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T regulatory cells and the control of alloimmunity: from characterisation to clinical application

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T regulatory cells (Treg) play an important role in the induction and maintenance of immunological tolerance. Recent findings in experimental transplant models combined with the development of functional reporter mice have opened new avenues to study Treg biology and their therapeutic potential. In particular, recent advances in understanding Treg function and lineage stability revealed unexpected plasticity of this lineage. Nevertheless, pre-clinical and pilot clinical trials using Treg cells as cellular therapies have been initiated suggesting the

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Introduction

The appropriate balance between effector and T regulatory cells (Treg) is indispensable for the maintenance of self-tolerance and of functional immune responses *in vivo*. In the transplant setting, numerous findings during the last 25 years have demonstrated the importance and therapeutic potential of Treg in the active control of rejection responses. Although the existence of various cell populations with regulatory/suppressive activity, such as IL-10 secreting Tr-1 cells, $CD28^{-}CD8^{+}$ cells or B regulatory cells, has been demonstrated, in this review we will focus on classical $CD25^{+}CD4^{+}$ Treg cells.

Treg mediate suppressive effects by several mechanisms including anti-inflammatory cytokines (IL-10, TGF-β and IL-35), direct cytotoxic effect (granzyme B and galectin-1), metabolic disruption (adenosine production and IL-2 deprivation) and modulation of dendritic cell function (CTLA-4, LAG3 and IDO induction) as discussed in [1]. Numerous regulatory mechanisms described to date suggest that multiple, redundant mechanisms are required for optimal Treg function *in vivo*.

Treg can be divided into two populations: thymic-derived naturally occurring $CD25^{+}CD4^{+}$ cells (nTreg) [2] and induced or adaptive Treg (iTreg) that are either differentiated from $CD25^{-}CD4^{+}$ nonregulatory cells or expanded from $CD25^{+}CD4^{+}$ cells in response to the antigen [3]. These cells differ in origin, antigen experience, ctor fer-ential expression of transcription factor Helios has been attributed to thymic-derived nTregs and proposed as a marker to differentiate between nTregs and iTregs [4••]. Both nTreg and iTreg have been demonstrated to play an important role in transplant tolerance.

In this review, we explore recent advances in understanding Treg function and lineage stability and its impact on tolerance induction protocols. Next, we focus on the current attempts to expand human Treg for clinical application as a cellular therapy in organ and cell transplantation. Finally, we discuss the arising or potential issues relating to the therapeutic application of Treg and proposed solutions.

FoxP3 expression in Treg

Expression of the transcription factor FoxP3 is essential for the development and function of Treg [5,6]. It was demonstrated that ectopic expression of Foxp3 in conventional T cells was sufficient to confer suppressive activity, repress IL-2 and IFNγ production and upregulate Treg-associated molecules such as CTLA-4 and GITR [2,7]. Furthermore, expression of FoxP3 in mature Treg is necessary for the maintenance of Treg-specific transcription profile and of Treg function [8].

Epigenetic regulation of FoxP3 expression

Several epigenetic markers, such as histone acetylation and methylation, and cytosine residue methylation in CpG dinucleotides, have been reported at the Foxp3 locus [9•]. In particular, a unique CpG-rich island within an evolutionarily conserved region upstream of exon 1, named TSDR (Treg-specific demethylation region), was demonstrated to be unmethylated in natural Treg but heavily methylated in other CD4+ T cells [10•,11]. Demethylation of these CpG sites resulted in strong and stable induction of FoxP3. In human, upon *in vitro* expansion of Treg, CpG methylation increased correlating with loss of FoxP3 expression and emergence of

pro-inflammatory cytokines [12•]. Interestingly, $CD45RA^+FoxP3^+$ naïve Treg showed no increase in CpG methylation after 3-week culture, whereas $CD45RA^-FoxP3^+$ memory-like Treg from the same donors lost CpG demethylation status and converted into non-Treg cells. Recent advances in our understanding of the complex regulation of FoxP3 expression have led to new methods of analysing Treg based on quantitative DNA methylation analysis of FoxP3 locus [13•], which may add a useful test for quality assessment of *ex vivo* manipulated Treg cells.

