definitive conclusions on the role of Th2 cells in GvHD from this small study was difficult, Th2 and non-Th2 recipients showed similar rates of acute and chronic GvHD with a similar organ distribution.

Th17

Th17 & Th1/Th2 revisited in autoimmune diseases

Classically, autoimmune diseases have been assumed to be associated with dysregulated Th1 responses on the basis of findings from animal models of autoimmunity such as experimental autoimmune encephalomyelitis (EAE). EAE is a mouse model of multiple sclerosis. However, accumulating evidence argues against the role of Th1 cells in the pathogenesis of EAE. EAE was more severe in IFN-γ-deficient mice than wild-type mice [29], whereas IL-23p19-deficient mice, in which Th1 cells were not reduced, were resistant to EAE [30]. Subsequent studies pointed to the role of IL-23 as an inducer of IL-17 and demonstrated that IL-17-producing Th17 cells are primarily responsible for EAE induction [31,32]. Deficiency of IL-17 or ROR-γt, a key regulator of Th17 development, attenuates EAE [32,33]. Similarly, blockade of IL-17 ameliorated experimental arthritis [34]. In patients with rheumatoid arthritis, IL-17 levels were elevated in synovial fluids [35]. Infiltration of Th17 cells was observed in the biopsied samples of chronically inflamed tissues in patients with psoriasis, Crohn's disease, rheumatoid arthritis and asthma [36]. These results clearly indicate that Th17 immunity plays a central role in many autoimmune diseases.

Differentiation & function of Th17 cells

Th17 cells are characterized by their expression of proinflammatory cytokines IL-17, IL-21, and IL-22, cytokines involved in neutrophilia, production of antimicrobial peptides and tissue repair [13,31,37,38]. IL-17 belongs to a family of six members: IL-17A, IL-17B, IL-17C, IL-17D, IL-17E (also known as IL-25) and IL-17F. Among them, IL-17A and IL-17F, which form heterodimers, are by far the best characterized cytokines [39]. IL-17 is also produced by innate immune cells in an inflammatory milieu, and may have a central role in the initiation of IL-17-dependent immune responses even before Th17 development [40].

Th17 cells differentiate from naive $CD4^+$ T cells by stimulation with antigens in the presence of TGF-β and IL-6 or IL-21 in mice (FIGURE 1) [13–15,38,41–43]. In humans, different Th17 differentiation factors have been initially reported, but factors inducing Th17 cells in mice and humans seem to be identical [41,44–49]. IL-21 is produced by Th17 cells, and thus, it is a part of a positive feedback loop for amplifying Th17 immunity [38]. Naive T cells do not express IL-23R, except on activation, and IL-23 stabilizes differentiating Th17 cells and promotes further maturation of Th17 cells in part by inducing IL-22 in these cells [13,50]. Specific transcriptional factors such as ROR-γt, ROR-α and STAT3 define the Th17 transcriptional program because they are indispensable for Th17 differentiation [33,43,51–53]. In addition, Th17 cells can be induced in memory T cells by IL-23 and IL-1β [13,31,49].

Th17 cells are found in the systemic circulation, secondary lymphoid organs and tissues, particularly in the intestinal mucosa where they protect the host from microorganisms that invade through the epithelium [54]. Th17 differentiation is influenced by innate immunity and by the composition of the intestinal microbiota. Stimulation with binding of bacteria-derived peptidoglycan to Toll-like receptor 2 on dendritic cells (DCs) in the gut induces production of IL-23 and IL-1β, leading to Th17 differentiation [41]. Lumenal ATP and flagellin are critically involved in this process [55,56]. Th17 cells contribute to host defense against certain pathogens by inducing expression of antimicrobial factors and recruiting neutrophils to mucosal surface [57]. They also play a role in maintaining intestinal ecology with commensal bacterial flora [58]. On the flip side, Th17 differentiation is influenced by the composition of the intestinal microbiota; the commensal microbe segmented filamentous bacterium increases Th17 cells in the lamina propria of mice, whereas segmented filamentous bacterium is absent in mice with few Th17 cells [54]. However, such a specific bacterium that can induce Th17 differentiation in the lamina propria of humans remains to be identified. Importantly, broad receptor distribution of IL-17 and IL-22 results in massive tissue responses to Th17 cells; these cells trigger local tissues to release IL-6, TNF-α, IL-1β and chemokines and recruit neutrophils and macrophages to inflammatory sites [57].

Regulatory T cells

Regulatory T-cell subsets

T-cell-mediated immunoregulation is one of the main mechanisms responsible for maintaining antigen-specific operational tolerance *in vivo*, controlling T-cell homeostasis, and

the homeostatic expansion of T cells in lymphopenic hosts. Although many lymphocyte populations that can suppress antigen-specific immune responses have been described, the best described *bona fide* populations of regulatory cells are $CD4^+25^+FOXP3^+$ Tregs [16,17]. FOXP3 is an oligomeric component of a large supermolecular complex and critical regulator of the development and function of Tregs [59,60]. Certain *FOXP3* gene mutants are associated with X-linked autoimmunity–allergic dysregulation syndrome and immunodysregulation, polyendocrinopathy and enteropathy X-linked syndrome in humans [61,62]. Individuals with these disorders fail to develop Tregs and experience varied symptoms that include diarrhea, dermatitis, insulin-dependent diabetes, thyroiditis and anemia, with massive T-cell infiltration into the affected tissue. There are other types of $CD4^+FOXP3^-$ Tregs; Tr1 cells are generated *in vitro* by antigenic stimulation in the presence of IL-10 and secrete IL-10 and TGF-β [63]. A subset of $CD8^+$ T cells also suppresses $CD4^+$ T-cell responses with phenotypic and functional features similar to $CD4^+$ Tregs in mice and human [64–67]. However, little is known regarding the role of $CD8^+$ suppressor cells in GvHD and this article will focus only on $CD4^+$ Tregs.

Development & function of Tregs

Tregs can be divided into two populations: naturally occurring Tregs (nTregs) that develop in the thymus and induced Tregs (iTregs) that are derived from $CD4^+FOXP3^-$ T-cell population in the periphery (Figure 1) [68]. Thymic development of nTregs requires high-affinity interactions between their TCR and self-peptide–MHC complexes expressed in thymic stromal cells. Differentiation of conventional $CD4^+$ T cells (Tcons) into iTregs in the periphery represents the outcome of cytokine-mediated activation of specific STAT proteins and the induction of lineage-specific transcriptional factors, and depends upon TGF-β.

Tregs are indispensable in maintaining self-tolerance and immune homeostasis, establishing tolerance to allografts and promoting fetomaternal tolerance. The disadvantage of Tregs is dampening of antitumor T-cell responses [69]. Tregs suppress effector activities of differentiated $CD4^+$ and $CD8^+$ T cells and the function of NK cells, NKT cells, B cells, macrophages and DCs. Tregs must be activated through their TCR to be functionally suppressive and IL-2 signaling is required for their expansion and function [70,71]. Tregs suppress immune responses through contact-dependent mechanisms and the production of soluble factors, including TGF-β, IL-10 and IL-35 [17]. Contact-dependent suppression is mediated through multiple molecules such as cytotoxic T lymphocyte antigen (CTLA)4, lymphocyte activation gene 3, granzyme A and CD95. Naive Tregs do not express these molecules, except on activation. While CTLA-4 expression on Tregs is critical for suppression by modulating APCs, Tregs can also suppress effector T cells through direct contact even in the absence of APCs [72]. The mechanisms of cytokine-mediated suppression include FOXP3 induction in Tcons and attenuation of DC function by TGF-β and IL-10 [68].

Human $Foxp3^+$ $CD4^+$ T cells are composed of three phenotypically and functionally distinct subpopulations: $CD45RA^+FOXP3^{lo}$ naive Tregs, $CD45RO^+Foxp3^{hi}$ effector Tregs, and $CD45RO^+FOXP3^{lo}$-activated Tcons that are pathogenic because they produce proinflammatory cytokines [73–75]. Thus, FOXP3 is not always a reliable marker for human Tregs: $Foxp3^+$ T cells are heterogenous, including nonregulatory T cells and naive Tregs that only weakly express Foxp3.

