Therapeutic opportunities for manipulating T_{Reg} cells in autoimmunity and cancer

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Abstract | Forkhead box P3 (FOXP3)-expressing regulatory T (T_{Reg}) cells have a pivotal role in the regulation of immune responses and in the maintenance of immunological self-tolerance. These cells have emerged as attractive targets for strategies that allow the steering of immune responses in desired directions — arming the immune system to destroy infected cells and cancer cells or downregulating it to limit tissue destruction in autoimmunity. Efforts to understand the generation, activation and function of T_{Reg} cells should permit the development of therapeutics for reprogramming the immune system. In this Review, we discuss insights into the generation of T_{Reg} cells, their involvement in disease and the molecular basis of the dominant tolerance exerted by $FOXP3^+$ T_{Reg} cells that could permit their safe and specific manipulation in humans.

Recessive tolerance
A form of passive tolerance involving clonal deletion (negative selection) and clonal inactivation (anergy), both of which represent cell-intrinsic mechanisms.

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The absence of a pathological immune response to self-tissues is due to two main tolerance mechanisms: recessive tolerance and dominant tolerance. These occur centrally within the thymus and peripherally in the body. Recessive tolerance takes place in the form of apoptotic death (negative selection) or anergy of immature and mature lymphocytes[1–4], whereas dominant tolerance is executed by a committed lineage of regulatory T (T_{Reg}) cells. These cells are characterized by the expression of the high-affinity interleukin-2 (IL-2) receptor α-chain (IL-2Rα; also known as CD25) and the X-linked gene forkhead box P3 (*FOXP3*), encoding the transcription factor FOXP3, which serves as a lineage specification factor for the development and function of $CD4^+CD25^+$ T_{Reg} cells[5,6] (BOX 1).

$FOXP3^+$ T_{Reg} cells have attracted considerable attention as they can 'tame' their inflammatory counterparts by direct contact-dependent inhibition of antigen-presenting cells and effector T cells or by the release of anti-inflammatory cytokines such as IL-10 or transforming growth factor-β (TGFβ). They have been shown to maintain their regulatory functions for a long period of time — even in the absence of antigens that induced their generation — and are stable and transferable[5–8], thereby permitting the prospective generation of these cells to prevent and suppress unwanted immunity.

The specificity of T_{Reg}-mediated suppression results from the co-recruitment of T_{Reg} and effector T cells in antigen-draining lymph nodes and at the effector site within peripheral tissues. T_{Reg} cells harbouring specificity for a particular T cell receptor (TCR) have the ability to suppress several effector cells with distinct TCR specificities when colocalized at the same antigen-presenting cell. This phenomenon is called bystander suppression[9–11].

The crucial impact of dominant tolerance in the prevention of experimental autoimmunity has been supported by studies showing that autoimmunity in mice as a result of thymectomy in the neonatal period can be prevented by the transfer of $FOXP3^+$ T_{Reg} cells from healthy donors[12]. Conversely, the acute elimination of $FOXP3^+$ T_{Reg} cells in healthy mice can lead to death resulting from multi-organ autoimmunity within 10 days[13,14]. The pivotal role of human $FOXP3^+$ T_{Reg} cells in maintaining immunological self-tolerance is illustrated by the fatal autoimmune disease IPEX (immunodysregulation, polyendocrinopathy, enteropathy, X-linked syndrome), which is caused by mutations in the *FOXP3* gene[1,15]. Patients with IPEX syndrome produce a broad range of autoantibodies and develop insulin-dependent diabetes, thyroiditis, eczema, haemolytic anaemia and inflammatory bowel disease (IBD), and without bone marrow transplantation they die at an early age.

Failures of immune-regulatory pathways have been investigated in the context of several inflammatory and autoimmune diseases, particularly type 1 diabetes, multiple sclerosis, systemic lupus erythematosus, rheumatoid arthritis, IBD, psoriasis and myasthenia gravis (reviewed in REF. 16). The regulatory deficiencies can be rooted in

Box 1 | Characterization of human T_{Reg} cells

Human regulatory T (T_{Reg}) cells were first isolated from peripheral blood and characterized as $CD4^+CD25^{high}$ T cells by several groups in 2001 (REFS 29,158–162). Forkhead box P3 (FOXP3), first identified in mice in 2003 (REFS 5,6,163), was later confirmed as a specific marker for human T_{Reg} cells[164]. Human FOXP3 is expressed as two main isoforms: FOXP3A (which is an orthologue of mouse FOXP3) and FOXP3B. The FOXP3B isoform is a splicing variant that lacks exon 2, which encodes the proline-rich domain that forms part of the Leu-X-X-Leu-Leu motif that is required for binding to the transcription factor retinoid-related orphan receptor-α (RORα)[165]. It also lacks the amino-terminal residues that are required for interaction with the transcription factor nuclear factor of activated T cells (NFAT) and the resulting transcriptional repression[166,167]. FOXP3 is able to specifically suppress the transactivation domains of NFAT, thereby blocking its ability to induce the endogenous expression of target genes, including key cytokine genes such as interleukin-2 (*IL2*) and *IL4*. Studies in highly purified naive $CD4^+CD25^-CD45RA^+$ T cells have shown that both isoforms of FOXP3 confer a suppressive function when they are highly overexpressed[168–170]. However, the cellular distribution and expression ratio of these two human FOXP3 isoforms are not yet fully understood.

Recent studies have demonstrated that in contrast to mouse T_{Reg} cells, human T_{Reg} cells exhibit phenotypic and functional heterogeneity and can be subdivided into subsets based on the expression of surface markers. The subdivision can be achieved by deriving a signature of CD25, CD127, CD45RA, CD45RO, cytotoxic T lymphocyte antigen 4 (CTLA4), inducible T cell co-stimulator (ICOS) and the level of FOXP3 expression[171–173].

Dominant tolerance
The active suppression of an immune response by suppressor cells including regulatory T (T_{Reg}) cells. By contrast, deletional tolerance and induction of anergy are forms of passive tolerance. Dominant tolerance is transferable to naive recipients, whereas passive tolerance is not.

Negative selection
Also known as clonal deletion. The intrathymic elimination of $CD4^+CD8^+$ double-positive and single-positive thymocytes that express T cell receptors with a high affinity for self-antigens.

Fork-head box P3
(FOXP3). Forkhead or winged helix transcription factor that is expressed primarily in regulatory T (T_{Reg}) cells and controls their function. Mutations in the *FOXP3* gene result in life-threatening autoimmunity in humans as well as in experimental mice.

Bystander suppression
A situation whereby regulatory T cells of a particular specificity are able to suppress several effector cells with different specificities when colocalized at the same antigen-presenting cell.

the specific cytokine milieu at the site of inflammation, where the activation status of local antigen-presenting cells may influence T_{Reg} or pathogenic T cell induction and function[16–18]. Type 1 diabetes and other autoimmune diseases are thought to develop when T cells with specificity for weakly binding TCR agonists, which may include self-antigens, evade thymic negative selection and then mount an autoimmune attack in the periphery[19,20–24] (BOX 2).

There is also evidence for an involvement of T_{Reg} cells in the pathology of allergy; several studies found impaired $CD4^+CD25^+$ T_{Reg} cell-mediated inhibition of allergen-specific T helper type 2 (T_H2) responses in allergic patients during hayfever season[25,26] or in individuals who reveal prominent T_H2 responses to other allergens[27]. Furthermore, clinical trials in which $CD4^+CD25^+$ T cells were depleted from the peripheral blood of healthy individuals showed that this approach results in enhanced proliferative and T_H2 cytokine-mediated responses to various allergens, including milk, nickel and grass[26,28,29]. This implies that $CD4^+CD25^+$ T_{Reg} cells have an active role in suppressing allergen-specific T_H2 responses in healthy individuals.