Treg lineage stability

FoxP3 epigenetic analysis and the development of functional reporter mice questioned the dogma of natural Treg lineage stability. An elegant study by Zhou *et al.* examined the stability of Treg cells by tracing cells that induced and downregulated FoxP3 during their life span [14••]. The authors found that cells that at some point expressed FoxP3 and lost its expression shared their TCR repertoire both with $FoxP3^+$ Treg cells and with conventional T cells suggesting that they originated from both nTreg and iTreg. These 'ex-Treg' had an activated-memory phenotype and produced pro-inflammatory cytokines. Notably, an autoimmune microenvironment favoured loss of FoxP3, and 'ex-Treg' cells from diabetic mice were able to transfer diabetes [14••]. Notably for the transplant setting, it was also demonstrated that some peripheral $FoxP3^+CD4^+$ cells lose their FoxP3 expression and start producing IFNγ and IL-17 after transfer to a lymphopenic host [15•].

Cellular therapy with Treg

Mouse pre-clinical models

Many strategies exist for the *in vivo* or *ex vivo* generation and/or expansion of Treg. The most common *in vivo* approaches are based on the fact that exposure to antigen increases Treg frequency and/or potency by either expanding naturally occurring Treg or inducing the generation of adaptive Treg from cells that do not originally possess regulatory activity [16•]. Generation of Treg can be achieved by attenuation of activating signals during antigen presentation. In the mouse, donor-specific transfusion (DST) combined with a nondepleting anti-CD4 antibody generates $CD25^+CD4^+$ cells able to prevent skin graft rejection [17]. Moreover *in vitro* culture of mouse $CD4^+$ or $CD25^-CD4^+$cells in the presence of alloantigen and anti-CD4 antibody results in the enrichment of $CD62L^+CD25^+$ cells effective in controlling graft survival [18]. Interestingly, conditioning of $CD4^+$ cells in the presence of interferon-γ (IFN-γ) and immature DC can also generate $FoxP3^+$ cells that are able to protect both skin and islet transplants from rejection [19•,20]. Notably, alloantigen-reactive Treg from *in vivo* tolerised mice demonstrate increased levels of IFN-γ production transiently after antigen-specific reactivation through T cell receptor [21•]. *In vivo*, IFNγ produced locally where the Treg are present, the draining lymph nodes and the graft [22•], creates a microenvironment that influences the function of other cells in the vicinity, including the Treg themselves where evidence for the activation of IFNγ signalling pathways has been reported [21•].

Another approach to enrich Treg *in vivo* is to create Treg-favouring conditions. In the transplantation setting, patients are treated with diverse immunosuppressive drug combinations, which may have a different impact on Treg. It was demonstrated that calcineurin inhibitors (CNI), especially cyclosporine A, are detrimental to Treg, whereas the mTOR inhibitor rapamycin was shown to be beneficial for Treg both in terms of *in vivo* generation and function in mouse models [23] and in *in vitro* cultures of human Treg [24]. It was recently demonstrated that adoptive transfer of a low number of alloantigen-specific Treg under a cover of low dose of rapamycin induced long-term survival of heart transplant in unmanipulated host, an outcome otherwise difficult to obtain [25]. Interestingly, in terms of alloantigen-specificity of Treg two recent papers have independently demonstrated that regulatory cells specific for both directly (by donor APC) and indirectly (by host APC) presented alloantigens prolonged graft survival with substantially greater efficacy than Treg with only direct anti-donor specificity [26•,27•]. Noteworthy, successful attempt to achieve long-term acceptance of islet allografts without immunosuppression was demonstrated by Webster *et al.* who *in vivo* expanded Treg by injecting mice with IL-2/anti-IL-2 monoclonal antibody complexes [28•].

Human Treg

Human Treg are currently less well characterised and understood than mouse Treg, so a thorough understanding of their biology is vital before clinical applications can be initiated. It is also important to highlight that there are substantial differences between human and mouse Treg; most notably the differences in FoxP3 expression between mouse and human. In human, FoxP3 is also expressed by activated nonregulatory T cells as well as by Treg, and activated nonregulatory cells also upregulate CD25 expression. Thus not all $CD25^+FOXP3^+CD4^+$ will be genuine Treg and therefore isolation strategies based on $CD25^{hi/+}CD4^+$ are likely to be imperfect. Other markers are therefore needed to enrich Treg from human peripheral blood mononuclear cells. Recently, it has been demonstrated that $CD127^{lo}CD25^+CD4^+$ T cells are characterised by a higher percentage of $FoxP3^+$ cells with a more pronounced suppressive capacity [29,30]. Expansion of $CD127^{lo}CD25^+CD4^+$ cells resulted in high yield of regulatory cells which maintained high FoxP3 expression [31••]. Importantly, as we recently demonstrated in a clinically relevant humanised model of transplant arteriosclerosis, *ex vivo* expanded $CD25^{hi}CD4^+$ and $CD127^{lo}CD25^+CD4^+$ Treg cells have been very effective in inhibiting vasculopathy, with $CD127^{lo}CD25^+CD4^+$

