Abstract: Foxp3 + regulatory T cells (Tregs) maintain immune tolerance, prevent autoimmunity and modulate immune responses during infection and cancer. Recent studies have revealed considerable heterogeneity and plasticity within the Treg compartment, depending on the immunological context, which may result in Tregs losing their suppressive function in inflammatory environments. We review how dysfunctional Tregs contribute to disease pathogenesis in inflammatory conditions and how inappropriate regulatory responses may hamper protective immunity in the context of infection and cancer. We also discuss how Tregs might be targeted therapeutically to re-establish a proper balance between regulatory and effector responses in autoimmunity, infections, and cancer.

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Regulatory T cells: balancing protection versus pathology

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Summary

Foxp3+ regulatory T cells (Tregs) maintain immune tolerance, prevent autoimmunity and modulate immune responses during infection and cancer. Recent studies have revealed considerable heterogeneity and plasticity within the Treg compartment, depending on the immunological context, which may result in Tregs losing their suppressive function in inflammatory environments. We review how dysfunctional Tregs contribute to disease pathogenesis in inflammatory conditions and how inappropriate regulatory responses may hamper protective immunity in the context of infection and cancer. We also discuss how Tregs might be targeted therapeutically to re-establish a proper balance between regulatory and effector responses in autoimmunity, infections, and cancer.

Key words: regulatory T cells; autoimmunity; infections; cancer

Introduction

Regulatory T cells (Tregs) are a subset of CD4+ T cells that are crucial for immune homeostasis. Tregs are defined by their expression of the transcription factor forkhead-box protein P3 (Foxp3), which is essential for their development and suppressive function [1]. Loss of Foxp3 function leads to severe lymphoproliferative disease and autoimmunity, and highlights the essential role of Tregs in maintaining immune tolerance [2, 3]. In addition to preventing autoimmunity and inflammatory diseases, Tregs ensure a controlled immune response upon pathogen encounter and thereby prevent immune pathology. Conversely, excessive suppression by Tregs can hamper pathogen clearance and promote chronic infection [4, 5]. In addition, Tregs can also restrain anti-tumour immune responses and thus promote tumour progression [6]. Properly balanced Treg function and activation is therefore essential to prevent immune pathology but allow for protective immune responses against tumours and pathogens.

To accomplish these tasks, Tregs must adapt to their immune environment and specialise into subsets with distinct functional properties that work together to ensure an adequate immune response while maintaining immune tolerance [7]. However, the plasticity required for this specialisation also bears the risk of instability, and recent studies have revealed the ability of Tregs to lose suppressive and acquire effector function [8, 9]. These observations reveal a potential causative role of unstable or dysfunctional Tregs in inflammatory settings. Therefore, understanding the factors that control Treg stability, plasticity and function is an important step towards improving safety and efficacy of therapeutic applications directed against Tregs. In this review, we discuss the role of Tregs in autoimmunity, infectious diseases, and cancer by addressing: the importance of a fine-tuned regulatory response for maintaining a healthy balance between effector and regulatory T cell responses; Treg specialisation and heterogeneity and its impact on Treg function and stability in inflammatory settings; therapeutic approaches used to manipulate Treg numbers and function.

Regulatory T cells in autoimmunity

The importance of Tregs in preventing autoimmunity becomes evident in patients with IPEX (immunodysregulation polyendocrinopathy enteropathy X-linked syndrome), who suffer from multi-organ autoimmunity because of a mutation in the FOXP3 gene and hence impaired Treg function [2, 3]. In addition, perturbations in the pathways of the Treg suppressive network, including the interleukin (IL)-2 / IL-2 receptor α-chain (IL-2Rα, CD25) axis or the co-inhibitory receptor CTLA-4 (cytotoxic T-lymphocyte-associated protein 4), predispose to autoimmune disorders [10, 11]. Whereas it is generally accepted that Tregs are essential for maintaining self-tolerance, studies addressing whether altered Treg frequencies and/or function affect the disease course or severity of e.g. type 1 diabetes [12], systemic lupus erythematosus [13], or rheumatoid arthritis [14] have led to largely confounding findings. However, differences in the markers used to define the Treg population and hence the purity and composition of the analysed Treg population might in part account for the divergent findings, and highlight the importance of a standardised set of markers that allows for cross-comparison between independent studies (table 1). Nowadays a general consensus exists that in humans Foxp3-expressing Tregs are most accurately defined as CD4+CD25+CD127cells [15, 16].
The role of Tregs defined on the basis of these markers has been studied most extensively in multiple sclerosis. Multiple sclerosis patients with relapsing-remitting disease have reduced frequencies of Tregs with impaired suppressive capacity [17, 18]. Decreased expression levels of FoxP3 [17] as well as of CTLA-4 [19] detected in multiple sclerosis patients were attributed to Treg dysfunction. Importantly, treatment of multiple sclerosis with interferon-beta (IFN-β) [17] and glatiramer acetate [20] restores Treg numbers and FoxP3 expression levels, supporting a protective role of Tregs in multiple sclerosis.