In contrast to allergy and autoimmunity, evidence from patients with cancer suggests that increased T_{Reg} cell activity may be associated with poor immune responses to tumour antigens and can contribute to immune dysfunction. Recent data indicate that T_{Reg}-mediated immune suppression is one of the crucial mechanisms of immune evasion by the tumour and the main obstacle of successful tumour immunotherapy[30]. In line with these arguments, high numbers of $CD4^+CD25^+$ T_{Reg} cells have been found in patients with lung, pancreatic, breast, liver and skin cancer, both in the blood and in the tumour itself[30–33]. Moreover, it has become evident that the interaction between tumours and their microenvironment is critical not only during oncogenesis and tumour progression but also in the context of anticancer therapies. Recent studies have shown that numerous anticancer agents, beyond their cytostatic properties, have the capacity to stimulate the innate and adaptive immune system, resulting — in some cases — in long-term protective memory T cell responses or in the inhibition of T_{Reg} cell function or survival, thereby facilitating tumour eradication[34–36].

The central role of $FOXP3^+$ T_{Reg} cells in immune homeostasis has led to a quest for novel therapeutic strategies that can target these cells to treat several diseases. In the case of autoimmune diseases, the path to effective therapeutic targeting is likely to require an understanding of the mechanisms of autoimmunity that underlie the breakdown of the tolerance that is normally maintained by T_{Reg} cells. The induction of specific T_{Reg} cells in this context could allow the modulation of the immune response for clinical benefit while limiting long-term general immune suppression. In the case of cancer, new approaches for cancer immunotherapy will be facilitated by the identification of crucial molecular and cellular pathways involved in tumour growth and maintenance, including the role of T_{Reg} cells in the evasion of tumour immunity as well as the development of agents that specifically inhibit these pathways. Here, we discuss the current knowledge on the generation and manipulation of $FOXP3^+$ T_{Reg} cells and consider how these insights can be used for the design of suitable compounds that permit the induction of antigen-specific tolerance for the safe and specific prevention of autoimmunity or the ablation of specific T_{Reg} cells to boost immune responses in patients with cancer. The targeting of $FOXP3^-$ T_{Reg} cell populations with suppressive potential — such as IL-10-producing type 1 T_{Reg} cells — is beyond the focus of this Review and has been discussed elsewhere in the literature[37].

Strategies for the manipulation of T_{Reg} cells

T_{Reg} cell manipulation as a crucial component of immune therapy is based on numerous different approaches. These include strategies to enhance T_{Reg} cell generation — for example, through cytokines or subimmunogenic antigen application — and strategies to manipulate the activation, expansion, survival or suppressive function of T_{Reg} cells by interfering with intracellular signalling pathways or microRNAs (miRNAs). Moreover, the epigenetic regulation of the *FOXP3* locus can be targeted (FIG. 1).

Cytokines. Cytokines function as primary regulators of the differentiation, activity and function of T cells. Some of these modulators, such as TGFβ and IL-2, have been shown to be critical for the regulation of T_{Reg} cell generation.

TGFβ was demonstrated to be of crucial importance for the development, function and survival of T cells[38]. It was shown to promote the generation of $FOXP3^+$ T_{Reg} cells *in vitro,* but FOXP3 expression in these cells was found to be unstable[39]. TGFβ signalling induces the phosphorylation of SMAD2 and SMAD3 transcription factors[40]. *FOXP3* gene expression is regulated by a core

Box 2 | **Prevention of type 1 diabetes in a mouse model by T_{Reg} cell induction**

Mouse models of type 1 diabetes such as non-obese diabetic (NOD) mice have shown that insulin is an essential autoantigen[174,175], and NOD mice that express a mutant insulin gene product that is not recognized by T cells do not develop type 1 diabetes[176]. Furthermore, in mice and humans, the T cell response to insulin is highly focused on a segment of the insulin B-chain comprising residues 9–23 (REFS 177–180). The human epitope is identical to that of mouse insulin.

Initial experiments to test whether extrathymic forkhead box P3-positive (FOXP3$^+$) regulatory T (T_{Reg}) cell induction by subimmunogenic delivery of natural insulin B-chain epitopes can interfere with the development of murine type 1 diabetes revealed only a minor efficacy of T_{Reg} cell generation and a slight delay in diabetes progression[61]. As one possible means to explain the poor efficacy of T_{Reg} cell induction by natural insulin B-chain epitopes in murine type 1 diabetes, recent studies have suggested that the insulin B-chain peptide is presented by I-A^{g7} molecules in an unfavoured low-affinity binding register, which results in weak agonistic activity of the peptide presented by the major histocompatibility complex (MHC)[23,181]. When bound in this unfavourable register, an arginine residue of the insulin epitope faces another arginine in the positively charged p9 pocket of I-A^{g7} — a highly unfavourable match[23,181–183]. Although this represents a compelling model, it is challenged by others who suggest that binding occurs mainly in different likewise low-affinity registers[184]. Importantly, crystal structures of the human type 1 diabetes susceptibility alleles human leukocyte antigen DQ8 (HLA-DQ8) and HLA-DQ2 as well as the homologous molecule in the NOD mouse, I-A^{g7}, reveal striking structural overlap between the MHC peptide-binding pockets[185], which suggests that there are similar peptide presentation events of insulin epitopes in the human form of type 1 diabetes.

In NOD mice we devised a strongly agonistic mimetope of the natural insulin B-chain epitope in order to establish efficient insulin-specific FOXP3$^+$ T_{Reg} cell conversion. The strongly agonistic insulin mimetope induced much stronger proliferation as well as conversion of naive T cells into FOXP3$^+$ T_{Reg} cells when used under appropriate subimmunogenic conditions[61]. Furthermore, application of the insulin mimetope under subimmunogenic conditions protected NOD mice from developing type 1 diabetes for 40 weeks and longer[4,61]. Analyses of FOXP3 expression by immunohistochemistry in pancreatic cryosections from NOD mice of various treatment groups showed that, in contrast to the natural insulin epitope, T_{Reg} cell generation with the strongly agonistic insulin B-chain mimetope caused elevated numbers of FOXP3$^+$ T_{Reg} cells. Likewise, in NOD FOXP3 green fluorescent protein (GFP)-tagged reporter mice, subimmunogenic T_{Reg} cell induction with the strongly agonistic insulin B-chain variant led to increased percentages and absolute numbers of GFP-positive cells in pancreatic lymph nodes. By contrast, treatment with the natural insulin epitope resulted in a lower increase in numbers of FOXP3$^+$ T_{Reg} cells[61].

For the efficient targeting of T_{Reg} cell induction in autoimmune disorders such as type 1 diabetes, we therefore propose that as well as supporting escape from 'recessive' tolerance, weakly agonistic self-antigens such as insulin epitopes in type 1 diabetes also lack the ability to efficiently induce 'dominant' tolerance through extrathymic induction of FOXP3$^+$ T_{Reg} cells that control the peripheral immune response. It is anticipated that the mimetope-based T_{Reg} cell conversion strategy could be most efficient as a primary vaccination with the goal of inducing insulin-specific FOXP3$^+$ T_{Reg} cells in predisposed individuals who are at a genetic risk of developing diabetes.