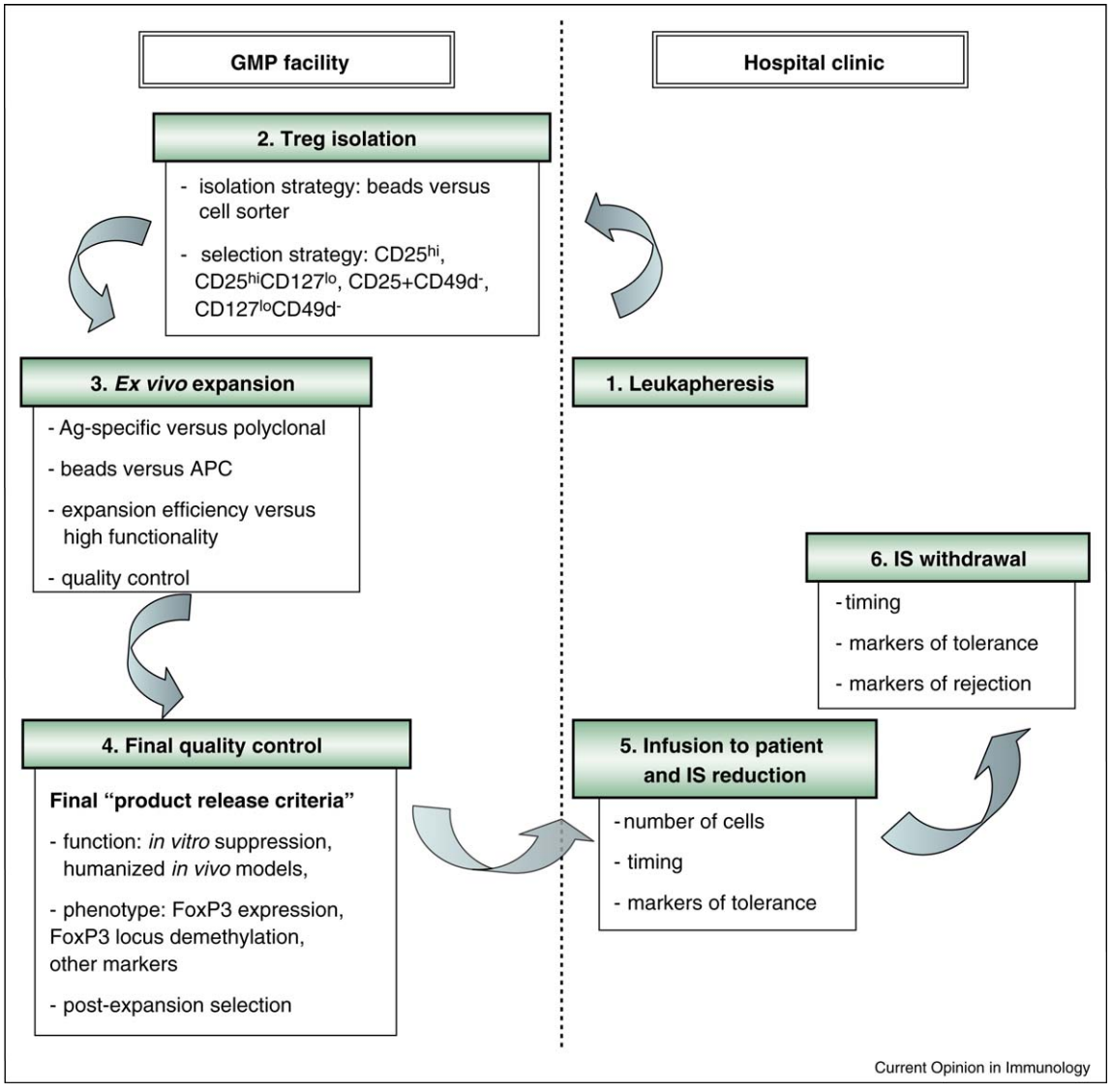
cells being five times more efficient than conventional Treg [32••]. Interestingly, another study subdivided Treg into two subtypes: resting naïve $CD25^{+}CD45RA^{+}FoxP3^{lo}$ and activated $CD25^{hi}CD45RA^{-}FoxP3^{hi}$ regulatory cells. A third population of $FoxP3^{+}$ cells phenotyped as $CD25^{+}CD45RA^{-}FoxP3^{lo}$ was demonstrated to consist of cytokine-secreting, non-suppressive cells [33•]. Notably, whereas both regulatory subpopulations were highly suppressive *in vitro*, only resting Treg were able to proliferate *in vitro* and *in vivo*, converting into activated CD45RA negative Treg.