Plasticity of effector & Tregs subsets

Regulatory and effector T-cell subsets are found to be more malleable than originally thought on the basis of the more stringent commitment of Th1 and Th2 cells, and flexibility in T-cell commitment is not an exception [76]. Th17 cells have the potential to convert toward a Th1 profile in mice and humans [77]. Th17 cells isolated from a certain strain of mice convert into Th1 cells after transfer into another strain of mice probably because of the difference in cytokine environment *in vivo* [78]. Th17 cells can shift to a Th1 phenotype in the presence of IL-12 [79]. This conversion occurs through expression of Th1 transcription factors STAT4 and T-bet that also antagonize Th17 development [13].

A common requirement of TGF-β in the differentiation of Th17 cells and iTregs suggests that these subsets may be developmentally linked and Th17 and Treg development programs are reciprocally interconnected. TGF-β is a pleiotropic cytokine that affects multiple cell lineages by promoting or opposing their differentiation, survival and proliferation. TGF-β inhibits Th1 and Th2 differentiation, whereas it induces Th17 and Treg differentiation [80]. Transcriptional factors involved in driving FOXP3 expression may indirectly regulate Th17 development, because FOXP3 inhibits the function of Th17-associated

transcriptional factor ROR-γt [81]. The local cytokine milieu is of great importance for Treg function and differentiation. Treg generation from antigen-stimulated T cells is promoted by the environment rich in TGF-β and retinoic acid at the expense of Th17 differentiation [82]. Tregs are reciprocally induced by exclusion of IL-6 from the Th17-skewing condition in mice and humans [83], whereas Tregs are converted to IL-17-producing cells when reactivated under Th17-polarizing conditions [84–88] or even under normal conditions [89]. A lymphopenic environment also leads to the loss of FOXP3 expression in Tregs and their differentiation into T follicular helper cells in Peyer's patches [90]. Furthermore, Foxp3$^+$ T cells that produce IL-17, IFN-γ or IL-4, and those express ROR-γt or T-bet have been demonstrated in mice and humans [81,91–97].

For the maintenance of the developmentally established Treg function program, continuous expression of Foxp3 is an absolute requirement. Committed Tregs are resistant to such conversion to effector T cells upon adoptive transfer into lymphopenic hosts, whereas a relatively minor fraction of uncommitted CD25$^-$ or CD25^{lo} FOXP3$^+$ T cells can lose FOXP3 expression and divert into effector T-cell lineages [98]. By contrast, a recent study using inducible labeling and tracking the fate of Tregs *in vivo* demonstrated that Tregs are remarkably stable under physiological and inflammatory conditions, and that Treg homeostasis is maintained by self-renewal of established mature Tregs [99]. Although that study does not exclude the possibility that recently generated FOXP3$^+$ cells can differentiate into effector T cells in a lymphopenic or proinflammatory environment, it argues against plasticity of once committed Tregs. This issue is fundamentally important for those studies directed to manipulate immune responses in a therapeutically useful manner.

Th17 & Tregs in experimental GvHD

Th17

Research on Th17 in GvHD is rapidly developing. The role of Th17 responses in GvHD has been evaluated using IL-17-deficient mice; however, it has not yet been completely established. Yi *et al.* showed that infusion of IL-17-deficient donor T cells induced more severe GvHD than that of wild-type T cells [100]. This was in association with enhanced Th1 differentiation of donor T cells by increased production of IL-12 from DCs in the absence of IL-17. By contrast, two other studies failed to show the effects of IL-17 on GvHD mortality [101,102]. Differences between experimental designs such as strain combinations, intensity of conditioning irradiation and T-cell dose may result in inconsistent results. Furthermore, ROR-γt-deficient T cells can also induce acute GvHD as severe as that observed with wild-type T cells [103]. IL-17 is not required for transition from acute to chronic GvHD [104]. These results suggest that Th17 differentiation is at least dispensable for GvHD. Yi *et al.* further showed that liver and gut GvHD is largely Th1 dependent, whereas skin GvHD is more Th17 dependent and lung GvHD is Th2 dependent [105]. Thus, each T-helper subset may play a differential role in inducing tissue-specific GvHD; Th17 responses may be involved in skin GvHD.

Recently, Hill *et al.* demonstrated that donor pretreatment with granulocyte colony-stimulating factor (G-CSF) induced Th17 differentiation of donor T cells after PBSCT [106]. PBSCT from IL-17-deficient donors treated with G-CSF significantly reduced skin scleroderma in association with reduced infiltration of macrophages that are important sources of TGF-β, a mediator of fibrosis [107,108]. These findings are highly relevant clinically as PBSCT from G-CSF-mobilized donors is a risk for chronic GvHD compared with bone marrow transplant in humans [109]. However, the role of IL-17 and TGF-β in human chronic GvHD has not yet been identified.

The ability of *ex vivo*-polarized Th17 cells to induce GvHD in each target organ has also been evaluated [103,110]. Th17 cells were generated by repetitive stimulation of naive CD4$^+$ T cells with CD3/CD28 in the presence of TGF-β, TNF-α, IL-6, IL-1β and IL-23, as well as anti-IL-2 monoclonal antibodies (mAbs) and anti-IFN-γ mAbs [110]. On transfer into lethally irradiated mice, these Th17 cells migrated and expanded in GvHD target organs and lymphoid tissue, and subsequently induced more severe pathological lesions of the skin and lung, but equivalent pathological lesions of the liver and intestine in comparison with naive CD4$^+$ T cells [110]. Unexpectedly, however, IL-17 blockade ameliorated skin GvHD but had no impact on the mortality and morbidity of systemic GvHD. These Th17 cells produced TNF-α and IFN-γ in addition to IL-17A and IL-17F after transfer. Neutralization of TNF-α significantly reduced the mortality and morbidity of systemic GvHD. Another study also confirmed such a central role of TNF-α in systemic GvHD mediated by polarized Th17 cells [111]. Iclozan *et al.* generated

Th17 cells by stimulating $CD4^+$ T cells with anti-CD3 mAbs in the presence of TGF-β, IL-6, anti-IL-4 and anti-IFN-γ mAbs [103]. These Th17 cells had superior expansion and migration capabilities in GvHD target organs, which correlated with their increased pathogenicity, compared with Th1 or naive T cells.

Taken together, it seems that Th17 cells are sufficient but not necessary for GvHD induction. However, these studies in which *ex vivo*-polarized T cells were infused have limitations and results from these studies should be carefully interpreted. In these studies, Th17 cells are polyclonally expanded and therefore antigen nonspecific. This is not clinically relevant because antigen-specific Th17 cells are supposedly generated *in vivo* after transplantation. Furthermore, incubation of T cells *in vitro* might result in incomplete polarization and partial anergy. Upon transfer, these cells may fail to survive and proliferate or cause secondary conversion to a mixed phenotype or to a different regulatory or effector T-cell subset, as has been stated in previous studies of polarized Th1 and Th2 cells. *In vitro*-generated Th17 cells do not essentially produce IFN-γ, but can produce IFN-γ upon restimulation without Th17-polarizing cytokines. On transfer, such Th17 cells did not maintain their cytokine profiles because they produced large amounts of IFN-γ, although IFN-γ was not required for Th17 cell-mediated GvHD in these studies [103,110]. Whether the production of IFN-γ by Th17-skewed cells is part of the evolution of the T-cell subset *in vivo* or whether IFN-γ is produced by a preformed Th1-like component that subsequently expands is unclear.

IL-6, IL-21 & IL-23 in GvHD

IL-6 is one of the central mediators of inflammation. It has a pivotal function in dictating whether T cells differentiate into Tregs or Th17 cells. IL-6-deficient mice do not develop a Th17 response and their peripheral repertoire is dominated by Tregs [42]. Serum IL-6 levels are often elevated during GvHD in mice and humans [112,113]. *IL-6* gene polymorphisms that lead to altered IL-6 expression levels are linked to GvHD severity [114]. Although IL-6 elevation is not specific for GvHD and the association of *IL-6* gene polymorphism and GvHD severity is controversial, these results suggest that IL-6 could be a target molecule to treat GvHD. A recent study showed that blockade of the IL-6 signaling pathway with anti-IL-6R mAbs ameliorates GvHD in association with augmentation of thymic-dependent and thymic-independent production of Tregs with a concomitant reduction of Th1 and Th17 cells in GvHD target organs [112]. By contrast, Tawara *et al.* reported that anti-IL-6R mAb-mediated attenuation of GvHD was independent of the direct effects on effector T-cell expansion or donor Tregs [113].