The development and use of human-haemato-lymphoid-system mice will provide an excellent accessible system permitting predictive *in vivo* immunology research[184] for further validation of the proposed strategy of FOXP3$^+$ T_{Reg} cell conversion based on the subimmunogenic application of strongly agonistic variants of crucial self-antigens. It has been shown that humanized immunocompromised NOD/SCID/gamma c$^{-/-}$ mice harbour a highly diverse immune T cell receptor repertoire, which is essential for mounting an efficient yet not self-destructive adaptive immune response[186]. The replacement of mouse MHC molecules by human MHC components has been a major advance in increasing the utility of these 'humanized' mice as this permits the generation and maintenance of robust human T cell responses [187].

promoter and at least three enhancers (conserved non-coding sequence 1 (CNS1) to CNS3)[41]. Recent data have suggested that, during evolution, a CNS1-dependent mechanism of extrathymic T_{Reg} cell differentiation emerged in placental animals to enforce maternofetal tolerance[42]. In T_{Reg} cell conversion assays performed *in vitro*, SMAD3 first binds to the CNS1 enhancer before dissociating from CNS1 and temporarily binding to the promoter[43]. Recent studies with *Foxp3*-mutant mice, in which the binding of SMAD3 to CNS1 was abolished, have revealed that *in vitro* T_{Reg} cell conversion assays in the presence of TGFβ do require the binding of SMAD3 to the enhancer, whereas *in vivo* SMAD3 binding to CNS1 is dispensable for T_{Reg} cell generation, with the exception of the gut[44]. In humans, the role of TGFβ in T_{Reg} cell generation from naive T cells is still unclear[45].

Type 1 diabetes
A disorder caused by the autoimmune-mediated destruction of pancreatic islet β-cells, resulting in hyperglycaemia and insulin deficiency at diagnosis.

IL-2 signalling is essential for T_{Reg} cell homeostasis *in vivo*, which has been extensively studied in both IL-2- and IL-2R-deficient mice[46,47]. Recent studies provided evidence that the injection of some IL-2-specific monoclonal antibodies (anti-mouse IL-2 monoclonal antibodies S4B6, JES6-5 and MAB602) into mice resulted in an enhanced biological activity of pre-existing IL-2, as observed by a massive increase in the proliferation of CD8$^+$ T cells[48]. These studies supported the concept that the increase in CD8$^+$ T cell proliferation occurred through the formation of immune complexes. In further experiments it was demonstrated that the injection of mice with IL-2 combined with a particular IL-2-specific monoclonal antibody (JES6-1) rapidly and specifically expanded FOXP3$^+$ T_{Reg} cells[49,50]. The induced T_{Reg} cell expansion was found to be transient, and expanded

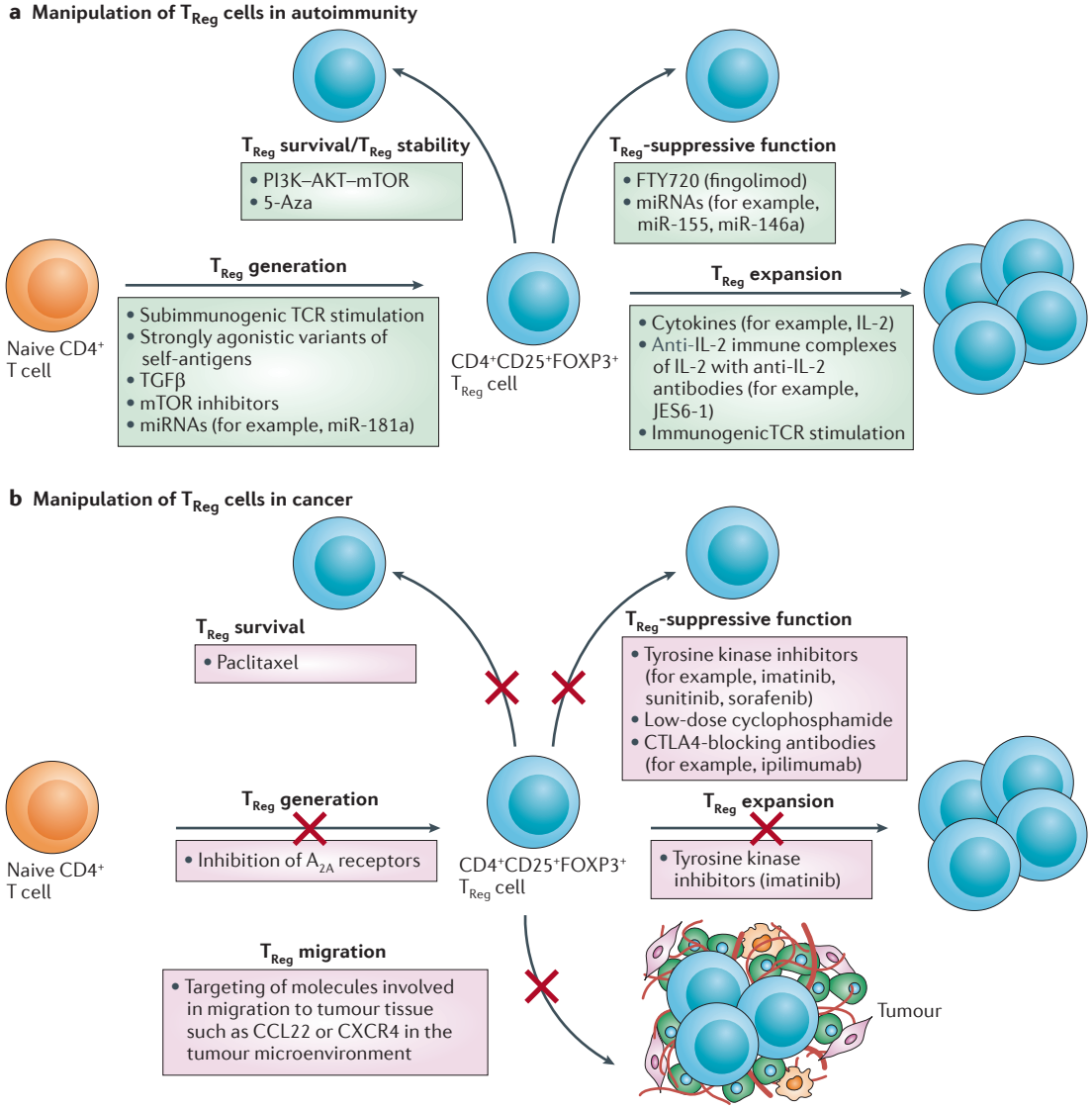

Figure 1 | **General strategies for the manipulation of regulatory T cells in autoimmunity and cancer.** **a** | Approaches to enhance regulatory T (T_{Reg}) cell generation include subimmunogenic T cell receptor (TCR) stimulation using strongly agonistic variants of self-antigens as well as transforming growth factor-β (TGFβ), inhibitors of mammalian target of rapamycin (mTOR) and microRNAs (miRNAs). T_{Reg} cell expansion can be achieved using cytokines such as interleukin-2 (IL-2) as well as by immunogenic antigen application. Manipulation of T_{Reg} cell survival and stability can be achieved using phosphoinositide 3-kinase (PI3K)–AKT–mTOR inhibitors or DNA methyltransferase 1 inhibitors such as 5-aza-2-deoxycytidine (5-Aza), whereas enhancement of T_{Reg}-suppressive functions can be accomplished using drugs such as fingolimod (FTY720) or by targeting miRNAs. **b** | The induction of T_{Reg} cells might be hindered by novel reagents inhibiting adenosine A_{2A} receptors. Blockade of T_{Reg} cell expansion can be achieved using tyrosine kinase inhibitors such as imatinib, which can also help to limit the suppressive function of T_{Reg} cells. Likewise, T_{Reg}-mediated immune suppression can be hindered by the application of low doses of cyclophosphamide or by cytotoxic T lymphocyte antigen 4 (CTLA4)-blocking antibodies such as ipilimumab. Taxanes such as paclitaxel have been found to specifically impair the survival of T_{Reg} cells. Further approaches include the targeting of molecules that are involved in T_{Reg} cell migration to tumour tissue, such as tumour microenvironment CC motif chemokine 22 (CCL22) or CXC chemokine receptor 4 (CXCR4). These strategies have the overall goal of promoting T cell-mediated tumour destruction.

T_{Reg} cells were shown to be highly activated; they harboured superior suppressive functions and survived for 1–2 weeks.