Within their reduced Treg pool, patients with multiple sclerosis show an accumulation of Th1-like Tregs that produce interferon-gamma (IFN-γ) [21] (Th1: type 1 T helper cell; table 2). The production of IFN-γ in these Tregs was accompanied by upregulation of the Th1 master transcription factor T-bet and loss of suppressive function. Blockade of IFN-γ could re-establish the suppressive capacity of the Tregs while the initial induction of the Th1-like phenotype was dependent on IL-12 signalling. Importantly, IFN-β treatment reduced frequencies of IFN-γ-producing Th1-Tregs in multiple sclerosis patients to normal levels [21]. Similar findings were reported in patients with type 1 diabetes [22], supporting a protective role of Tregs in the disease.

Mouse models in which Tregs lack the ability to co-express FoxP3 together with effector Th lineage-specific transcription factors revealed the ability of Tregs to specialise during the course of inflammatory responses. Driven by the corresponding cytokine environment, co-expression of T-bet or Stat3 was shown to be indispensable for control of Th1 or Th17 effector T cell responses, respectively [23, 24]. In the mouse model, delayed induction of the IL-12Rβ2 chain prevented Th1-specialised Tregs from fully differentiating into potentially pathogenic IFN-γ-producing Tregs [25]. The appearance of IFN-γ-producing Tregs in patients with multiple sclerosis suggests that this pathway is defective in these patients. A recent study reported the accumulation of Tregs expressing T-bet in the central nervous system of mice during experimental autoimmune encephalomyelitis, the mouse model of multiple sclerosis. However, in contrast to the initial reports, T-bet deficiency in Tregs had no impact on the control of the proinflammatory T cell response in this study [26]. These findings indicate that specialisation might not be universally required for Treg function and homing. It will thus be important to evaluate whether Treg specialisation is beneficial or potentially harmful in different inflammatory diseases.

In patients with rheumatoid arthritis, Tregs are counterintuitively enriched in the synovial fluid, the site of inflammation [27, 28], raising the question of whether they are dysfunctional and thus unable to prevent disease. When examined for their functional properties, Tregs isolated from the synovial fluid of patients with rheumatoid arthritis retained their ability to suppress effector T cell proliferation in vitro [27, 28], but displayed defects in suppression of effector cytokine production (table 2) [14]. Furthermore, the inflammatory environment in rheumatoid arthritis, particularly the presence of tumour necrosis factor-alpha (TNF-α), led to a loss in the suppressive function of Tregs [14, 29]. Whether this is also the case in other autoimmune settings is still unclear [30]. Parallel to the IFN-γ-producing Th1-Tregs observed in multiple sclerosis, Tregs from rheumatoid arthritis patients have the potential to secrete the proinflammatory cytokine IL-17 when re-stimulated ex vivo [31]. However, in contrast to IFN-γ-producing Th1-Tregs, they retain their ability to suppress effector cell proliferation in vitro [32, 33]. IL-17-secreting FoxP3+ Tregs were first described under steady-state conditions, and co-express FoxP3 and the Th17 master transcription factor RORγt (retinoic acid receptor-related orphan receptor gamma-i) [32-34]. Despite the fact that they retain their suppressive capacity, FoxP3+ RORγt+ Tregs secrete IL-17 in response to IL-1β or IL-6 and thereby potentially contribute to inflammation [32, 33]. The relevance of these Th17-like Treg cells in autoimmune inflammation still remains unclear, but again highlights the importance of considering Treg heterogeneity and subset distribution in disease settings.