A number of different strategies for the isolation/enrichment of human Treg have been described in the literature, but to date there is no consensus as to which strategy produces the optimal population for use in cell therapy applications. The critical steps and the questions awaiting answers in the process of developing clinically approved Treg cellular therapy are outlined in Figure 1.

Clinical application of human Treg

One of the obstacles in the implementation of clinical protocols using Treg is their low frequency in the peripheral blood leading to the need for *ex vivo* multiplication of the cells prior to their use *in vivo*. The most commonly used expansion protocol at present is based on stimulation by anti-CD3/anti-CD28 beads in the presence of high doses of recombinant IL-2, supplemented in some protocols with rapamycin. This protocol results in the efficient expansion of polyclonal Treg, generating sufficient numbers of cells for cellular therapy [34•]. However, the expansion is antigen non-specific without any enrichment

Figure 1



Steps in preparation and clinical application of Treg cells. In transparent boxes questions awaiting answers in the process of developing Treg cellular therapy. IS—immunosuppression; GMP—good manufacturing practices.

step for the cells of interest. More appealing for clinical application, is the concept of expanding or generating antigen-specific Treg, in the setting of transplantation, donor alloantigen-reactive Treg. Interestingly, human Treg expanded with allogeneic PBMC, were found to be more suppressive *in vitro* than polyclonaly-driven cells and, surprisingly, expanded at a similar rate as polyclonaly-stimulated cells [35].

Clinical trials

There are several ongoing clinical trials with the application of Treg cellular therapy. To date regulatory cells have been adoptively transferred into haematopoietic stem cell transplant (HSCT) recipients in Germany, USA and Italy [36•]. In one of such trials, led by Matthias Edinger from Regensburg, Germany, freshly isolated bead-selected donor Treg were infused into HSCT recipients. So far nine patients have been included in the study with no side effects (M. Edinger, personal communication). In a study led by Massimo Martelli, 22 patients were inoculated with freshly isolated donor Treg followed by infusion of haematopoietic stem cells and donor conventional T cells. This strategy improved immune recovery after HSCT without causing graft versus host disease (GVHD) (Di Ianni *et al.*, 51st ASH Annual Meeting and Exposition, New Orleans, LA, December 2009). Another clinical trial in the University of Minnesota, USA, led by Bruce Blazar and co-workers, used *ex vivo* expanded bead-enriched, third-party, partially HLA-matched Treg cells from umbilical cord blood (UCB). These UCB Treg cells were administered into myeloablated or nonmyeloablated recipients of two unrelated UCB units [36•].

Whereas aforementioned studies utilised Treg to prevent GVHD, Trzonkowski *et al.* [34•] have reported their findings from two patients with GVHD who were treated with anti-CD3/anti-CD28 bead expanded $CD25^{+}CD127^{lo}$ Treg. One of the patients, with chronic GVHD, responded to the therapy with alleviation of the symptoms and reduction of immunosuppression. However, in the case of the second patient with acute, grade IV GVHD, only transient improvement was observed. Notably, in the case of the patient treated successfully with cellular Treg therapy, only 1×10^5 Treg cells/kg body weight was sufficient to achieve clinical improvement. Published reports and unpublished results from other ongoing studies indicate that Treg cellular therapy is proving to be effective in clinical situations. Currently, further clinical studies are being planned to apply Treg therapy in solid organ transplantation.

Questions arising

IL-17 production by FoxP3+ cells and Treg lineage stability

The ability of *in vitro* expanded Treg to convert into cytokine producing, nonregulatory cells upon prolonged TCR stimulation [12•] has led to concerns about the efficacy and safety of *ex vivo* Treg expansion protocols. Importantly, it was demonstrated that a proportion of circulating Treg have the capacity to secrete IL-17 and express RORγt [37,38]. These IL-17 producing cells are of memory phenotype and express CCR6, a marker associated with Th17 cells. Interestingly, some authors have shown that IL-17 producing $CCR6^{+}$ Treg are as equally suppressive as $CCR6^{-}$ Treg [37,38], whereas others demonstrated a loss of suppressive function in $FoxP3^{+}IL\text{-}17^{+}$ clones after strong TCR stimulation in the presence of APC [39]. Although the function and regulatory properties of these cells *in vivo* are still debatable, the possibility that they may elicit unwanted responses when transferred to patients cannot be excluded. However, several markers of 'true' Treg have been described such as IL-1R2, LAP and GARP that may allow additional post-expansion purification steps to be introduced into clinical protocols to reduce the risk of introducing cells that do not have regulatory function [40•,41•]. Alternatively, the use of a pure, conversion resistant Treg subpopulation ($CD25^{+}CD45RA^{+}CD127^{lo}$) as a starting population for expansion [12•] would also reduce any safety concerns.