Two recent clinical studies in patients with chronic graft-versus-host disease (GVHD) have found that low-dose IL-2 application is suited to preferentially expand T_{Reg} cells in humans. Twenty-nine patients were treated with low-dose IL-2, which resulted in enhanced T_{Reg} cell numbers in all patients without altering effector T cell numbers. Around half of the individuals showed clinical improvement. Some patients even presented with amelioration in GVHD manifestations that had been

considered to be irreversible. Importantly, IL-2 application did not impair other immune functions[51]. In another study, ten patients with autoimmune vasculitis induced by hepatitis C virus infection were treated with low-dose IL-2. All patients developed increased proportions of T_{Reg} cells, with nine patients showing clinical improvement. Notably, the authors did not report any adverse effects associated with the IL-2 therapy[52].

Self-antigens. Strategies using self-antigens for the specific induction of T_{Reg} cells are intensively studied as they bear the promise of specifically targeting the harmful effects of self-reactive immune cell functions while preserving the ability of the immune system to fight off infections. It is thought that the ability to tightly control and limit the activation of peripheral self-reactive T cells is crucial for the avoidance of autoimmunity such as that observed in type 1 diabetes (BOX 2). The advantage of peptide-specific therapy over other forms of therapy is that it lacks potential metabolic activity and can limit the range of the response to the desired pathogenic epitopes without enhancing the possibility of the hyperactivation of self-reactive T cells. It has been suggested that the route, schedule of administration and ligand dose crucially affect the efficacy of self-antigen-specific tolerance induction[53].

Studies on T_{Reg} cell generation and function *in vitro* as well as in various mouse models have aided the identification and development of targets for efficient T_{Reg} cell manipulation. Such models have shown that TCR ligation is required for the differentiation of naive $CD4^+$ T cells into $FOXP3^+$ T_{Reg} cells — a process that can be induced intra-[54,55] or extrathymically[11,49,56–58]. Currently, it is still unclear whether intrathymic versus extrathymic T_{Reg} cells are generated based on different or overlapping biological functions and whether the requirements for intrathymic versus extrathymic T_{Reg} cell induction are distinct. Moreover, the numerical contributions of intrathymically versus extrathymically induced $FOXP3^+$ T_{Reg} cells to the total T_{Reg} pool under steady-state conditions or in the course of an immune challenge are not yet understood in detail. Several lines of evidence, however, support the hypothesis that extrathymically induced T_{Reg} cells are of crucial importance in organs such as the gut, where continuous T cell stimulation occurs under normal conditions by microbiota in the presence of locally produced TGFβ and retinoic acid derived from dendritic cells[59].

Studies in mice have shown that TCR ligand affinity affects the extrathymic generation of $FOXP3^+$ T_{Reg} cells *in vivo,* and that $FOXP3^+$ T_{Reg} cells induced by low-affinity ligands do not persist[60]. The ideal settings for the extrathymic *in vivo* generation of a stable population of murine $FOXP3^+$ T_{Reg} cells were found to require the delivery of strongly agonistic TCR ligands under subimmunogenic conditions[11,49,56–58,60,61]. By contrast, even immunogenic high doses of weakly agonistic ligands failed to generate a persistent population of $FOXP3^+$ T_{Reg} cells, supporting the concept that TCR ligand density cannot compensate for limited agonistic activity in determining the efficacy of the induction and persistence of $FOXP3^+$ T_{Reg} cells. This scenario might be of special relevance in autoimmune diseases (for example, type 1 diabetes) in which weakly agonistic ligands have been found to limit the induction of $FOXP3^+$ T_{Reg} cells. Strong T cell activation negatively influenced extrathymic T_{Reg} cell induction owing to a cell-cycle-dependent sustainment of a silenced state of the *FOXP3* locus[62].

The most efficient *de novo* conversion into $FOXP3^+$ T_{Reg} cells by subimmunogenic stimulation was achieved in T cells that proliferated least extensively[58]. Animal models have also shown that T_{Reg} cells that are extrathymically induced can be expanded by antigen delivery[58,63]. It was found that re-encounter of an antigen, even if this occurs in an immunogenic context, does not induce the elimination of T_{Reg} cells or reduce their activity[63,64], but instead leads to T_{Reg} cell proliferation[58]. Using TCR-transgenic mouse models and subimmunogenic antigen application, extrathymically induced T_{Reg} cells were shown to exhibit a long lifespan accompanied by stable FOXP3 expression[39].

These findings indicate that quantitative and qualitative differences in the strength of the interactions between TCR and major histocompatibility complex (MHC) peptides crucially affect signalling events downstream of ligand recognition by the TCR and thereby instruct the cell fate decision in peripheral T cells[65]. It is assumed that subimmunogenic conditions do not activate the pathway involving phosphoinositide 3-kinase (PI3K), AKT and mammalian target of rapamycin (mTOR)[60,66,67] (as discussed below), which is in agreement with studies indicating that constitutive activation of AKT limits the stable induction of FOXP3 expression[68].

In humans, *in vitro* studies using naive $CD4^+$ T cells have shown that CD25 and FOXP3 expression is induced following strong cell activation through the TCR. However, the upregulation of FOXP3 in response to such experimental activation is transient, only induces low levels of FOXP3 and does not confer suppressive capacities[69]. Stable and high expression of FOXP3 is required in order to maintain the suppressive capacities of human T_{Reg} cells[70,71].

More studies are required to gain an improved understanding of how the subimmunogenic application of self-antigens for the efficient and stable induction of $FOXP3^+$ T_{Reg} cells can be best achieved in autoimmune diseases. These efforts might also include novel strategies for the application of self-antigens — for example, the use of dissolving microneedle patches[72], which were recently tested for the administration of insulin to individuals with type 1 diabetes[73].

The PI3K–AKT–mTOR network. The serine/threonine kinase mTOR has been shown to have a central role in the regulation of the immune response, and the PI3K–AKT–mTOR signalling pathway was found to suppress the induction of FOXP3 expression *in vitro* and *in vivo*[66]. Efficient upregulation of FOXP3 expression requires proteins such as the E3 ubiquitin protein ligase CBLB[74] or phosphatase and tensin homolog (PTEN), which antagonize PI3K function and inhibit downstream

signalling through AKT[4,67]. However, the specific conditions required for the optimal induction of FOXP3 following inhibition of the AKT–mTOR pathway still remain to be fully understood.

Conversely, constitutive activation of AKT was shown to suppress the stable induction of FOXP3 expression[68]. Animal models have shown that $CD4^+$ T cells that lack mTOR fail to differentiate into effector T cells under appropriate skewing conditions. Instead, following their activation, mTOR-deficient T cells differentiate into $FOXP3^+$ T_{Reg} cells[75]. Recently, it was shown that signalling through mTOR complex 1 (mTORC1) results in T_H1 and T_H17 cell differentiation, whereas signalling through mTORC2 promotes T_H2 cell differentiation[75].

The activity of the PI3K–AKT–mTOR network can be manipulated by pharmacological compounds, several of which are approved for clinical use. Rapamycin — an allosteric inhibitor of mTOR that was approved as an immunosuppressant in 1999 — was originally identified as an antifungal compound derived from *Streptomyces hygroscopicus*, found in soil samples collected from Easter Island (Rapa Nui) in 1965 (REF. 76). However, rapamycin had very limited antibiotic properties, but closer inspection revealed its potent immunosuppressive characteristics[77]. Various studies demonstrated that application of the mTOR inhibitor rapamycin and its analogue everolimus (Afinitor; Novartis) resulted in a specific promotion of $FOXP3^+$ T_{Reg} cell induction *in vitro*[49,78] and *in vivo*[49,57]. These drugs also increased the stability of FOXP3 expression and enhanced the survival of T_{Reg} cells[49].