The pathological importance of Treg plasticity and the ability of Tregs to adapt effector function under inflammatory conditions is well demonstrated in cancer. Checkpoint inhibitors block signalling through inhibitory receptor-related orphan receptors such as PD-1 or CTLA-4 and can thus reverse T cell exhaustion, which favours immune evasion of tumour cells or pathogens. Blockade of these pathways can additionally support the function of effector T cells by reducing the suppressive capacity of Tregs. CTLa-4 = cytotoxic T-lymphocyte-associated protein 4; IL = interleukin; PD-1 = programmed death 1; Th = T helper cell; Treg = regulatory T cell.
conditions were investigated in the mouse model of collagen-induced arthritis. Exposure of CD25<sup>hi</sup>Foxp3<sup>+</sup> T cells to high levels of IL-6, as encountered during arthritis, resulted in a loss of Foxp3 expression in this still plastic population and thus converted them into IL-17-secreting T cells driving pathology [35]. This highlights the importance of Treg stability when addressing the imbalance between effector T cells and Tregs in inflammatory diseases. A number of studies have focused on the question of whether Tregs are stable or if they can convert into other potentially pathogenic T cell phenotypes. In addition to sustained expression of Foxp3 and CD25, the unique epigenetic signature of Tregs, marked by hypomethylation of CpG motifs in key genes such as the Foxp3 conserved noncoding sequence 2 (CNS2), Il2ra (CD25), Ikgf4 (Eos) and Ctl4, ensures Treg stability and maintenance of a functional Treg phenotype [36–38]. The epigenetic programming of Tregs may even ensure their long-term stability when Foxp3 expression is transiently lost [39, 40]. However, this topic is still somewhat controversial and some studies suggest that Tregs can permanently lose Foxp3 expression under inflammatory conditions and convert to “ex-Tregs” exhibiting a pathogenic phenotype [41, 42]. Whether these “ex-Tregs” actually underwent epigenetic reprogramming or rather represent precursors that only transiently upregulated Foxp3 is also not entirely clear. Regardless of this debate, a better understanding of Treg stability is a prerequisite for the development of Treg cell therapy, especially for treatment in inflammatory settings.

On the basis of these observations it is clear that in both Th1- and Th17-dominated autoimmune settings, Treg numbers as well as their function are critical determinants for disease progression. Therapeutic approaches should thus aim at restoring a healthy balance between effector cells and functional Tregs (fig. 1B). Th17 and Treg cells share the dependence on transforming growth factor-beta (TGF-β) for their development, but the presence of IL-6 drives their differentiation towards the Th17 lineage [43–45]. Interference with the IL-6-derived signal, for example with a blocking antibody to the IL-6 receptor (tocilizumab), thus inhibits Th17 but supports Treg differentiation. This therapeutic approach has successfully been used in rheumatoid arthritis, where treatment resulted in an increase in Treg but not Th17 numbers, restoring the Treg/Th17 balance [46]. Ongoing studies on IL-2 treatment, as key cytokine in Treg development and maintenance, have demonstrated a specific activation and expansion of Tregs, but not effector T cells, when low doses of IL-2 are administered, suggesting a more general approach for modulation of the T effector / Treg balance to treat autoimmune diseases [47]. Similarly, adoptive immunotherapy with ex-<i>vivo</i> expanded CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>+</sup> polyclonal Tregs is another interesting approach to restoring the T effector / Treg balance in multiple autoimmune diseases [48]. However, many open issues regarding the stability and functionality of Tregs expanded <i>in vitro</i> still need to be addressed before this approach could become broadly applicable.

In summary, Tregs are a very heterogeneous population harbouering subsets with a high level of plasticity and possibly variable thresholds for stability under inflammatory conditions. The assessment of Tregs defined by their stability and suppressive properties in the relevant inflammatory setting of each autoimmune disease will be key in ensuring the efficacy of re-establishing the T effector / Treg balance through therapeutic approaches.