IL-21 is produced by Th17 cells and its receptors are broadly expressed on T, B and NK cells. Inhibition of IL-21 signaling on donor T cells attenuates GvHD, particularly in the intestine [102,115,116]. Bucher *et al.* showed an increased number of $Foxp3^+$ Tregs in the intestine of anti-IL-21 mAb-treated recipients of $Foxp3^-$ T cells, suggesting Treg conversion [115]. However, Oh *et al.* suggested that attenuated GvHD is caused by an impairment of effector T-cell differentiation, rather than by an increase in Tregs [102]. Although multiple mechanisms seem to contribute to GvHD amelioration, no study has showed impaired Th17 differentiation in the absence of IL-21 signaling on donor $CD4^+$ T cells.

IL-23 is not involved in the initial Th17 differentiation but is necessary for the generation of a completely functional Th17 response. Expression of *IL-23* and *IL-23R* genes was upregulated during GvHD in mouse colon [117]. IL-23 production by donor-derived APCs is critical for intestinal GvHD, but not for liver or lung GvHD [117]. However, IL-23-mediated intestinal GvHD is dependent on donor-derived IFN-γ, but not on IL-17 [117], although another study showed reduced serum levels of IL-17 in the absence of donor IL-23 [118]. A subsequent study demonstrated that inhibition of IL-23 signaling reduced intestinal GvHD without losing a GVL response [119].

Tregs

A role of Tregs in GvHD was initially noted in experiments of costimulatory blockades [120]. Blockade of the CD40/CD40L pathway or B7 pathways ameliorated acute GvHD. This effect was not observed when Tregs were removed from the donor inoculum [120]. The suppressive effects of Tregs on GvHD are not restricted to costimulatory blockades, but rather a general phenomenon. Removal of Tregs from the donor graft dramatically accelerates GvHD [121,122]. Conversely, addition of freshly isolated donor-type Tregs significantly delays GvHD, especially at high regulatory-to-effector T-cell ratios (1:1) [121–125]. In addition, donor or host Tregs were able to ameliorate ongoing chronic GvHD [126,127]. These results revealed the purification of sufficient numbers of Tregs could be

a major limitation for applying this strategy to humans. To date, the only realistic use of Tregs in humans would be to expand them *ex vivo*.

In this context, the ability of *ex vivo*-expanded Tregs to inhibit GvHD has been tested [120,121,124,128]. Donor-derived Tregs were isolated and expanded in culture with host-derived APCs or with CD3/CD28 stimulation and high-dose IL-2 [124]. Exogenous IL-2 is critical because Tregs do not produce sufficient amounts of IL-2 for their own expansion. Injection of *ex vivo*-expanded Tregs significantly ameliorated GvHD induced by Tcons, without interfering with stem cell engraftment and functional immune reconstitution with diverse TCR Vβ repertoire [124,129]. Improved immune reconstitution is mediated by inhibition of GvHD-induced damage of the thymic and secondary lymphoid microenvironment [129]. Tregs can also be generated *in vitro* by stimulating FOXP3$^-$ T cells in the presence of immunosuppressive agents or tolerizing DCs. These iTregs show potent suppressive activity *in vitro* and induce tolerance in transplantation [130–132] and autoimmunity [133,134] *in vivo*, although one study demonstrated that iTregs quickly reverted to FOXP3$^-$ T cells and failed to prevent GvHD upon transfer [135]. STATs are critical transcriptional factors involving iTreg generation. Infusion of CD4$^+$ T cells constitutively expressing active STAT5b induces attenuated GvHD by enhancing the expansion and function of Tregs [136]. STAT3 ablation enhances Treg reconstitution through thymus-dependent and thymus-independent pathways [137]. These results suggest that pharmacological or genetic modulation of the STAT protein in T cells may be a useful approach to generate efficient iTregs. *In vivo* induction of iTregs may be an alternative approach. Injection of *ex vivo*-generated regulatory DCs also protect against chronic GvHD by enhancing generation of iTregs from FOXP3$^-$ donor T cells *in vivo* [138].

After infusion, Tregs colocalize with alloreactive T cells and expand in secondary lymphoid organs. Alloreactive donor T cells are activated by alloantigens expressed on host APCs in secondary lymphoid organs [2,3]. Stimulation of donor Tregs by host APCs is also necessary for the induction of GvHD protection, suggesting a double-edged role of host APCs in inducing and regulating GvHD [139]. Tregs suppress the initial activation and functional maturation of alloreactive donor T cells in secondary lymphoid organs. A recent study using an *in vitro* human skin explant GvHD model showed that donor-derived Tregs effectively suppressed CD8-mediated skin damage only when added to the culture during initial effector T-cell priming with alloantigens [140]. Therefore, Treg therapy may be more effective when given as GvHD prophylaxis than as a treatment of established GvHD. Several costimulatory pathways such as CD28/B7, OX40/OX40L, 4-1BB/4-1BBL, CD30/CD153 and PD1/PDL1 are critical for Treg-mediated suppression [141–143]. However, these pathways are also important for Tcon activation and exhaustion of effector T cells in GvHD [11,144].

Upon stimulation, Tregs rapidly switch expression of adhesion molecules and chemokine receptors, acquire a new migratory capacity and migrate to GvHD target tissues [145]. The important T-cell homing receptor CD62L is required by Tregs to suppress Tcons at the priming sites (secondary lymphoid organs) [128,146]. On the other hand, CCR5$^-$ Tregs fail to migrate to GvHD target tissues [147]. CD103$^+$ Tregs that express high levels of CCR5 but low levels of CD62L directly migrate to GvHD target tissues and strongly suppress GvHD upon transfer [127].

A recent study in a mouse model suggested an association between progressive loss of Tregs during the course of acute GvHD with subsequent development of chronic GvHD by allowing expansion of pathogenic Th1 and Th17 cells [148]. On the other hand, thymic injury during acute GvHD allows the emergence of autoreactive T cells that escape from negative thymic selection and cause chronic GvHD, even in the presence of functional Tregs [149]. Whether such an imbalance between regulatory and effector T cells induced during acute GvHD could be a cause of chronic GvHD remains to be determined in humans.

Whether a graft-versus-leukemia (GVL) effect is maintained when Tregs are envisaged for the control of GvHD is a critical point to be determined. Since Tregs suppress the initial activation of alloreactive donor T cells in secondary lymphoid organs, Treg therapy probably abrogates GVL activity of donor T cells. However, Treg therapy did not result in a complete loss of GVL activity in several animal studies [124,125,150]. This is probably due to an incomplete inhibition of alloreactive T-cell activation that allows induction of at least some GVL effects mediated through the perforin–granzyme pathway. This pathway plays a major role in GVL but may play a lesser role in Treg-mediated suppression of GvHD [151]. Factors related to leukemia, such as leukemia burden, its aggressiveness or the localization of its growth, could also determine whether GVL activity would be lost in Treg therapy [124].

Recent experimental and clinical studies suggest that exposure of the fetus to noninherited maternal antigens (NIMAs) during pregnancy has an impact on allogeneic SCT performed later in life [152–154]. In mice, 'child-to-mother' bone marrow transplant from a NIMA-exposed donor reduced morbidity and mortality of GvHD in an antigen-specific manner [155]. Tolerogenic NIMA effects were mediated by exposure to NIMAs both *in utero* and through breastfeeding after birth, and required Tregs [156]. Substantial numbers of maternal cells cross the placenta to reside in fetal lymph nodes, inducing the development of Tregs that suppress fetal antimaternal immunity and persist at least until early adulthood in humans [157].

Tregs could suppress alloresponses through antigen-specific suppression, linked suppression or infectious tolerance. Linked suppression is the phenomenon of suppressing responses to a specific antigen by copresenting it simultaneously with another antigen, against which tolerance has previously been established. iTregs recognizing ovalbumin (OVA) prevent GvHD mediated by alloantigen-specific polyclonal effector T cells only when allogeneic recipients express OVA [132]. Tolerance can be infectious. Coactivation of Tregs with Treg cell-depleted $CD4^+$ T cells results in anergized $CD4^+$ T cells that in turn inhibit the activation of Tcons [158]. In the presence of Tregs that actively maintain allograft tolerance, naive T cells are newly recruited to the graft and differentiate into graft-specific Tregs [159]. Whether Tregs exert their Treg function through such a linked suppression or infectious tolerance remains to be determined in humans.