Everolimus, also known as RAD-001, is a hydroxyethyl ether derivative that has been developed for oral administration. Rapamycin- or everolimus-mediated enhancement of $FOXP3^+$ T_{Reg} cell induction was shown to be caused by the interference of the drugs with the activation of the PI3K–mTOR signalling pathway and by their ability to limit cellular proliferation and dampen T cell activation mediated by the ATP-gated P2X purinergic receptor 7, which controls calcium influx[49]. Moreover, rapamycin or everolimus may affect activated conventional T cells more prominently than T_{Reg} cells by inhibiting the proliferation or survival of the former and thus enriching the latter[79].

It was suggested that rapamycin specifically limited mTORC1 activity without affecting mTORC2. Based on these findings, rapamycin was thought to promote T_{Reg} cell differentiation by blocking the activation of mTORC1. Recent findings, however, have shown that mTOR inhibitors such as rapamycin or rapalogues such as everolimus can inhibit mTORC2 as well, leading to lowered activity of AKT *in vitro* and *in vivo*[80–83]. These data are in accordance with recent results on the impact of FOXO transcription factors in regulating FOXP3 expression[84,85] (FIG. 2). Phosphorylation by AKT was demonstrated to inactivate FOXO transcription factors by excluding them from the nucleus. Likewise, mTORC2-dependent phosphorylation of FOXO1 and FOXO3A results in their inactivation by promoting their sequestration in the cytoplasm[66]. It became apparent that both of these transcription factors are crucial for the induction of FOXP3 expression[84,85], and several conserved FOXO consensus binding motifs were identified in the promoter region of the *FOXP3* locus[84,85].

Accordingly, in the presence of mTORC2 activation, FOXO1- and FOXO3A-mediated FOXP3 expression is limited. Rapamycin- or everolimus-mediated inhibition of mTORC2 and AKT activity may therefore facilitate the maintenance of active FOXO transcription factors within the nucleus in order to promote the induction of FOXP3. Therefore, it has been hypothesized that ATP-competitive inhibitors of the kinase domain of mTOR that are suited to inhibit both mTORC1 and mTORC2, such as WYE132 or Torin 2 (which are currently in development), might also specifically induce T_{Reg} cells[86]; future studies are required to address this question.

The PI3K–AKT–mTOR signalling pathway can also be targeted with drugs that affect downstream factors such as FOXO proteins and support their nuclear translocation[84,85,87]. As an example, pyrazolopyrimidine derivatives were found to be potent FOXO nuclear relocators as well as biochemical inhibitors of PI3K. A combination of virtual screening and molecular modelling provided insights into a structure–activity relationship, which indicated the preferred substituents on the pyrazolopyrimidine scaffold for optimal activity. Further studies permitted the synthesis of ETP-45658, which was demonstrated to function as a potent and selective inhibitor of PI3K[88]. Its potential role in T_{Reg} cell induction warrants further studies.

It is of interest that rapamycin derivatives such as temsirolimus have also been developed as anticancer agents based on their strong cytostatic effects. As discussed above, the induction of FOXP3 following antigenic stimulation was demonstrated to be most efficient when T cells proliferate least extensively[49,58]. Therefore, agents that limit T cell proliferation can hinder cell-cycle-dependent recruitment of DNA methyltransferase 1 (DNMT1) to the *FOXP3* locus, opposing its inactivation[62,89]. However, studies in mice have suggested that the continuous application of mTOR inhibitors such as rapamycin after immune stimulation impedes the proliferation of both effector T cells and T_{Reg} cells[90].

The S1PR1–mTOR axis. Sphingosine-1-phosphate receptor 1 (S1PR1) — a G protein-coupled receptor for the bioactive lipid sphingosine-1-phosphate (S1P) — is a critical regulator of T cell egress from the thymus and secondary lymphoid organs[91]. It has become clear that mice with haematopoietic cells lacking S1PR1 have no T cells in the periphery because mature T cells are unable to exit the thymus[91].

Studies performed *in vitro* and *in vivo* in mice have revealed that S1PR1 activates AKT and mTOR in T_{Reg} cells, which deliver a cell-intrinsic negative signal to restrain T_{Reg} cell generation, survival and suppressive activity[92]. In conventional $CD4^+$ T cells, S1PR1 was shown to be dispensable for acute mTOR activation but required for sustained mTOR activity during the differentiation of these cells into effector T cells. S1PR1-dependent activation of mTOR was demonstrated to hinder the generation of T_{Reg} cells while promoting T_H1 cell differentiation in a

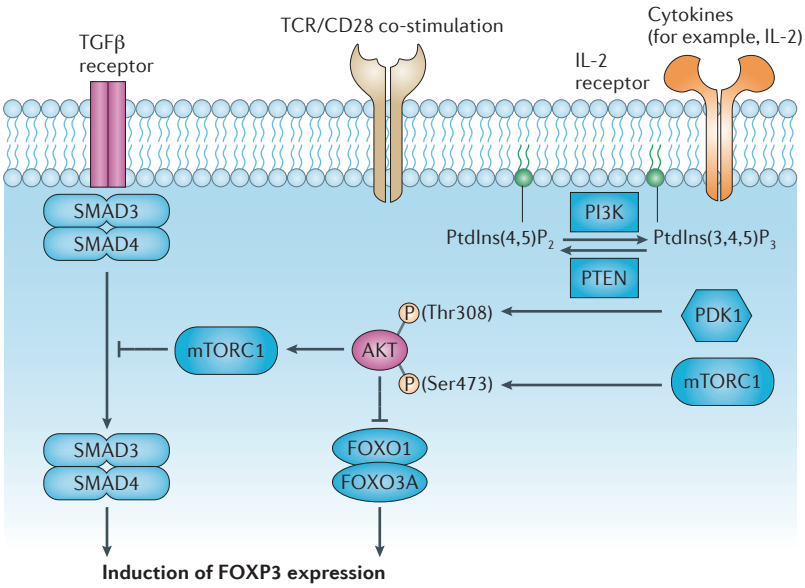

Figure 2 | **PI3K–AKT–mTOR signalling to block the generation of FOXP3$^+$ T_{Reg} cells.** T cell receptor (TCR) stimulation, CD28 co-stimulation and cytokine signalling contribute to the activation of the signalling network mediated by phosphoinositide 3-kinase (PI3K), AKT and mammalian target of rapamycin (mTOR). AKT is activated upon phosphorylation of Thr308 by 3-phosphoinositide-dependent kinase 1 (PDK1) and phosphorylation of Ser473 by mTOR complex 2 (mTORC2). mTORC2 is crucial for the full activation of AKT by the phosphorylation of Ser473, and therefore AKT can be both upstream of mTORC1 and downstream of mTORC2. AKT induces the activation of mTORC1, which inhibits regulatory T (T_{Reg}) cell generation by antagonizing SMAD signalling. Active AKT phosphorylates forkhead box O (FOXO) proteins, which triggers their exclusion from the nucleus and their sequestration into the cytoplasm, where they cannot access their target genes, such as FOXP3. IL-2, interleukin-2; PtdIns(3,4,5)P_3, phosphatidylinositol-3,4,5-trisphosphate; PtdIns(4,5)P_2, phosphatidylinositol-4,5-bisphosphate; PTEN, phosphatase and tensin homolog; TGFβ, transforming growth factor-β.

reciprocal manner[6,92] (FIG. 3). Inhibition of T_{Reg} cell generation following mTOR activation was shown to be at least partially due to the fact that mTOR antagonizes SMAD3, which — as outlined above — can be involved in the induction of FOXP3 downstream of TGFβ signalling[92,93].

FTY720, also known as fingolimod (Gilenya; Novartis), was demonstrated to induce the sequestration of T cells in lymphoid organs by interacting with S1PR1. FTY720 is a structural analogue of S1P and a recently developed immune-modulator that has been approved for the treatment of relapsing–remitting multiple sclerosis. It is known to also bind to S1PR3, S1PR4 and S1PR5 but it does not bind to S1PR2. It has been shown that FTY720 treatment downregulates S1PR1 expression in mice and creates a temporary pharmacological S1PR1-null state in lymphocytes, providing an explanation for FTY720-induced lymphocyte sequestration[91].