### Regulatory T cells in infectious diseases

Pathogens and nascent transformed cells that can progress to cancer pose a constant threat to the host, which must be kept in check through appropriate immune responses. However, excessive responses resulting from failure to adequately control the magnitude and extent of the response can result in collateral damage of affected tissues and organs, also known as immunopathology [49]. A number of

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**Table 1: Marker combinations to identify regulatory T cells (Tregs)**

| Marker combination | Represented population | Organism | Comments | References |
|--------------------|------------------------|----------|----------|------------|
| CD4<sup>+</sup>CD25<sup>+</sup> | Tregs and activated effector T cells | Mouse/ human | Activated effector T cells upregulate CD25. | |
| CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> | Tregs | Mouse | Exclusive marker combination to identify Tregs. | |
| CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup>CD127<sup>+</sup> | Primarily Tregs | Human | Activated effector T cells transiently upregulate Foxp3. | |

**Table 2: Subsets of human regulatory T cells (Tregs) and their potential role in autoimmune disease.**

| Markers | Function | References |
|---------|----------|------------|
| Foxp3+CD25+Other markers | Suppression | Effector cytokines | Comments | |
| ++ | CD45RA<sup>-</sup>CTLA-4<sup>-</sup> | +++ | – | Resting or naive Tregs |
| +++ | CD45RA<sup>-</sup>CTLA-4<sup>-</sup> | +++ | – | Effector or activated Tregs |
| ++ | CD45RA<sup>-</sup>CTLA-4<sup>-</sup> | – | IL-2, IFN-γ | Treg precursor, may lose Foxp3 expression |
| +++ | CD45RA<sup>-</sup>CCR6<sup>-</sup>CD45RA<sup>-</sup>HLA-DR<sup>-</sup>CCR6<sup>-</sup>CD45RA<sup>-</sup>CD27<sup>-</sup>CCR6<sup>-</sup> | +++ | IL-17 (express RORγt) | |
| +++ | CD45RA<sup>-</sup>CD127<sup>-</sup> | + | IFN-γ (express T-bet, CXCR3) | Subset increased in MS and T1D patients. |

CTLA-4 = cytotoxic T-lymphocyte-associated protein 4; CXCR3 = C-X-C motif chemokine receptor type 3; FoxP3 = forkhead-box protein P3; IFN = interferon; IL = interleukin; MS = multiple sclerosis; RORγt = retinoic acid receptor-related orphan receptor gamma-t; T1D = type 1 diabetes
Regulatory T cells in cancer

Immune surveillance is essential for eradication of aberrant cells and prevention of their progression to cancer. Based on their regulatory function, an involvement of Tregs in tumour progression was long suspected [66]. In some human cancers, high prevalence of Tregs and low numbers of CD8⁺ effector T cells are associated with poor prognosis (reviewed in [6]). To date there is still relatively little known about the function and plasticity of tumour-associated Tregs. Nevertheless, Tregs are thought to inhibit potent anti-tumour responses under certain conditions and many approaches to the development of cancer immunotherapies focused on the induction of anti-tumour immunity by reducing the suppressive capacity of Tregs. A now widely utilised cancer immunotherapy is high-dose IL-2. This has been successfully applied for treatment of metastatic melanoma and renal cell carcinoma [67, 68]. Since IL-2 can promote effector T cell differentiation and stimulate natural killer (NK) cells, the prolonged survival in treated patients may be attributed to increased anti-tumour immune responses [69]. A drawback of this treatment lies in the high doses of IL-2 required, which can lead to serious side effects [69]. However, high doses seem to be necessary for the induction of anti-tumour immune responses, as low doses preferentially expand Tregs owing to their expression of the high-affinity IL-2 receptor, which includes the IL-2 receptor α-chain (CD25) [47]. In contrast, effector T cells are mostly restricted to expressing the low-affinity IL-2 receptor and thus require higher concentrations of IL-2 for activation (fig. 1C). The differential expression of high versus low affinity IL-2 receptors in effector T cells and Tregs might, under certain circumstances, even interfere with the responsiveness of patients.
to high-dose IL-2 treatment. For instance, one study reported the expansion of an activated Treg subset in melanoma patients treated with high-dose IL-2 [70]. This study also came to the conclusion that more prominent expansion of this Treg subset predicted poorer responsiveness towards high-dose IL-2 therapy. To counteract these unwanted effects, it may be beneficial to combine IL-2 treatment with depletion or inhibition of Tregs. This could potentially increase the efficacy of IL-2 in triggering an anti-tumour response and hence might even allow for administration of less toxic IL-2 dosages. The principle of Treg depletion as a possible cancer treatment was already explored over a decade ago, and was shown to slow tumour growth and enhance anti-tumour immunity [71, 72]. However, when considering Treg depletion via the CD25-blocking antibody daclizumab, it is important to note that CD25 is also expressed on activated effector T cells and, depending on the timing and dose, daclizumab might also deplete effector T cells. Furthermore, Treg depletion generally harbours the risk of causing autoimmunity. Selective stimulation of specific lymphocyte subsets by use of IL-2-antibody complexes has also been suggested as an alternative to classic IL-2 immunotherapy in order to minimise its shortcomings [69]. Depending on the antibody clone, IL-2-antibody complexes direct activity of IL-2 towards CD25+ cells such as Tregs or selectively expand CD122high effector cells in mice [73, 74]. Therefore, the use of IL-2-antibody complexes might facilitate a more tailored approach to balancing the Treg/effector T cell ratio therapeutically. Targeting of immune checkpoint inhibitors represents another promising approach to activate anti-tumour immune responses (fig. 1C). Current immunotherapies are directed against the co-inhibitory receptors CTLA-4 and PD-1, and exhibit unprecedented efficacy in several cancer indications [75]. Combination therapy against CTLA-4 (ipilimumab) and PD-1 (nivolumab) was shown to have synergistic effects in melanoma patients and significantly prolonged progression-free survival [76]. In the mouse model, this combined treatment resulted in increased tumour infiltration of cytotoxic T cells, reduced frequencies of Tregs and, ultimately, tumour regression in up to 75% of mice [77]. These results highlight the similarity between the T cell responses in cancer and chronic infection, where the same approach results in superior viral clearance [58]. Importantly, these co-inhibitory molecules are also highly expressed on Tregs, where they contribute to Treg suppressive function. Immune checkpoint blockade is therefore likely to both interfere with Treg function and enhance effector responses, and thereby promote the effector response through two synergistic pathways. This dual action of checkpoint inhibitors was investigated in detail in a recent set of studies on the novel co-inhibitory receptor TIGIT (T cell immunoglobulin and immunoreceptor tyrosine-based inhibition motif [ITIM] domain), which recently gained attention as another potential checkpoint inhibitor for cancer therapy. Like CTLA-4 and PD-1, TIGIT is expressed on effector as well as regulatory T cells, and mechanistic studies in mice revealed that anti-TIGIT therapy affects both CD8+ T cell responses as well as Tregs, supporting an impact of checkpoint inhibitors on both effector as well as regulatory T cell function [78–80].