In summary of this part, apparently, any Th-cell lineage has the potential to mediate GvHD, whereas Tregs limit the disease. The fine balance between Th1, Th2, Th17 and Treg subsets may be associated with the severity, manifestation and tissue distribution of GvHD.

Th17 & Tregs in human GvHD

Th17

The initial phase of GvHD involves cytokine release during preconditioning of SCT recipients and cytokine gene polymorphisms are associated with GvHD development and its severity. Carvalho *et al.* analyzed the associations between variants in *IL-17A*, *IL-17F* and *IL-23R* genes and clinical outcome in T cell-depleted allogeneic SCT [160]. Donor polymorphism of *IL-17A* and *IL-23R* genes was the most important prognostic factor for survival and GvHD. This suggested the potential usefulness of Th17 genotyping in donor selection, although validation studies are required to confirm this association. Clinical studies assessing the correlation between Th17 cells with GvHD have shown conflicting results. Th17 cells are increased in peripheral blood of patients with acute GvHD in one study [161], but not another study [162]. Ratio of Th17 to Treg was increased in the biopsied samples of GvHD tissues in one study [161], whereas it was decreased in another study [163]. Th17 cells were not increased in the skin in contrast to a significant increase in IFN-γ-producing cells at the onset of acute GvHD [162].

Ustekinumab is a clinical-grade antibody against p40, a common component of IL-12 and IL-23, and their use lead to a blockade of Th1 and Th17 differentiation. The efficacy and safety of ustekinumab has been demonstrated in Phase III trials for treatment of psoriasis [164–166]. It has also been tested in Phase II studies for multiple sclerosis, sarcoidosis and Crohn's disease [167,168]. On the basis of results from animal studies, blockade of Th1 and Th17 are also considered promising for GvHD prevention [19,105,148]. The anti-IL-17 mAb LY2439821 has been tested for treatment of rheumatoid arthritis [169]. However, results from animal studies indicate that blockade of the IL-17 pathway alone may have limited effects on GvHD.

Modulation of the Th17/Treg balance by cytokine blockade is a realistic and attractive strategy to prevent GvHD. Tocilizumab, a humanized mAb against IL-6R, has been shown to be effective against several autoimmune and inflammatory diseases such as rheumatoid arthritis and Castleman's disease [170–172]. Although an animal study demonstrated that IL-6 blockade attenuated acute GvHD by increasing Tregs [112], whether IL-6 blockade could shift from Th17 responses toward Tregs in humans remains to be determined. A single case report suggested the effectiveness of tocilizumab in a patient with refractory gastrointestinal GvHD [173]. Tocilizumab may also be effective for treatment of sclerodermatous skin lesion in chronic GvHD because a recent study demonstrated its efficacy for the skin lesion in patients with systemic sclerosis [174].

Tregs

Although the beneficial effect of Treg appears to be clear in the context of ameliorating GvHD in experimental studies, data from clinical studies show controversies in terms of Treg function in human patients. The association between the

number of Tregs infused or Treg reconstitution post-transplant and clinical outcome is not clear so far [175–177]. In earlier studies, where $CD4^+$ Tregs were defined as expressing only CD25, higher numbers of $CD4^+25^+$ cells correlated with more acute and chronic GvHD probably due to expression of CD25 on activated effector T cells [178,179]. Daclizumab is a humanized mAb against CD25. In a randomized study, the addition of daclizumab to corticosteroids did not reduce GvHD [180]. This unexpected outcome may be observed because of the elimination or suppression of Tregs by daclizumab. The source of stem cells impacts the Treg content. G-CSF preferentially mobilizes $CD62L^{lo}$ Tregs that have poor suppressive activity [181,182]. T-cell lineage in umbilical cord blood is biased toward immune tolerance but the function of cord blood Tregs appears to be similar to that of adult Tregs [183].

Recent studies thus analyzed FOXP3 expression and demonstrated reduced numbers of $FOXP3^+CD4^+CD25^+$ T cells in peripheral blood of patients with acute and chronic GvHD [184–186]. Such an association was also found after T-cell-depleted SCT, but not after CD25-depleted SCT [187,188]. By contrast, an increased frequency of a skin-homing Treg population (cutaneous lymphocyte antigen [CTL^+]) or a gut-homing Treg population ($\alpha4\beta7^+$) in peripheral blood was associated with a reduced risk of skin or gut GvHD, respectively [189]. Immunohistochemical studies of biopsy samples taken from GvHD target organs demonstrated a decreased ratio of $FOXP3^+$ cells to $CD8^+$ T cells in patients with intestinal GvHD [190,191], although a subsequent study failed to show this association [163]. Increase in $FOXP3^+$ cells in the skin was associated with better treatment response [192]. However, in most studies, the patients were sampled at a single time point. Studies repetitively measuring Treg number during the first year after SCT clearly showed the Treg number varied widely depending on the sampling time points [186,193–195]. Moreover, as previously mentioned, the findings that $FOXP3^+$ T cells in humans are functionally heterogeneous including non-Treg cells, and that a subset of $FOXP3^-$ T cells has a suppressive function may be related to these disparate findings. Thus, these results need to be validated in a larger, multicenter cohort.

Tregs display greater proliferation and metabolic activity than Tcons in the steady state [196,197]. Thus, Tregs are more sensitive to irradiation and cyclophosphamide, both are used as a preparative regimen of SCT [198]. After allogeneic SCT, the initial phase of T-cell reconstitution is primarily dependent on peripheral expansion of mature T cells present in the stem cell graft. Tregs can also be generated from a $FOXP3^-$ T-cell population in lymphopenic host mice [199]. After acute lymphopenia-induced homeostatic proliferation, Treg reconstitution can occur through thymus-dependent generation from donor hematopoietic progenitor cells. Although the relative contribution of thymic and extrathymic pathways to the long-term reconstitution of Tregs remains to be determined, thymic generation of naive Tregs was markedly impaired in adult patients [195].

Treg reconstitution appears to occur at least as fast as Tcons after SCT [187,188,194,195]. During the lymphopenic period after transplantation, Tregs underwent more rapid expansion than Tcons, acquired a predominantly activated/memory phenotype and increased susceptibility to Fas-mediated apoptosis [195]. Thus, a Treg pool was preferentially declined and resulted in a prolonged imbalance between Tregs and Tcons in patients with extensive chronic GvHD. This issue, however, may be still controversial because another study demonstrated an increased ratio of Tregs to Tcons in patients with chronic GvHD following a reduced intensity conditioning and *in vivo* T-cell depletion with alemtuzumab [200]. A recent study demonstrated a positive correlation between Treg recovery and cytomegalovirus-specific $CD8^+$ T cells [186]. This may be a reflection of better immune reconstitution in the absence of GvHD.

Stimulation of Treg reconstitution may be a promising approach to induced tolerance. Rapamycin promotes Treg differentiation and as a treatment accumulates Tregs in the skin [201]. Extracorporeal photopheresis is clinically used to treat GvHD. Studies in mice and humans demonstrated that the transfer of white blood cells exposed to UV radiation increased peripheral blood Tregs and serum levels of TGF-β [202–205].

Clinical application of Tregs

Accumulating evidence from experimental animal studies suggest that adoptive transfer of Tregs is a promising strategy in preventing or treating GvHD in humans. In animal studies, a ratio of nearly 1:1 of Tregs:Tcons is required for effective GvHD suppression [121,123–125]. Since alloreactive T cells presumably exist at a high frequency and obtaining a sufficient number of relatively rare Tregs from a single donor is difficult, *ex vivo* expansion of Tregs is largely necessary

to achieve large-scale deletion of alloreactive T cells *in vivo* in order to create transplantation tolerance. Achieving maximum purity of Tregs is also critically important in order to avoid the risk of GvHD exacerbation by contaminating effector T cells.