Analysis of chiral analogues of FTY720 showed that FTY720-phosphate (FTY720-P) is the biologically active form and that it is phosphorylated primarily by sphingosine kinase 2 (SPK2)[94] to produce *S*-FTY720-P[95]. Experiments in rats and humans have demonstrated that only the biologically active *S*-configured enantiomer becomes phosphorylated, whereas the *R*-enantiomer is not phosphorylated[96]. Whether FTY720 acts as an agonist or a functional antagonist, or both, has been controversial[95,97]. FTY720 has been shown to increase the functional activity of FOXP3$^+$ T_{Reg} cells[3,98,99] and to promote their generation *in vivo*[92,93]. These effects of FTY720 appeared to mimic S1PR1 deficiency, which suggests that FTY720 may act as a functional antagonist of S1PR1 in T_{Reg} cells. Therefore, it appears reasonable to further explore the S1PR1–mTOR axis in T_{Reg} cells for the development of new therapeutics.

As FTY720-mediated immune regulation is mainly mediated by its interaction with S1PR1 (REF. 91), strategies have emerged to develop next-generation S1PR modulators that selectively target S1PR1. As one example, the S1PR1-selective agonist SEW2871 is structurally unrelated to S1P but is capable of activating multiple signals that are triggered by S1P[100]. Both SEW2871 and S1P were shown to activate extracellular signal-regulated kinase (ERK), AKT and RAC signalling pathways and induce S1PR1 internalization and recycling[100]. SEW2871 stimulates lymphocyte trafficking *in vitro* and induces lymphopenia in mice via an S1PR1-dependent mechanism[101]. Thus far, its impact on T_{Reg} cell generation has not been investigated.

Another S1PR1-selective agonist, KRP-203, has structural similarity to FTY720. In animal models of organ transplantation, KRP-203 prolonged skin and heart allograft survival and attenuated chronic rejection[102]. Notably, because KRP-203 selectively targets S1PR1 without affecting S1PR3, the use of KRP-203 could potentially limit some of the adverse effects associated with FTY720 (REF. 103). Moreover, several antagonists that interfere with the activation of S1PR by S1P — such as the S1PR1 antagonist 3-amino-4-(3-hexylphenylamino)-4-oxobutylphosphonic acid (W146) — have recently been described[104–106]. However, although these compounds are useful for basic research, it has been suggested that they are not suitable as drugs owing to their toxicity[107,108].

miRNAs. miRNAs are a class of evolutionarily conserved small non-coding RNAs that control gene expression by targeting mRNAs for degradation or translational repression. miRNA control has emerged as a crucial regulatory principle in the mammalian immune system[109]. This was demonstrated in studies involving the T cell-specific deletion of a conditional allele of Dicer 1, encoding an RNase III enzyme that is essential for the generation of mature miRNAs. Dicer-deficient T cells had a decreased proliferative potential and showed increased apoptosis in response to activation. Moreover, it became clear that the Dicer-dependent miRNA pathway regulates effector CD4$^+$ T cell differentiation[110,111] and is required for both the generation and suppressive function of T_{Reg} cells. Ablation of either Dicer or Drosha, both miRNA precursor-processing enzymes, in T_{Reg} cells resulted in early-onset fatal autoimmunity that was identical to the lethal autoimmune symptoms observed in FOXP3-deficient mice devoid of T_{Reg} cells[112,113]. These studies demonstrated a central role for the Dicer-dependent miRNA

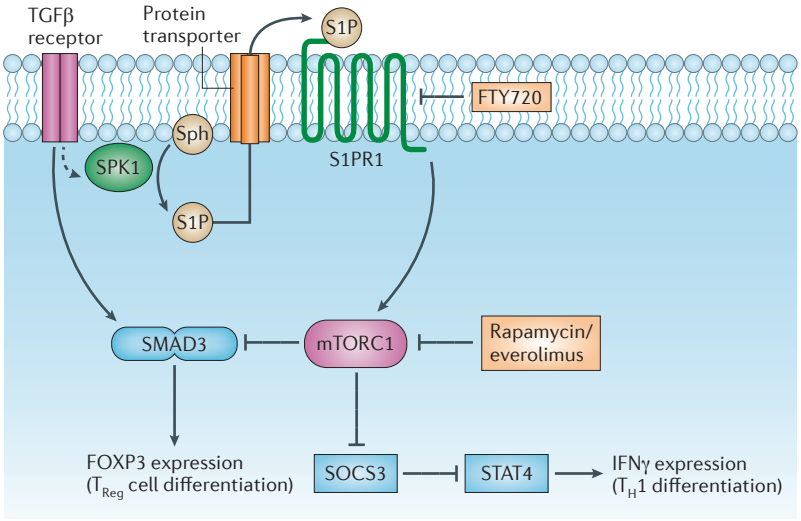

Figure 3 | **Possible scheme for S1P–S1PR1-dependent regulation of T_{Reg} versus T_H1 cell differentiation.** Transforming growth factor-β (TGFβ) induces the expression of sphingosine kinase 1 (SPK1) and the initiation of the axis involving sphingosine-1-phosphate (S1P), S1P receptor 1 (S1PR1) and mammalian target of rapamycin (mTOR) following the phosphorylation of sphingosine (Sph) to S1P. Among other signals, mTOR — and specifically mTOR complex 1 (mTORC1) — can be activated by S1P. mTORC1 inhibits SMAD signalling, thereby antagonizing regulatory T (T_{Reg}) cell differentiation. mTORC1 blocks the induction of suppressor of cytokine signalling 3 (SOCS3), which is an important player in the regulation of signal transducer and activator of transcription 4 (STAT4) and promotes T_H1 cell differentiation[92]. FOXP3, forkhead box P3; IFNγ, interferon-γ.

pathway in T_{Reg} cell biology, but our understanding of the role of individual miRNAs in T_{Reg} cell generation and function is still incomplete.

Previous studies in mice had identified a set of differentially expressed miRNAs in T_{Reg} cells and non-T_{Reg} cells[110] — such as miRNA-155 (miR-155), which is highly expressed in T_{Reg} cells. *In vivo* analyses of miR-155-deficient animals demonstrated the crucial involvement of this miRNA in T_{Reg} cell homeostasis in the presence of limiting amounts of IL-2, whereas it appeared to be dispensable under non-competitive lymphopenic settings[114]. These conclusions were drawn from bone marrow transplantation experiments involving miR-155-deficient and miR-155-sufficient bone marrow transfer into lymphopenic and lymphoreplete recipients. It became clear from these sets of experiments that miR-155 is needed to maintain the proliferative fitness of T_{Reg} cells in lymphoreplete mice, in which T_{Reg} cells compete for limiting growth factors such as IL-2, but not in lymphopenic mice, in which there is no competition for growth factors.

By contrast, miR-155 was shown to be largely dispensable for the suppressive function of T_{Reg} cells. These studies suggested that FOXP3-dependent regulation of miR-155 ensures efficient phosphorylation of signal transducer and activator of transcription 5 (STAT5) in the presence of limiting amounts of IL-2 and thereby supports the competitive fitness of T_{Reg} cells. STAT5, which is activated downstream of the IL-2R and other common γ-chain cytokine receptors, was proposed to represent a transcription factor that is involved in the direct regulation of FOXP3 expression[115]. Moreover, STAT5 was shown to directly bind to the FOXP3 promoter and a FOXP3 CNS element. Furthermore, studies by Burchill[116] and Yao[117] demonstrated that, following the induced ablation of a conditional *Stat5* allele in $CD4^+CD8^+$ double-positive thymocytes, a substantial reduction in $CD4^+$ single-positive FOXP3-expressing thymocytes could be seen.