Another way to prevent conversion into Th17 cells may be to use pharmacologic intervention during *ex vivo* culture. Recently, retinoic acid (RA) has been described as inhibiting IL-17 polarisation and to promote FoxP3 expression [42]. Another pharmacologic agent, already in use, as discussed above, is rapamycin which was demonstrated to improve suppressive activity and FoxP3 expression in *ex vivo* expanded human Treg, especially when isolated with magnetic beads [24].

Introduction of additional factors to the *in vitro* Treg cultures that maintain FoxP3 expression in *ex vivo* manipulated cells appears to be an attractive way of ensuring their effectiveness and safety for the patient. Additionally, obtaining cells with higher per cell activity would potentially allow a significant reduction in the number of Treg required for each clinical application.

Cancer and infection immunity

One of the concerns regarding the application of Treg in transplant recipients is the possibility of inhibition of antitumour and antiviral immunity. Theoretically, infusion with large numbers of potent suppressor cells may present a serious obstacle to the induction of effective immune responses towards infectious pathogens and a reduction in immune surveillance against tumour cells. As is often the case with the immune system, reality may not fit with theoretical predictions. For example, *in vivo* ablation of Treg was recently demonstrated to lead to accelerated fatal infection during mucosal herpes simplex virus infection, suggesting that in some situations Treg facilitate early protective responses to local viral infection [43].

Notably, it has also been demonstrated in mice that antigen-specific, *in vivo* induced Treg which were able to induce tolerance in primary and secondary allograft recipients did not affect anti-influenza response even when bystander regulation was deliberately induced [44].

Numerous studies suggest that the accumulation of Treg at tumour sites may affect anti-tumour immunity, therefore infusion of substantial numbers of Treg may particularly influence the response towards already existing early tumours. Notably, high numbers of Treg in the blood have been recently associated with increased risk of new tumour development in kidney transplant recipients with non-malignant squamous cell carcinoma [45]. On the other hand, immunosuppressive agents currently being used are themselves associated with increased risk of cancer [46]. Therefore, careful screening and monitoring of transplant recipients eligible for Treg cellular therapy should be performed before and after infusion in any pilot clinical study.

Conclusion

Recent progress in understanding Treg biology and the development of experimental mouse models has highlighted potential avenues in the translation of research-based knowledge to the clinic. Insights into the biological role of FoxP3, the effects of immunosuppression on Treg and new protocols to expand or induce Treg provide a knowledge base for developing clinical strategies to achieve long-term graft survival without life-long immunosuppression.

Conflict of interest

Authors declare no conflict of interest.

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References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Vignali DA, Collison LW, Workman CJ: **How regulatory T cells work**. *Nat Rev Immunol* 2008, **8**:523-532.

2. Hori S, Nomura T, Sakaguchi S: **Control of regulatory T cell development by the transcription factor Foxp3**. *Science* 2003, **299**:1057-1061.

3. Karim M, Kingsley CI, Bushell AR, Sawitzki BS, Wood KJ: **Alloantigen-induced CD25+CD4+ regulatory T cells can develop in vivo from CD25−CD4+ precursors in a thymus-independent process**. *J Immunol* 2004, **172**:923-928.

4. •• Thornton AM, Korty PE, Tran DQ, Wohlfert EA, Murray PE, Belkaid Y, Shevach EM: **Expression of Helios, an Ikaros transcription factor family member, differentiates thymic-derived from peripherally induced Foxp3+ T regulatory cells**. *J Immunol* 2010, **184**:3433-3441.

This study demonstrates that Helios, a member of the Ikaros transcription factor family, is preferentially expressed by thymic-derived natural Tregs but not antigen-specific FoxP3+ T cells induced *in vivo* so it may serve as a marker of nTregs.

5. Bennett CL, Christie J, Ramsdell F, Brunkow ME, Ferguson PJ, Whitesell L, Kelly TE, Saulsbury FT, Chance PF, Ochs HD: **The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3**. *Nat Genet* 2001, **27**:20-21.