The FOXP3$^+$ T-cell population in humans is heterogeneous and includes non-Treg cells [75]. FOXP3 can be weakly expressed in activating effector cells. Thus, FOXP3 is not always a reliable marker for human Tregs. Moreover, intracellular staining for FOXP3 expression is not adequate for practical use when trying to isolate viable cells. Novel surface markers specific for Tregs have not yet been identified. Currently, antibody-coated magnetic bead separation is most often utilized for Treg isolation to first deplete non-CD4$^+$ T cells, followed by a positive selection to enrich for CD25$^+$ cells via a subsaturating concentration of CD25 antibody to capture the CD25^{hi} fraction [206,207]. Alternative isolation strategies include depletion of CD127$^+$ cells or selection of CD45RA$^+$ or CD49d$^+$ cells [208–211]. In culture, repetitive *in vitro* stimulation of Tregs results in a loss of stable FOXP3 expression [212]. CD45RA$^-$FOXP3^{hi} memory Tregs are the main source of converting cells, whereas CD45RA$^+$FOXP3^{lo} naive Tregs show less conversion. Loss of FOXP3 expression in memory Tregs appears to be the result of a cell intrinsic program initiated in those cells in response to strong and repetitive *in vitro* stimulation [212]. Therefore, CD4$^+$ CD25^{hi} CD45RA$^+$ naive Tregs may be an ideal subset choice for Treg therapy. Depletion of CD127$^+$ cells from the CD4$^+$ CD25^{hi} starting population may not improve the homogeneity of the cell product with respect to FOXP3 expression after *in vitro* expansion [212]. Hoffmann *et al.* provide instructions for isolation, polyclonal expansion, and functional characterization of human Tregs in detail [211].

High numbers of Tregs can be obtained by polyclonal or antigen-specific expansion. However, even adequately purified Tregs can give rise to a substantial fraction of T cells that produce proinflammatory cytokines in culture. Thus, maintaining Treg purity in expansion is important. Interaction between the sphingosine 1-phosphatase ($S1P_1$)–mTOR signaling selectively controlled the reciprocal differentiation of Th1 cells and Tregs [213]. The mTOR inhibitor rapamycin and $S1P_1$ antagonist FTY720 target this pathway to favor Treg differentiation. Addition of rapamycin to Treg expansion cultures blocks Treg differentiation into nonregulatory T cells [214–216]. Accordingly, Treg proliferation levels were found in patients who received rapamycin for acute GvHD prophylaxis compared with those who received other regimens [195]. In addition, administration of FTY720 has been shown to produce GvHD suppressive activity in mice models by Treg-dependent and -independent mechanisms [217–219]. Histone/protein deacetylases (HDACs) regulate chromatin remodeling and the function of transcription factors. FOXP3 activity is also regulated by HDACs and other transcriptional coregulators [220,221]. HDAC inhibitors promote the generation and function of Tregs [222,223]. Reddy *et al.* demonstrated that HDAC inhibitor vorinostat regulate experimental acute GvHD by multiple mechanisms, although it is not clear whether vorinostat might affect Treg function [224,225]. A Phase II clinical trial is ongoing to determine if vorinostat can reduce the prevalence of acute GvHD following matched related-donor SCT after reduced intensity conditioning.

One of the major concerns of Treg therapy is whether the infused Tregs can survive *in vivo*. Since activated Tregs are prone to die by apoptosis, strategies that clonally expand antigen-specific Tregs while inhibiting excessive activation in culture are required. However, most Treg expansion processes cause Treg maturation. Another concern is whether the infused Tregs can continue to be functionally stable *in vivo*, especially under proinflammatory conditions such as those found early after SCT; the cytokine environment is important for Treg function and differentiation. FOXP3 undergoes post-translational changes as a consequence of extrinsic cellular signals. Stability of FOXP3 expression is associated with demethylation of the Treg-specific demethylated region. Epigenetic imprinting in this region is critical for the establishment of a stable Treg lineage [226]. iTregs generated *de novo* in a TGF-β-dependent process do not display FOXP3 DNA demethylation despite Foxp3 expression, whereas FOXP3 DNA is continuously demethylated in nTregs [227]. Quantitative DNA methylation analysis of Foxp3 is a novel screening method for counting stable Tregs in peripheral blood and tissues and can be used to estimate *in vivo* expansion of functional Tregs after transfer [228].

The suppressive effects of Tregs are not always antigen-specific even after antigen-specific expansion. To achieve efficient therapeutic effects without general immunosuppression, generation of antigen-specific Tregs is required. For this purpose, Tregs are generated in the presence of host

APCs in culture [124]. Such antigen-specific Tregs are much more potent than irrelevant Tregs in controlling GvHD without inhibiting immune reconstitution in mice [124]. However, whether Tregs are antigen-specific in humans where the situation is not as simple as in mice remains unclear. Even once activated by a particular antigen, Tregs can suppress effector T cells irrespective of whether they share antigen-specificity with the Tregs *in vitro* [229]. However, such a bystander suppression by Treg has not been evident *in vivo*. A recent study showed that Tregs can prevent allograft rejection without compromising immunity to viral antigens *in vivo* [230].

Defining the optimal timing of administration in order to achieve the most efficient effects of Treg therapy is also critically important. Treg therapy initiated after GvHD is established appears to have little value for therapeutic application as shown in an *in vitro* skin explant model [140]. Accordingly, Tregs should be expanded before SCT in each case, making Treg therapy cost ineffective and impractical.

An additional potential obstacle to successful use of Tregs is an adverse impact of immunosuppressive agents on Tregs. Calcineurin inhibitors such as cyclosporine and tacrolimus inhibit IL-2 transcription and thereby have an inhibitory effect on Tregs that are dependent on IL-2 [231]. By contrast, other agents such as rapamycin and mycophenolate mofetil, which act through alternative mechanisms, may be permissive for Treg expansion and function [231]. Thus, selection of immunosuppressive drugs that have less suppression on Tregs than on Tcons may be crucial for successful Treg therapy. The net effect of GvHD prophylaxis with a combination of calcineurin inhibitor and rapamycin or mycophenolate mofetil on Treg reconstitution, however, remains unclear. Alternative approaches include use of calcineurin inhibitor-free GvHD prophylaxis as a platform of Treg therapy or infusion of IL-2 to support *in vivo* expansion of Tregs. The latter strategy was tested in a recent clinical trial; administration of low-dose IL-2 after $CD4^+$ donor lymphocyte infusion (DLI) enhanced Treg expansion [232].

Several clinical trials are currently ongoing to examine the adoptive transfer of Tregs as prevention against GvHD in humans. The first-in-man clinical results of the treatment of GvHD with *ex vivo*-expanded Tregs was reported from Poland [210]. $CD4^+CD25^{hi}CD127^+$ cells were sorted from the SCT donor buffy coat, polyclonally expanded with anti-CD3/anti-CD28 beads with IL-2, and infused into two patients suffering from GvHD. The therapy was effective in a patient with chronic GvHD, but not in one with acute GvHD. Umbilical cord blood can be a readily accessible source of Tregs. Results of a prospective and multicenter clinical trial to evaluate the safety of cord blood-derived Tregs were also reported [233]. $CD25^{hi}$ cells were isolated using anti-CD25 magnetic beads and the CliniMACS® device from cryopreserved cord blood and expanded similarly. Expansion efficiency was much better in cord blood Tregs than adult Tregs. Upon infusion, no infusional toxicities were observed in 23 patients who underwent double unit cord blood transplantation. Apparently no increase of acute GvHD, infection and disease relapse occurred. The group from Perugia in Italy conducted adoptive immunotherapy with Tregs after HLA-haploidentical PBSCT [234]. Freshly isolated $CD4^+CD25^{hi}$ cells with CliniMACS were infused 4 days before infusion of Tcons together with purified $CD34^+$ PBSCs. Treg therapy made administration of Tcons feasible in a haploidentical setting, with a low incidence of GvHD and cytomegalovirus reactivation. Conversely, the safety and efficacy of Treg-depleted DLI was studied in 17 adult patients with malignancy relapse after SCT [235]. This strategy was safe and induced GvHD and GVL in patients not responding to a classical DLI.