Similarly to miR-155, miR-146a was identified to be mainly expressed in T_{Reg} cells[118] and induced following the activation of effector T cells and myeloid cells. In effector T cells, miR-146a was demonstrated to function as a negative regulator to limit signalling mediated by TNF receptor-associated factor 6 (TRAF6) as well as IL-1R-associated kinase 1 (IRAK1) and IRAK2 in inflammatory conditions[119,120]. Recent studies have demonstrated an indispensable role of miR-146a in the suppressive function of T_{Reg} cells in mice *in vivo*[118]. Mechanistically, it has been shown that massive activation of STAT1, which is a key transcription factor required for T_H1 effector cell differentiation, is controlled in T_{Reg} cells by miR-146a. This mechanism controls interferon-γ (IFNγ)-dependent T_H1-mediated immune pathology and prevents T_{Reg} cells from turning into IFNγ-producing T_H1-like cells[118].

In contrast to miR-155 and miR-146a, which act downstream of cytokine signalling, miR-181a was found to have an important role in modulating TCR antigen recognition and signalling thresholds[121,122]. Overexpression of miR-181a in mature $CD4^+$ T cells was shown to increase ERK activity and to convert antagonistic peptides into agonists. The future design of miRNA antagonists or agonists and their co-delivery with antigens *in vivo* could possibly facilitate $CD4^+$ T_{Reg} cell induction in humans by modulating antigen recognition thresholds. As miRNAs control — in a dose-dependent manner — cellular states or responses such as T_{Reg} cell function and fitness by regulating several proteins simultaneously, they could be attractive targets for future drug discovery and medical interventions aimed at T_{Reg} cells.

Epigenetic regulation of the* FOXP3 *locus. The generation and stability of T_{Reg} cells can also be directly controlled by the epigenetic regulation of the *FOXP3* locus. The human *FOXP3* locus contains four conserved non-coding regions that are the sites of epigenetic regulation of FOXP3 transcription. The T_{Reg}-specific demethylated region (TSDR) — a CpG-rich intronic enhancer region — contains a T_{Reg}-specific pattern of CpG methylation. In humans and mice, the TSDR is completely demethylated in T_{Reg} cells but is fully methylated in resting conventional T cells, $FOXP3^{low}$ activated conventional T cells and in T_{Reg} cells induced *in vitro* by treatment with TGFβ. It is assumed that the methylation status of the TSDR region determines the long-term stability of FOXP3 transcription.

It was shown that forced CpG demethylation through short-term treatment with the DNMT1 inhibitor 5-aza-2-deoxycytidine (5-Aza) and mTOR inhibitors such as rapamycin or everolimus can induce stable FOXP3 expression in conventional T cells. At high

TNF receptor-associated factor 6
(TRAF6). A protein that is associated with, and mediates, signal transduction from members of the tumour necrosis factor (TNF) receptor superfamily, and also from members of the Toll-like receptor and interleukin-1 receptor family. TRAF6 also interacts with various protein kinases, including interleukin-1 receptor-associated kinase 1 (IRAK1).

IL-1R-associated kinase 1
(IRAK1). Along with IRAK2, IRAK1 is a putative serine/threonine kinase that becomes associated with the interleukin-1 receptor (IL-1R) upon stimulation and is responsible for IL-1-induced upregulation of the transcription factor nuclear factor-κB.

doses, 5-Aza and rapamycin have a strong cytostatic effect, mediating anticancer properties. Related to this dose-dependent dual role of 5-Aza and rapamycin, it is interesting to note that the induction of FOXP3 following subimmunogenic antigen application is inversely correlated with cellular proliferation[39,49,58]. Consequently, compounds that inhibit cellular proliferation can antagonize the cell-cycle-dependent recruitment of DNMT1 to the *FOXP3* locus and thereby permit an active state of the *FOXP3* gene[62,89,123]. Accordingly, pharmacological inhibition of DNMT1 was found to increase both the induction and, crucially, the stability of *FOXP3* expression[8,39,62,89]. Future studies are required in which such tests are performed in an autoimmune disease setting.

Targeting T_{Reg} cells in cancer

T_{Reg} cell-mediated suppression of tumour-associated antigens has been proposed as a potential mechanism to explain the failure of antitumour immunity, and tumour-induced expansion of T_{Reg} cells was found to be an obstacle to successful cancer immunotherapy[124]. T_{Reg} cells have been shown to selectively interfere with the release of cytolytic granules by cytotoxic T lymphocytes (CTLs) in a reversible and TGFβ-dependent manner, thereby attenuating CTL-mediated cytotoxicity without detectably affecting CTL priming or differentiation[125]. This finding has implications for the design of therapeutic strategies that rely on the modulation of ongoing immune responses. Local or systemic interference with suppressor pathways of CTL activity, such as cytokines[126], Toll-like receptor agonists[127,128] or T_{Reg} cell depletion[129], may be effective in tumour therapy, in which immunity is desired.

Beyond their cytostatic characteristics, some conventional chemotherapeutic agents were found to affect the adaptive immune system, resulting in the inhibition of T_{Reg} function or viability and thereby enhancing antitumour immunity[34–36]. In line with these observations, administration of low-dose cyclophosphamide was demonstrated to selectively suppress inhibitory cell subsets including T_{Reg} cells, which could help induce anticancer immunity[130,131]. Paclitaxel — the taxane-based chemotherapeutic that is currently most widely used in the clinic — was found to specifically impair cytokine production and viability in $FOXP3^+$ T_{Reg} cells but not in $FOXP3^-$ $CD4^+$ effector T cells[132]. These findings indicate that some chemotherapeutic compounds possess immunosuppressive properties and that their therapeutic potential might, at least partially, be due to the modulation of antitumour immunity, including the impairment of T_{Reg} cells[34–36].

The immunogenic effects of more specific molecularly targeted therapeutic anticancer agents are also being recognized. However, the underlying molecular mechanisms of these immunomodulatory outcomes are far from being understood in detail[34]. For example, it was found that imatinib (Gleevec; Novartis), a tyrosine kinase inhibitor, blocked STAT3 and STAT5 signalling, decreased T_{Reg} cell frequency and impaired their suppressive functions at clinically relevant doses *in vivo*[133]. Other tyrosine kinase inhibitors that may induce an antitumour immune response by modulating T_{Reg} function include sunitinib (Sutent; Pfizer) and sorafenib (Nexavar; Bayer/Onyx), which limited infiltration by T_{Reg} cells while inhibiting STAT3 activity[134,135]; this was shown to decrease FOXP3 expression and to prevent the acquisition of suppressive functions in T_{Reg} cells[136]. Disrupted STAT signalling has been associated in several cases with decreased immune suppression by T_{Reg} cells, thereby promoting an efficient anticancer immune response[137]. These results support the concept that some of the targeted anticancer agents impair T_{Reg} cells, thereby antagonizing tumour-mediated immune suppression[34,138].

Ipilimumab (Yervoy; Bristol-Myers Squibb), an antibody that is specific for cytotoxic T lymphocyte antigen 4 (CTLA4), blocks an important inhibitory signal for activated T cells, thereby bolstering T cell responses and potentiating tumour destruction[139]. Even though CTLA4 is expressed by activated $CD8^+$ T cells, its major physiological role appears to be mediated by distinct effects on two major subsets of $CD4^+$ T cells: the inhibition of T_H cell activity and the enhancement of the immunosuppressive capacity of T_{Reg} cells. *CTLA4* is a target gene of FOXP3, and T_{Reg} cell-specific CTLA4 knockout or blockade of CTLA4 in T_{Reg} cells inhibits the ability of these cells to regulate autoimmunity and antitumour immunity. Thus, in considering the mechanism of action of CTLA4-specific antibodies, both enhancement of effector T cell activity and inhibition of T_{Reg} cell-dependent immune suppression should be discussed[139,140].