6. Wildin RS, Ramsdell F, Peake J, Faravelli F, Casanova JL, Buist N, Levy-Lahad E, Mazzella M, Goulet O, Perroni L *et al.*: **X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy**. *Nat Genet* 2001, **27**:18-20.

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

8. Williams LM, Rudensky AY: **Maintenance of the Foxp3-dependent developmental program in mature regulatory T cells requires continued expression of Foxp3**. *Nat Immunol* 2007, **8**:277-284.

9. • Lal G, Bromberg JS: **Epigenetic mechanisms of regulation of Foxp3 expression**. *Blood* 2009, **114**:3727-3735.

In this review, the authors discuss mechanisms of regulation of FoxP3 expression with the emphasis on the role of CpG DNA methylation and histone acethylation and methylation in Treg development and function.

10. • Lal G, Zhang N, van der Touw W, Ding Y, Ju W, Bottinger EP, Reid SP, Levy DE, Bromberg JS: **Epigenetic regulation of Foxp3 expression in regulatory T cells by DNA methylation**. *J Immunol* 2009, **182**:259-273.

In this study authors identified a unique CpG island within an upstream enhancer of FoxP3 gene that was methylated in peripheral $CD4^+$ T cells and TGFβ-induced Treg but not in natural Treg.

11. Floess S, Freyer J, Siewert C, Baron U, Olek S, Polansky J, Schlawe K, Chang HD, Bopp T, Schmitt E *et al.*: **Epigenetic control of the foxp3 locus in regulatory T cells**. *PLoS Biol* 2007, **5**:e38.

12. • Hoffmann P, Boeld TJ, Eder R, Huehn J, Floess S, Wieczorek G, Olek S, Dietmaier W, Andreesen R, Edinger M: **Loss of FOXP3 expression in natural human CD4+CD25+ regulatory T cells upon repetitive in vitro stimulation**. *Eur J Immunol* 2009, **39**:1088-1097.

This paper shows increase in CpG methylation in FoxP3 locus and loss of FoxP3 expression in human Treg after prolonged *in vitro* expansion.

13. • Wieczorek G, Asemissen A, Model F, Turbachova I, Floess S, Liebenberg V, Baron U, Stauch D, Kotsch K, Pratschke J *et al.*: **Quantitative DNA methylation analysis of FOXP3 as a new method for counting regulatory T cells in peripheral blood and solid tissue**. *Cancer Res* 2009, **69**:599-608.

This study demonstrates a new method for Treg monitoring in blood or tissues by measuring DNA methylation in FoxP3 locus.

14. •• Zhou X, Bailey-Bucktrout SL, Jeker LT, Penaranda C, Martinez-Llordella M, Ashby M, Nakayama M, Rosenthal W, Bluestone JA: **Instability of the transcription factor Foxp3 leads to the generation of pathogenic memory T cells in vivo**. *Nat Immunol* 2009, **10**:1000-1007.

In this study, authors demonstrated the existence in healthy mice of cells that downregulated or completely lost Foxp3 expression. These 'exFoxp3' T cells had an activated-memory T cell phenotype, produced inflammatory cytokines and led to the rapid onset of diabetes upon adoptive transfer.

15. • 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 USA* 2009, **106**:1903-1908.

This study reported that a minor fraction of FoxP3+ cells lose its FoxP3 expression after adoptive transfer to lymphopenic recipients. The majority of FoxP3-down-regulated cells lost Treg function and produced proinflammatory cytokines.

16. • Long E, Wood KJ: **Regulatory T cells in transplantation: transferring mouse studies to the clinic**. *Transplantation* 2009, **88**:1050-1056.

This review discusses strategies to expand or induce alloantigen-reactive Treg *in vivo* and *in vitro* as well as possibilities to translate these protocols to clinical application.

17. Kingsley CI, Karim M, Bushell AR, Wood KJ: **CD25+CD4+ regulatory T cells prevent graft rejection: CTLA-4- and IL-10-dependent immunoregulation of alloresponses**. *J Immunol* 2002, **168**:1080-1086.

18. Oliveira V, Sawitzki B, Chapman S, Appelt C, Gebuhr I, Wieckiewicz J, Long E, Wood KJ: **Anti-CD4-mediated selection of Treg in vitro–in vitro suppression does not predict in vivo capacity to prevent graft rejection**. *Eur J Immunol* 2008, **38**:1677-1688.