Future perspective

The balance between regulatory and effector T cells may be an important determinant of GvHD induction or tolerance induction and the fine balance between T helper subsets *in vivo* may impact on clinical manifestation and tissue distribution of GvHD. This assumption suggests that effective strategies to suppress GvHD include selective inhibition of effector T cells while maintaining or enhancing Treg function and reconstitution, as well as manipulation of the balance between T helper subsets to treat organ-specific GvHD. In this context, adoptive Treg therapy has been initiated in SCT patients. These initial studies will provide data regarding safety and feasibility of Treg therapy. We will be paying particular attention to whether Treg therapy could increase the risk for opportunistic infection by their nonspecific immunosuppressive effects, whether it could promote leukemic relapse, and whether it would rather induce GvHD by the contaminated effector cells or by the *in vivo* conversion of Tregs to inflammatory T cells. In addition, development of novel methods to monitor *in vivo* survival and expansion of functional Tregs are required

to estimate impacts of Treg therapy on its outcome. Such a new technology also helps to determine the optimal timing and cell dose of Treg therapy. Selection of drugs for GvHD prophylaxis that do not impair Treg function is also crucial for successful implantation of Treg immunotherapy. Currently performed custom-made Treg preparations for each patient are not realistic for the widespread use of Treg immunotherapy. Improvement of Treg therapy thus requires much better understanding of the checks and balances the immune system to maintain tolerance and induce immunity by overcoming many problems that we now face. Certainly, Treg therapy has a great promise of avoiding general immunosuppression and many toxicities by currently using potent immunosuppressive agents. In order to modulate the balance of Th subsets *in vivo*, the use of biological products such as cytokine-neutralizing mAbs that has increasingly been used clinically in treating patients with cancer and autoimmune diseases, is a realistic and attractive strategy because cytokine environment is critically important for T helper and Treg differentiation. To establish highly effective Treg therapy or cytokine modulation, consideration of timing of administration is particularly important in the setting of highly inflammatory allogeneic SCT. However, as reduced intensity conditioning has been

Executive summary

The Th1/Th2 paradigm in graft-versus-host disease

- Differential activation of Th1 or Th2 cells has been suggested to play an important role in GvHD development.
- The long-held assumption that acute GvHD is a Th1-mediated disease, whereas chronic GvHD is a Th2-mediated disease, needs to be refined.

Th17

- Th17 cells are characterized by their expression of proinflammatory cytokines IL-17, IL-21 and IL-22.
- Th17 cells differentiate from naive $CD4^+$ T cells by stimulation with antigens in the presence of TGF-β and IL-6 or IL-21.
- Th17 cells contribute to host defense against certain pathogens by inducing expression of antimicrobial factors and recruiting neutrophils.
- Th17 cells play a role in maintaining intestinal ecology with commensal bacterial flora.
- Th17 immunity plays a central role in many autoimmune diseases.

Tregs

- Tregs are indispensable to maintain self-tolerance and immune homeostasis, establish tolerance to allografts and promote feto–maternal tolerance.
- Naturally occurring Tregs develop in the thymus while induced Tregs are derived from $CD4^+FOXP3^-$ T-cell population in the periphery.
- Tregs suppress immune responses through contact-dependent and cytokine-dependent mechanisms.
- $FOXP3^+$ T cells are heterogenous and include nonregulatory T cells.

Plasticity of effector & Treg cells

- There is a plasticity and flexibility of T-cell commitment to regulatory and effector T-cell subsets.
- Th17 cells have the potential to convert toward a Th1 profile.
- Th17 and Treg development programs are reciprocally interconnected.
- Stable expression of FOXP3 is required for the maintenance of the developmentally established Treg function program.

Th17 & Tregs in experimental GvHD

- The role of IL-17 in GvHD is still controversial.
- Th17 cells may be sufficient but not necessary for GvHD induction.
- Th17 may be involved in skin GvHD.
- Treg therapy suppresses GvHD only at high regulatory-to-effector T-cell ratios.
- Therapeutic approach to treat GvHD probably rely on nTregs, not iTregs.
- Tregs primarily suppress the initial activation of alloreactive donor T cells at priming sites.

Th17 & Tregs in human GvHD

- Clinical studies assessing the correlation between GvHD with Th17 or Tregs have shown conflicting results.
- Thymic generation of naive Tregs is markedly impaired in adults.
- Tregs undergo rapid expansion, acquire an activated/memory phenotype, and increase susceptibility to apoptosis during the lymphopenic period after transplantation.

Clinical application of Tregs

- *Ex vivo* expansion of Tregs is required to achieve large-scale deletion of alloreactive T cells *in vivo* in order to create transplantation tolerance.
- Achievement of a maximum Treg purity is required in order to avoid the risk of GvHD exacerbation by contaminating effector T cells.
- Survival of functionally stable Tregs *in vivo* after transfer remains a major concern of Treg therapy.
- Suppression of immunity against pathogens and leukemia remains to be estimated.
- The optimal timing for Treg therapy is not clear.

developed to minimize toxicity of conditioning regimen, advances in transplant medicine will synergistically facilitate development of such novel strategies.

Conversely, results from animal studies suggest that a combined blockade of Th1 and Th17 differentiation pathways of donor T cells may represent a promising strategy for the prevention or treatment of GvHD, while inhibition of either pathway seems to be insufficient to prevent GvHD [19,105,148]. In this context, novel strategies targeting Th1 and Th17 promoting cytokines, miRNAs, transcriptional factors such as T-bet and ROR-γt through administration of mAbs, small molecule inhibitors, or siRNA may be promising for the control of GvHD [236,237].

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties. No writing assistance was utilized in the production of this manuscript.

Bibliography

Papers of special note have been highlighted as:
* of interest
** of considerable interest

1 Ferrara JL, Levine JE, Reddy P, Holler E. Graft-versus-host disease. *Lancet* 373, 1550–1561 (2009).

2 Shlomchik WD, Couzens MS, Tang CB *et al.* Prevention of graft versus host disease by inactivation of host antigen-presenting cells. *Science* 285, 412–415 (1999).

3 Teshima T, Ordemann R, Reddy P *et al.* Acute graft-versus-host disease does not require alloantigen expression on host epithelium. *Nat. Med.* 8, 575–581 (2002).

4 Hill GR, Crawford JM, Cooke KJ, Brinson YS, Pan L, Ferrara JLM. Total body irradiation and acute graft versus host disease. The role of gastrointestinal damage and inflammatory cytokines. *Blood* 90, 3204–3213 (1997).

5 Zhang Y, Louboutin JP, Zhu J, Rivera AJ, Emerson SG. Preterminal host dendritic cells in irradiated mice prime CD8+ T cell-mediated acute graft-versus-host disease. *J. Clin. Invest.* 109, 1335–1344 (2002).

6 Koyama M, Hashimoto D, Aoyama K *et al.* Plasmacytoid dendritic cells prime alloreactive T cells to mediate graft-versus-host disease as antigen-presenting cells. *Blood* 113, 2088–2095 (2009).

7 Zhang Y, Joe G, Hexner E, Zhu J, Emerson SG. Host-reactive CD8+ memory stem cells in graft-versus-host disease. *Nat. Med.* 11, 1299–1305 (2005).

8 Anderson BE, McNiff J, Yan J *et al.* Memory CD4+ T cells do not induce graft-versus-host disease. *J. Clin. Invest.* 112, 101–108 (2003).

9 Zhang Y, Joe G, Hexner E, Zhu J, Emerson SG. Alloreactive memory T cells are responsible for the persistence of graft-versus-host disease. *J. Immunol.* 174, 3051–3058 (2005).

10 Korngold R, Sprent J. Negative selection of T cells causing lethal graft-versus-host disease across minor histocompatibility barriers: role of the H-2 complex. *J. Exp. Med.* 1114–1123 (1980).

11 Socie G, Blazar BR. Acute graft-versus-host disease: from the bench to the bedside. *Blood* 114, 4327–4336 (2009).

12 Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J. Immunol.* 136, 2348–2357 (1986).

13 Harrington LE, Hatton RD, Mangan PR *et al.* Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat. Immunol.* 6, 1123–1132 (2005).

* **Along with [14,15], describes an important role for TGF-β signaling in the differentiation of Th17 cells.**

14 Veldhoen M, Hocking RJ, Atkins CJ, Locksley RM, Stockinger B. TGFβ in the context of an inflammatory cytokine milieu supports *de novo* differentiation of IL-17-producing T cells. *Immunity* 24, 179–189 (2006).

* **Along with [13,15], describes an important role for TGF-β signaling in the differentiation of Th17 cells.**

15 Mangan PR, Harrington LE, O'Quinn DB *et al.* Transforming growth factor-β induces development of the Th17 lineage. *Nature* 441, 231–234 (2006).

