 responses in a Stat3-dependent manner. Science 2009 Nov 13;326(5955):986-991.

(172) Chung Y, Tanaka S, Chu F, Nurieva RI, Martinez GJ, Rawal S, et al. Follicular regulatory T cells expressing Foxp3 and Bcl-6 suppress germinal center reactions. Nat Med 2011 Jul 24;17(8):983-988.

(173) Cretney E, Xin A, Shi W, Minnich M, Masson F, Miasari M, et al. The transcription factors Blimp-1 and IRF4 jointly control the differentiation and function of effector regulatory T cells. Nat Immunol 2011 Apr;12(4):304-311.

(174) Murphy CA, Langrish CL, Chen Y, Blumenschein W, McClanahan T, Kastelein RA, et al. Divergent pro- and antiinflammatory roles for IL-23 and IL-12 in joint autoimmune inflammation. J Exp Med 2003 Dec 15;198(12):1951-1957.

(175) Cua DJ, Sherlock J, Chen Y, Murphy CA, Joyce B, Seymour B, et al. Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. Nature 2003 Feb 13;421(6924):744-748.

(176) Hansmann L, Schmidl C, Kett J, Steger L, Andreesen R, Hoffmann P, et al. Dominant Th2 differentiation of human regulatory T cells upon loss of FOXP3 expression. J Immunol 2012 Feb 1;188(3):1275-1282.

(177) Maddur MS, Miossec P, Kaveri SV, Bayry J. Th17 cells: biology, pathogenesis of autoimmune and inflammatory diseases, and therapeutic strategies. Am J Pathol 2012 Jul;181(1):8-18.

(178) Passerini L, Olek S, Di Nunzio S, Barzaghi F, Hambleton S, Abinun M, et al. Forkhead box protein 3 (FOXP3)

mutations lead to increased TH17 cell numbers and regulatory T-cell instability. J Allergy Clin Immunol 2011 Dec;128(6):1376-1379.e1.

(179) Bovenschen HJ, van de Kerkhof PC, van Erp PE, Woestenenk R, Joosten I, Koenen HJ. Foxp3+ regulatory T cells of psoriasis patients easily differentiate into IL-17A-producing cells and are found in lesional skin. J Invest Dermatol 2011 Sep;131(9):1853-1860.

(180) Liu T, Song CH, Liu AM, Xie C, Zhao F, Chen X, et al. Forkhead box P3+ T cells express interleukin-17 in nasal mucosa of patients with both allergic rhinitis and polyposis. Clin Exp Immunol 2011 Jan;163(1):59-64.

(181) Hovhannisyan Z, Treatman J, Littman DR, Mayer L. Characterization of interleukin-17-producing regulatory T cells in inflamed intestinal mucosa from patients with inflammatory bowel diseases. Gastroenterology 2011 Mar;140(3):957-965.

(182) Eastaff-Leung N, Mabarrack N, Barbour A, Cummins A, Barry S. Foxp3+ regulatory T cells, Th17 effector cells, and cytokine environment in inflammatory bowel disease. J Clin Immunol 2010 Jan;30(1):80-89.

(183) Zenewicz LA, Antov A, Flavell RA. CD4 T-cell differentiation and inflammatory bowel disease. Trends Mol Med 2009 May;15(5):199-207.

(184) Chao K, Zhong BH, Zhang SH, Gong XR, Yao JY, Chen MH. Imbalance of CD4(+) T cell subgroups in ulcerative colitis. Zhonghua Yi Xue Za Zhi 2011 Jun 21;91(23):1605-1608.

(185) Kryczek I, Wu K, Zhao E, Wei S, Vatan L, Szeliga W, et al. IL-17+ regulatory T cells in the microenvironments of chronic inflammation and cancer. J Immunol 2011 Apr 1;186(7):4388-4395.

(186) Hughson A, Bromberg I, Johnson B, Quataert S, Jospe N, Fowell DJ. Uncoupling of proliferation and cytokines from suppression within the CD4+CD25+Foxp3+ T-cell compartment in the 1st year of human type 1 diabetes. Diabetes 2011 Aug;60(8):2125-2133.

(187) Codarri L, Gyulveszi G, Tosevski V, Hesske L, Fontana A, Magnenat L, et al. RORgammat drives production of the cytokine GM-CSF in helper T cells, which is essential for the effector phase of autoimmune neuroinflammation. Nat Immunol 2011 Jun;12(6):560-567.

(188) Siffrin V, Radbruch H, Glumm R, Niesner R, Paterka M, Herz J, et al. In vivo imaging of partially reversible th17 cell-induced neuronal dysfunction in the course of encephalomyelitis. Immunity 2010 Sep 24;33(3):424-436.

(189) Tran DQ, Shevach EM. Therapeutic potential of FOXP3(+) regulatory T cells and their interactions with dendritic cells. Hum Immunol 2009 May;70(5):294-299.

(190) Hippen KL, Harker-Murray P, Porter SB, Merkel SC, Londer A, Taylor DK, et al. Umbilical cord blood regulatory T-cell expansion and functional effects of tumor necrosis factor receptor family members OX40 and 4-1BB expressed on artificial antigen-presenting cells. Blood 2008 Oct 1;112(7):2847-2857.

(191) Yamazaki S, Inaba K, Tarbell KV, Steinman RM. Dendritic cells expand antigen-specific Foxp3+ CD25+ CD4+ regulatory T cells including suppressors of alloreactivity. Immunol Rev 2006 Aug;212:314-329.

(192) Allan SE, Alstad AN, Merindol N, Crellin NK, Amendola M, Bacchetta R, et al. Generation of potent and stable human CD4+ T regulatory cells by activation-independent expression of FOXP3. Mol Ther 2008 Jan;16(1):194-202.

(193) Kleinewietfeld M, Starke M, Di Mitri D, Borsellino G, Battistini L, Rotzschke O, et al. CD49d provides access to "untouched" human Foxp3+ Treg free of contaminating effector cells. Blood 2009 Jan 22;113(4):827-836.

(194) Battaglia M, Stabilini A, Migliavacca B, Horejs-Hoeck J, Kaupper T, Roncarolo MG. Rapamycin promotes expansion of functional CD4+CD25+FOXP3+ regulatory T cells of both healthy subjects and type 1 diabetic patients. J Immunol 2006 Dec 15;177(12):8338-8347.

(195) de Groote ML, Verschure PJ, Rots MG. Epigenetic Editing: targeted rewriting of epigenetic marks to modulate expression of selected target genes. Nucleic Acids Res 2012 Nov 1;40(21):10596-10613.