19. • Feng G, Wood KJ, Bushell A: **Interferon-gamma conditioning ex vivo generates CD25+CD62L+Foxp3+ regulatory T cells that prevent allograft rejection: potential avenues for cellular therapy**. *Transplantation* 2008, **86**:578-589.
This paper provides evidence that IFNγ *ex vivo* conditioning generates functional Treg cells.

20. Feng G, Gao W, Strom TB, Oukka M, Francis RS, Wood KJ, Bushell A: **Exogenous IFN-gamma ex vivo shapes the alloreactive T-cell repertoire by inhibition of Th17 responses and generation of functional Foxp3+ regulatory T cells**. *Eur J Immunol* 2008, **38**:2512-2527.

21. • Wei B, Baker S, Wieckiewicz J, Wood KJ: **IFN-gamma triggered STAT1-PKB/AKT signalling pathway influences the function of alloantigen reactive regulatory T cells**. *Am J Transplant* 2009, **10**:69-80.
This study demonstrates that IFNγ signaling in alloantigen-reactive Treg influences their function *in vivo*.

22. • Carvalho-Gaspar M, Jones ND, Luo S, Martin L, Brook MO, Wood KJ: **Location and time-dependent control of rejection by regulatory T cells culminates in a failure to generate memory T cells**. *J Immunol* 2008, **180**:6640-6648.
This paper shows preferential accumulation of Treg within graft and graft-draining lymph nodes in tolerised recipients.

23. Gao W, Lu Y, El Essawy B, Oukka M, Kuchroo VK, Strom TB: **Contrasting effects of cyclosporine and rapamycin in de novo generation of alloantigen-specific regulatory T cells**. *Am J Transplant* 2007, **7**:1722-1732.

24. Battaglia M, Stabilini A, Roncarolo MG: **Rapamycin selectively expands CD4+CD25+FoxP3+ regulatory T cells**. *Blood* 2005, **105**:4743-4748.

25. Raimondi G, Sumpter TL, Matta BM, Pillai M, Corbitt N, Vodovotz Y, Wang Z, Thomson AW: **Mammalian target of rapamycin inhibition and alloantigen-specific regulatory T cells synergize to promote long-term graft survival in immunocompetent recipients**. *J Immunol* 2010, **184**:624-636.

26. • Joffre O, Santolaria T, Calise D, Al Saati T, Hudrisier D, Romagnoli P, van Meerwijk JP: **Prevention of acute and chronic allograft rejection with CD4+CD25+Foxp3+ regulatory T lymphocytes**. *Nat Med* 2008, **14**:88-92.
This paper demonstrated superiority of Treg with direct and indirect allospecificity over cells with direct allorecognition in preventing graft rejection.

27. • Tsang JY, Tanriver Y, Jiang S, Xue SA, Ratnasothy K, Chen D, Stauss HJ, Bucy RP, Lombardi G, Lechler R: **Conferring indirect allospecificity on CD4+CD25+ Tregs by TCR gene transfer favors transplantation tolerance in mice**. *J Clin Invest* 2008, **118**:3619-3628.
Together with the paper above, this paper demonstrated superiority of Treg with direct and indirect allospecificity over cells with direct allorecognition in preventing graft rejection.

28. • Webster KE, Walters S, Kohler RE, Mrkvan T, Boyman O, Surh CD, Grey ST, Sprent J: **In vivo expansion of T reg cells with IL-2-mAb complexes: induction of resistance to EAE and long-term acceptance of islet allografts without immunosuppression**. *J Exp Med* 2009, **206**:751-760.
This paper describes rapid *in vivo* expansion of Treg cells upon injection with IL-2-IL-2 mAb complexes. Such pre-treatment induced resistance to EAE and long-term acceptance of islet allograft in the absence of immunosuppression.

29. Liu W, Putnam AL, Xu-Yu Z, Szot GL, Lee MR, Zhu S, Gottlieb PA, Kapranov P, Gingeras TR, Fazekas de St Groth B *et al.*: **CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells**. *J Exp Med* 2006, **203**:1701-1711.

30. Seddiki N, Santner-Nanan B, Martinson J, Zaunders J, Sasson S, Landay A, Solomon M, Selby W, Alexander SI, Nanan R *et al.*: **Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells**. *J Exp Med* 2006, **203**:1693-1700.

31. •• Putnam AL, Brusko TM, Lee MR, Liu W, Szot GL, Ghosh T, Atkinson MA, Bluestone JA: **Expansion of human regulatory T-cells from patients with type 1 diabetes**. *Diabetes* 2009, **58**:652-662.
This paper demonstrates *ex vivo* expansion of human Treg cells from both healthy subjects and type I diabetes patients with no difference in expansion rate or suppressive capacity.