* **Along with [13,14], describes an important role for TGF-β signaling in the differentiation of Th17 cells.**

16 Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor α-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J. Immunol.* 155, 1151–1164 (1995).

17 Sakaguchi S, Miyara M, Costantino CM, Hafler DA. Foxp3+ regulatory T cells in the human immune system. *Nat. Rev. Immunol.* 10, 490–500 (2010).

18 Via CS. Kinetics of T cell activation in acute and chronic forms of graft versus host disease. *J. Immunol.* 146, 2603–2609 (1991).

19 Tawara I, Maeda Y, Sun Y *et al.* Combined Th2 cytokine deficiency in donor T cells aggravates experimental acute graft-vs-host disease. *Exp. Hematol.* 36, 988–996 (2008).

20 Jung U, Foley JE, Erdmann AA, Eckhaus MA, Fowler DH. CD3/CD28-costimulated T1 and T2 subsets: differential *in vivo* allosensitization generates distinct GVT and GVHD effects. *Blood* 102, 3439–3446 (2003).

21 Krenger W, Cooke KR, Sonis ST *et al.* Transplantation of polarized type 2 donor T cells reduces mortality caused by experimental graft-versus-host disease. *Transplantation* 62, 1278–1285 (1996).

22 Foley JE, Mariotti J, Ryan K, Eckhaus M, Fowler DH. Th2 cell therapy of established acute graft-versus-host disease requires IL-4 and IL-10 and is abrogated by IL-2 or host-type antigen-presenting cells. *Biol. Blood Marrow Transplant.* 14, 959–972 (2008).

23 Fowler DH, Kurasawa K, Smith R, Eckhaus MA, Gress RE. Donor CD4-enriched cells of Th2 cytokine phenotype regulate graft-versus-host disease without impairing allogeneic engraftment in sublethally irradiated mice. *Blood* 84, 3540–3549 (1994).

24 Murphy WJ, Welniak LA, Taub DD *et al.* Differential effects of the absence of interferon-γ and IL-4 in acute graft-versus-host disease after allogeneic bone marrow transplantation in mice. *J. Clin. Invest.* 102, 1742–1748 (1998).

25 Yang YG, Dey BR, Sergio JJ, Pearson DA, Sykes M. Donor-derived interferon γ is required for inhibition of acute graft-versus-host disease by interleukin 12. *J. Clin. Invest.* 102, 2126–2135 (1998).

26 Reddy P, Teshima T, Kukuruga M *et al.* Interleukin-18 regulates acute graft-versus-host disease by enhancing fas-mediated donor T cell apoptosis. *J. Exp. Med.* 194, 1433–1440 (2001).

27 Nikolic B, Lee S, Bronson RT, Grusby MJ, Sykes M. Th1 and Th2 mediate acute graft-versus-host disease, each with distinct end-organ targets. *J. Clin. Invest.* 105, 1289–1298 (2000).

28 Fowler DH, Odom J, Steinberg SM *et al.* Phase I clinical trial of costimulated, IL-4 polarized donor $CD4^+$ T cells as augmentation of allogeneic hematopoietic cell transplantation. *Biol. Blood Marrow Transplant.* 12, 1150–1160 (2006).

29 Ferber IA, Brocke S, Taylor-Edwards C *et al.* Mice with a disrupted IFN-γ gene are susceptible to the induction of experimental autoimmune encephalomyelitis (EAE). *J. Immunol.* 156, 5–7 (1996).

30 Cua DJ, Sherlock J, Chen Y *et al.* Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. *Nature* 421, 744–748 (2003).

31 Park H, Li Z, Yang XO, Chang SH *et al.* A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat. Immunol.* 6, 1133–1141 (2005).

32 Komiyama Y, Nakae S, Matsuki T *et al.* IL-17 plays an important role in the development of experimental autoimmune encephalomyelitis. *J. Immunol.* 177, 566–573 (2006).

33 Ivanov, II, McKenzie BS, Zhou L *et al.* The orphan nuclear receptor RORγt directs the differentiation program of proinflammatory IL-17$^+$ T helper cells. *Cell* 126, 1121–1133 (2006).

* **Demonstrates a key role for the transcriptional factor RORγt in TGF- and IL-6-dependent Th17 differentiation.**

34 Bush KA, Farmer KM, Walker JS, Kirkham BW. Reduction of joint inflammation and bone erosion in rat adjuvant arthritis by treatment with interleukin-17 receptor IgG1 Fc fusion protein. *Arthritis Rheum* 46, 802–805 (2002).

35 Kotake S, Udagawa N, Takahashi N *et al.* IL-17 in synovial fluids from patients with rheumatoid arthritis is a potent stimulator of osteoclastogenesis. *J. Clin. Invest.* 103, 1345–1352 (1999).

36 Pene J, Chevalier S, Preisser L *et al.* Chronically inflamed human tissues are infiltrated by highly differentiated Th17 lymphocytes. *J. Immunol.* 180, 7423–7430 (2008).

37 Liang SC, Tan XY, Luxenberg DP *et al.* Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides. *J. Exp. Med.* 203, 2271–2279 (2006).

38 Nurieva R, Yang XO, Martinez G *et al.* Essential autocrine regulation by IL-21 in the generation of inflammatory T cells. *Nature* 448, 480–483 (2007).

* **Along with** [42,43], **demonstrates a crucial role of IL-21 on Th17 differentiation.**

39 Wright JF, Guo Y, Quazi A *et al.* Identification of an interleukin 17F/17A heterodimer in activated human $CD4^+$ T cells. *J. Biol. Chem.* 282, 13447–13455 (2007).

40 Cua DJ, Tato CM. Innate IL-17-producing cells: the sentinels of the immune system. *Nat. Rev. Immunol.* 10, 479–489 (2010).

* **Excellent review of the literature on the role of innate immune system for the induction of adaptive Th17 immunity.**

41 van Beelen AJ, Zelinkova Z, Taanman-Kueter EW *et al.* Stimulation of the intracellular bacterial sensor NOD2 programs dendritic cells to promote interleukin-17 production in human memory T cells. *Immunity* 27, 660–669 (2007).

42 Korn T, Bettelli E, Gao W *et al.* IL-21 initiates an alternative pathway to induce proinflammatory Th17 cells. *Nature* 448, 484–487 (2007).

* **Along with** [38,43], **demonstrates a crucial role of IL-21 on Th17 differentiation.**

43 Zhou L, Ivanov, II, Spolski R *et al.* IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. *Nat. Immunol.* 8, 967–974 (2007).

* **Along with** [38,42], **demonstrates a crucial role of IL-21 on Th17 differentiation.**

44 Wilson NJ, Boniface K, Chan JR *et al.* Development, cytokine profile and function of human interleukin 17-producing helper T cells. *Nat. Immunol.* 8, 950–957 (2007).

45 Acosta-Rodriguez EV, Napolitani G, Lanzavecchia A, Sallusto F. Interleukins 1β and 6 but not transforming growth factor-β are essential for the differentiation of interleukin 17-producing human T helper cells. *Nat. Immunol.* 8, 942–949 (2007).

46 Manel N, Unutmaz D, Littman DR. The differentiation of human T(H)-17 cells requires transforming growth factor-β and induction of the nuclear receptor RORγt. *Nat. Immunol.* 9, 641–649 (2008).

47 Yang L, Anderson DE, Baecher-Allan C *et al.* IL-21 and TGF-β are required for differentiation of human Th17 cells. *Nature* 454, 350–352 (2008).

48 Cosmi L, De Palma R, Santarlasci V *et al.* Human interleukin 17-producing cells originate from a $CD161^+CD4^+$ T cell precursor. *J. Exp. Med.* 205, 1903–1916 (2008).

49 Korn T, Bettelli E, Oukka M, Kuchroo VK. IL-17 and Th17 cells. *Annu. Rev. Immunol.* 27, 485–517 (2009).

50 Ahern PP, Schiering C, Buonocore S *et al.* Interleukin-23 drives intestinal inflammation through direct activity on T cells. *Immunity* 33, 279–288 (2010).

51 Yang XO, Panopoulos AD, Nurieva R *et al.* STAT3 regulates cytokine-mediated generation of inflammatory helper T cells. *J. Biol. Chem.* 282, 9358–9363 (2007).