Initially, the approach of blocking CTLA4 in the cancer setting was questioned because there is no tumour specificity for the expression of CTLA4 ligands[124] and because CLTA4-knockout animals revealed a dramatic lethal autoimmune phenotype that was predictive of a high degree of immune toxicity associated with the blockade of this receptor. However, studies in preclinical models using tumour-bearing mice treated with CTLA4-specific antibodies showed that a therapeutic window could be achieved, inducing substantial antitumour responses without causing overt immune toxicities[141]. Ipilimumab has recently been approved by the US Food and Drug Administration (FDA) for the treatment of patients with advanced melanoma; it has also been approved by the European Medicines Agency (EMA) and regulatory agencies from various other countries.

Studies that aim to deplete T_{Reg} cells in patients with cancer are hampered by the fact that T_{Reg} cells do not express an exclusive surface molecule that can be targeted. Strategies for T_{Reg} cell depletion in patients with cancer have largely focused on agents that target the IL-2R, which is upregulated on T_{Reg} cells. The monoclonal antibody daclizumab (Zenapax; PDL BioPharma) targets IL-2Rα, and denileukin diftitox (Ontak; Esai) is a fusion protein of IL-2 and diphtheria toxin. Clinical studies in patients with melanoma and prostate cancer, where these agents have been combined with vaccines, have revealed conflicting results[142–146]. These data could probably be best explained by the fact that IL-2R is upregulated not only on T_{Reg} cells but also on activated effector T cells. Moreover, the timing of the two

Cytotoxic T lymphocyte antigen 4
(CTLA4). A member of the immunoglobulin superfamily. CTLA4 is expressed on the surface of T helper cells and transmits an inhibitory signal to T cells.

CC motif chemokine 22
(CCL22). A protein that is encoded by the *CCL22* gene in humans. CCL22 is secreted by dendritic cells and macrophages, and elicits its effects on its target cells by interacting with cell surface chemokine receptors such as CC chemokine receptor 4.

CXC chemokine receptor 4
(CXCR4). An α-chemokine receptor that is specific for CXC motif chemokine ligand 12 (CXCL12; also known as SDF1), a molecule with potent chemotactic activity for lymphocytes.

CXC motif chemokine ligand 12
(CXCL12; also known as SDF1). A small cytokine belonging to the chemokine family, members of which are often induced by pro-inflammatory stimuli such as lipopolysaccharides, tumour necrosis factor or interleukin-1.

application regimes appears to be critical[144,145]. Novel strategies are needed that permit the specific targeting of T_{Reg} cells and the elimination of their suppressive function.

Other potential approaches to control T_{Reg} cell function include the targeting of molecules that are involved in T_{Reg} cell migration. In ovarian cancer, CC motif chemokine 22 (CCL22) in the tumour microenvironment mediates the trafficking of T_{Reg} cells into the tumour, and CCL22 blockade was found to reduce T_{Reg} cell-mediated tumour trafficking[147]. In line with this finding, it became clear that the numbers of functional T_{Reg} cells are increased in the bone marrow microenvironment in patients who have prostate cancer with bone metastasis, and that CXC chemokine receptor 4 (CXCR4) and CXC motif chemokine ligand 12 (CXCL12) contribute to T_{Reg} cell migration to the bone marrow[148].

More recently, the adenosine A_{2A} receptor (encoded by the *ADORA2A* gene) received attention owing to its ability to inhibit T cell responses in part by upregulating FOXP3 expression in $CD4^+$ T cells[149]. Deletion of this receptor was found to result in enhanced pathological inflammatory responses to infection. This receptor could be of particular interest for targeting T_{Reg} cells in tumour immunity as the rate of cell death in tumours from cell turnover is high and these dying cells release adenosine. Based on the fact that the engagement of adenosine A_{2A} receptor by adenosine results in T_{Reg} cell induction, this could result in a self-amplifying loop within the tumour. Tumours were indeed found to grow more slowly in *Adora2a*-knockout mice[150]. Inhibition of adenosine A_{2A} receptor can be achieved using antibodies to block adenosine or using adenosine analogues. These drugs have been used in clinical trials for the treatment of Parkinson's disease; they have, however, not yet been clinically tested in patients with cancer[124].

Outlook and future directions

Fundamental insights into the role of T_{Reg} cells in immune tolerance, specifically the progress in our knowledge of antigen-specific $FOXP3^+$ T_{Reg} cell induction and function, provide a rich opportunity for the development of clinical protocols aimed at the specific prevention of unwanted immunity or the development of immunotherapeutic strategies for cancer.

Currently, the majority of strategies approved by the FDA for autoimmune diseases have focused on nonspecific immune suppression. Although this was found to be partially effective in inhibiting autoreactive immune cell function, these compounds have numerous side effects and long-term treatment is challenging.

Strategies that boost the development of T_{Reg} cells or induce their antigen-specific generation permit the specific blockade of the deleterious effects of self-reactive immune destruction while maintaining the ability of the immune system to clear non-self-antigens. Although some progress has been made in the development of self-antigen-specific tolerance induction, translating these strategies from the bench to the bedside has so far remained difficult. Results from clinical trials using natural self-antigens for the induction of self-tolerance — for example, natural insulin in the case of autoimmune type 1 diabetes — have mainly been disappointing[151–154], which might possibly be due to the fact that these approaches did not specifically explore the strategy of inducing $FOXP3^+$ T_{Reg} cells. The most promising finding has been with the oral administration of insulin to high-titre insulin autoantibody-positive children in the DPT-1 study, in which subsequent subgroup analysis of data revealed that there was a delay in the progression to type 1 diabetes in these patients[153]. In line with these findings, recent studies highlighted that oral immunotherapy was also suited for the treatment of egg allergy in children[155]. The results showed that oral immunotherapy can desensitize a high proportion of children with egg allergy and can induce sustained unresponsiveness in a clinically significant subset.

Additionally, the translation of antigen-specific tolerance strategies into tailored therapies for individual patients with autoimmune diseases has been hindered by several technical challenges. First, suitable target antigens need to be identified. Although this process is advanced in certain diseases, such as type 1 diabetes, these antigens are poorly characterized in other diseases, such as rheumatoid arthritis. Another limitation of the studies in autoimmune type 1 diabetes has been that none of these studies actually determined whether the choice of antigen, the time point and the route of administration indeed induced a tolerogenic response. Moreover, the approaches used did not explore the conditions that permit the specific conversion of naive T cells into $FOXP3^+$ T_{Reg} cells. It has been proposed that the efficacy of the tolerance induction regime may crucially depend on: the disease state, antigen dosage, route of administration, the study cohort that is treated as well as the choice of antigen — for example, insulin versus insulin B-chain peptides in the case of type 1 diabetes[156,157].

The development of strategies that inhibit the signalling intermediates of T cell activation (as discussed above) when combined with antigen-specific tolerance therapies presents an auspicious combinatorial approach that may enhance the efficacy of T_{Reg} cell induction while maintaining antigen specificity. Continued research to improve our understanding of the underlying molecular mechanisms of tolerance and to enhance the specificity and efficacy of each of these strategies, with a special focus on combinatorial approaches, will probably be required in order to deal with the complexity of the human immune system. Moreover, advanced patient screening via genomic and pharmacogenomic techniques will further support the establishment of successful T_{Reg} cell targeting in autoimmunity, thereby eventually providing tailored therapies in order to interfere with unwanted immune responses.

With regard to cancer, recent positive results of clinical trials with novel immunoactive drugs that — among others — target T_{Reg} cells, as well as the observation that classical chemotherapeutic agents can affect the generation of T_{Reg} cells, indicate that T_{Reg} cells may be an important target for new immunotherapeutic strategies.

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Competing interests statement
The authors declare no competing financial interests.

FURTHER INFORMATION
Carolin Daniel's homepage: http://www.helmholtz-muenchen.de/en/idf1/working-groups/type-1-diabetes-immunological-tolerance/index.html

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