32. •• Nadig SN, Wieckiewicz J, Wu DC, Warnecke G, Zhang W, Luo S, Schiopu A, Taggart DP, Wood KJ: **In vivo prevention of transplant arteriosclerosis by ex vivo-expanded human regulatory T cells**. *Nat Med* 2010, **16**:809-813.
This study compares *in vivo* capacity of expanded human Treg cells in a clinically relevant humanized mouse model of transplant arteriosclerosis.

33. • Miyara M, Yoshioka Y, Kitoh A, Shima T, Wing K, Niwa A, Parizot C, Taflin C, Heike T, Valeyre D *et al.*: **Functional delineation and differentiation dynamics of human CD4+ T cells expressing the FoxP3 transcription factor**. *Immunity* 2009, **30**:899-911.
In this study, functional and phenotypic dissection of human FoxP3$^+$ cells has been demonstrated.

34. • Trzonkowski P, Bieniaszewska M, Juscinska J, Dobyszuk A, Krzystyniak A, Marek N, Mysliwska J, Hellmann A: **First-in-man clinical results of the treatment of patients with graft versus host disease with human ex vivo expanded CD4+CD25+CD127-T regulatory cells**. *Clin Immunol* 2009, **133**:22-26.
In this study, clinical application of *ex vivo* expanded Treg cells in 2 GVHD patients has been reported.

35. Peters JH, Hilbrands LB, Koenen HJ, Joosten I: **Ex vivo generation of human alloantigen-specific regulatory T cells from CD4(pos)CD25(high) T cells for immunotherapy**. *PLoS ONE* 2008, **3**:e2233.

36. • Riley JL, June CH, Blazar BR: **Human T regulatory cell therapy: take a billion or so and call me in the morning**. *Immunity* 2009, **30**:656-665.
In this review, authors discuss preclinical studies in support for Treg cellular therapy and challenges for clinical application.

37. Ayyoub M, Deknuydt F, Raimbaud I, Dousset C, Leveque L, Bioley G, Valmori D: **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 USA* 2009, **106**:8635-8640.

38. Voo KS, Wang YH, Santori FR, Boggiano C, Wang YH, Arima K, Bover L, Hanabuchi S, Khalili J, Marinova E *et al.*: **Identification of IL-17-producing FOXP3+ regulatory T cells in humans**. *Proc Natl Acad Sci USA* 2009, **106**:4793-4798.

39. Beriou G, Costantino CM, Ashley CW, Yang L, Kuchroo VK, Baecher-Allan C, Hafler DA: **IL-17-producing human peripheral regulatory T cells retain suppressive function**. *Blood* 2009, **113**:4240-4249.

40. • Wang R, Kozhaya L, Mercer F, Khaitan A, Fujii H, Unutmaz D: **Expression of GARP selectively identifies activated human FOXP3+ regulatory T cells**. *Proc Natl Acad Sci USA* 2009, **106**:13439-13444.
This study describes new marker of activated human Treg.

41. • 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, **113**:5125-5133.
This study describes new markers of activated human Treg.

42. Elias KM, Laurence A, Davidson TS, Stephens G, Kanno Y, Shevach EM, O'Shea JJ: **Retinoic acid inhibits Th17 polarization**

and enhances FoxP3 expression through a Stat-3/Stat-5 independent signaling pathway. *Blood* 2008, **111**:1013-1020.

43. Lund JM, Hsing L, Pham TT, Rudensky AY: **Coordination of early protective immunity to viral infection by regulatory T cells**. *Science* 2008, **320**:1220-1224.

44. Bushell A, Jones E, Gallimore A, Wood K: **The generation of CD25+CD4+ regulatory T cells that prevent allograft rejection does not compromise immunity to a viral pathogen**. *J Immunol* 2005, **174**:3290-3297.

45. Carroll RP, Segundo DS, Hollowood K, Marafioti T, Clark TG, Harden PN, Wood KJ: **Immune phenotype predicts risk for posttransplantation squamous cell carcinoma**. *J Am Soc Nephrol* 2010, **21**:713-722.

46. Vajdic CM, McDonald SP, McCredie MR, van Leeuwen MT, Stewart JH, Law M, Chapman JR, Webster AC, Kaldor JM, Grulich AE: **Cancer incidence before and after kidney transplantation**. *JAMA* 2006, **296**:2823-2831.