As illustrated by both IL-2 treatment and the checkpoint inhibitor approaches, a key aspect in effective cancer immunotherapies lies in skewing the balance of Tregs and effector T cells towards an increased effector response. A better understanding of the dynamics of marker expression on both effector and regulatory T cells in the different disease settings, as well as a detailed assessment of their expression patterns in patients will be an important step to improve therapeutic efficacy and minimise side effects.

Conclusions

The studies discussed here emphasise the broad impact Tregs have on the development of tolerance versus immunity. Consequently their manipulation harbours enormous therapeutic potential for the treatment of a wide spectrum of diseases ranging from autoimmunity to chronic infection and cancer. However, in order to realise this potential, a better understanding of the underlying Treg biology is essential. In particular, deeper insight into the mechanisms that govern Treg stability, plasticity, and specialisation will be necessary to allow tailored manipulation of Treg numbers and function aimed at re-establishing a healthy balance between tolerance and protective immunity in this broad range of diseases.

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Figure 1

**Therapeutic targeting of the effector T cell–Treg balance.** Depicted are approaches that can restore a dysfunctional ratio of effector T cells and Tregs in different diseases to the healthy state (A). (B) In autoimmune diseases, self-tolerance is lost and autoreactive effector T cells expand. In Th17-driven autoimmune diseases, blocking of IL-6 signalling favours Treg and inhibits Th17 differentiation. Low-dose IL-2 therapy, which leads to expansion of Tregs and thus restores a healthy effector T cell–Treg balance represents a more general approach for treatment of autoimmunity. (C) Application of high-dose IL-2 results in expansion of effector T cells and induction of antitumor immune responses in cancer patients. Checkpoint inhibitors block signalling through inhibitory receptors such as PD-1 or CTLA-4 and can thus reverse T cell exhaustion, which favours immune evasion of tumour cells or pathogens. Blockade of these pathways can additionally support the function of effector T cells by reducing the suppressive capacity of Tregs.

CTLA-4 = cytotoxic T-lymphocyte-associated protein 4; IL = interleukin; PD-1 = programmed death 1; Th = T helper cell; Treg = regulatory T cell