* **Demonstrates STAT3 to be a critical regulator of Th17 immunity.**

52 Yang XO, Pappu BP, Nurieva R *et al.* T helper 17 lineage differentiation is programmed by orphan nuclear receptors ROR α and ROR γ. *Immunity* 28, 29–39 (2008).

53 Milner JD, Brenchley JM, Laurence A *et al.* Impaired Th17 cell differentiation in subjects with autosomal dominant hyper-IgE syndrome. *Nature* 452, 773–776 (2008).

54 Ivanov II, Atarashi K, Manel N *et al.* Induction of intestinal Th17 cells by segmented filamentous bacteria. *Cell* 139, 485–498 (2009).

* **Demonstrates that commensal bacteria play an important role in shaping the balance of T helper cell subsets.**

55 Atarashi K, Nishimura J, Shima T *et al.* ATP drives lamina propria Th17 cell differentiation. *Nature* 455, 808–812 (2008).

56 Uematsu S, Fujimoto K, Jang MH *et al.* Regulation of humoral and cellular gut immunity by lamina propria dendritic cells expressing Toll-like receptor 5. *Nat. Immunol.* 9, 769–776 (2008).

57 O'Connor W Jr, Zenewicz LA, Flavell RA. The dual nature of Th17 cells: shifting the focus to function. *Nat. Immunol.* 11, 471–476 (2010).

58 Cerf-Bensussan N, Gaboriau-Routhiau V. The immune system and the gut microbiota: friends or foes? *Nat. Rev. Immunol.* 10, 735–744 (2010).

59 Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 299, 1057–1061 (2003).

60 Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of $CD4^+CD25^+$ regulatory T cells. *Nat. Immunol.* 4, 330–336 (2003).

61 Bennett CL, Christie J, Ramsdell F *et al.* The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat. Genet.* 27, 20–21 (2001).

62 Wildin RS, Ramsdell F, Peake J *et al.* X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy. *Nat. Genet.* 27, 18–20 (2001).

63 Chen Y, Kuchroo VK, Inobe J, Hafler DA, Weiner HL. Regulatory T cell clones induced by oral tolerance: suppression of autoimmune encephalomyelitis. *Science* 265, 1237–1240 (1994).

64 Zhang ZX, Yang L, Young KJ, DuTemple B, Zhang L. Identification of a previously unknown antigen-specific regulatory T cell and its mechanism of suppression. *Nat. Med.* 6, 782–789 (2000).

65 Gilliet M, Liu YJ. Generation of human CD8 T regulatory cells by CD40 ligand-activated plasmacytoid dendritic cells. *J. Exp. Med.* 195, 695–704 (2002).

66 Bisikirska B, Colgan J, Luban J, Bluestone JA, Herold KC. TCR stimulation with modified anti-CD3 mAb expands $CD8^+$ T cell population and induces $CD8^+CD25^+$ Tregs. *J. Clin. Invest.* 115, 2904–2913 (2005).

67 Kim HJ, Verbinnen B, Tang X, Lu L, Cantor H. Inhibition of follicular T-helper cells by $CD8^+$ regulatory T cells is essential for self tolerance. *Nature* 467, 328–332 (2010).

68 Chen W, Jin W, Hardegen N *et al.* Conversion of peripheral $CD4^+CD25^-$ naive T cells to $CD4^+CD25^+$ regulatory T cells by TGF-β induction of transcription factor Foxp3. *J. Exp. Med.* 198, 1875–1886 (2003).

69 Mougiakakos D, Choudhury A, Lladser A, Kiessling R, Johansson CC. Regulatory T cells in cancer. *Adv. Cancer Res.* 107, 57–117 (2010).

70 Malek TR, Yu A, Vincek V, Scibelli P, Kong L. CD4 regulatory T cells prevent lethal autoimmunity in IL-2Rβ-deficient mice. Implications for the nonredundant function of IL-2. *Immunity* 17, 167–178 (2002).

71 Furtado GC, Curotto de Lafaille MA, Kutchukhidze N, Lafaille JJ. Interleukin 2 signaling is required for $CD4^+$ regulatory T cell function. *J. Exp. Med.* 196, 851–857 (2002).

72 Wing K, Onishi Y, Prieto-Martin P *et al.* CTLA-4 control over $Foxp3^+$ regulatory T cell function. *Science* 322, 271–275 (2008).

73 Gavin MA, Torgerson TR, Houston E *et al.* Single-cell analysis of normal and FOXP3-mutant human T cells: Foxp3 expression without regulatory T cell development. *Proc. Natl Acad. Sci. USA* 103, 6659–6664 (2006).

* **Demonstrates the upregulation of FOXP3 expression on naive $CD4^+$ T cells upon activation.**

74 Tran DQ, Ramsey H, Shevach EM. Induction of Foxp3 expression in naive human $CD4^+Foxp3$ T cells by T-cell receptor stimulation is transforming growth factor-β dependent but does not confer a regulatory phenotype. *Blood* 110, 2983–2990 (2007).

75 Miyara M, Yoshioka Y, Kitoh A *et al.* Functional delineation and differentiation dynamics of human $CD4^+$ T cells expressing the FoxP3 transcription factor. *Immunity* 30, 899–911 (2009).

** **Refines our understanding of human $FOXP3^+CD4^+$ T cells and has important implications for Treg immunotherapy.**

76 Bluestone JA, Mackay CR, O'Shea JJ, Stockinger B. The functional plasticity of T cell subsets. *Nat. Rev. Immunol.* 9, 811–816 (2009).

77 Annunziato F, Cosmi L, Santarlasci V *et al.* Phenotypic and functional features of human Th17 cells. *J. Exp. Med.* 204, 1849–1861 (2007).

78 Bending D, De La Pena H, Veldhoen M *et al.* Highly purified Th17 cells from BDC2.5NOD mice convert into Th1-like cells in NOD/SCID recipient mice. *J. Clin. Invest.* 119, 565–572 (2009).

79 Lee YK, Turner H, Maynard CL *et al.* Late developmental plasticity in the T helper 17 lineage. *Immunity* 30, 92–107 (2009).

80 Rubtsov YP, Rudensky AY. TGFβ signalling in control of T-cell-mediated self-reactivity. *Nat. Rev. Immunol.* 7, 443–453 (2007).

81 Zhou L, Lopes JE, Chong MM *et al.* TGF-β-induced Foxp3 inhibits Th17 cell differentiation by antagonizing RORγt function. *Nature* 453, 236–240 (2008).

82 Mucida D, Park Y, Kim G *et al.* Reciprocal Th17 and regulatory T cell differentiation mediated by retinoic acid. *Science* 317, 256–260 (2007).

83 Bettelli E, Carrier Y, Gao W *et al.* Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* 441, 235–238 (2006).

* **First to reveal reciprocal control of Th17 and Treg differentiation and provides important insight into Treg immunotherapy.**

84 Xu L, Kitani A, Fuss I, Strober W. Cutting edge: regulatory T cells induce $CD4^+CD25^-$ $Foxp3^-$ T cells or are self-induced to become Th17 cells in the absence of exogenous TGF-β. *J. Immunol.* 178, 6725–6729 (2007).

85 Yang XO, Nurieva R, Martinez GJ *et al.* Molecular antagonism and plasticity of regulatory and inflammatory T cell programs. *Immunity* 29, 44–56 (2008).

86 Koenen HJ, Smeets RL, Vink PM, van Rijssen E, Boots AM, Joosten I. Human $CD25^{high}Foxp3^{pos}$ regulatory T cells differentiate into IL-17-producing cells. *Blood* 112, 2340–2352 (2008).

87 Voo KS, Wang YH, Santori FR *et al.* Identification of IL-17-producing $Foxp3^+$ regulatory T cells in humans. *Proc. Natl Acad. Sci. USA* 106, 4793–4798 (2009).

88 Zhou L, Chong MM, Littman DR. Plasticity of $CD4^+$ T cell lineage differentiation. *Immunity* 30, 646–655 (2009).

89 Li L, Kim J, Boussiotis VA. IL-1β-mediated signals preferentially drive conversion of regulatory T cells but not conventional T cells into IL-17-producing cells. *J. Immunol.* 185, 4148–4153 (2010).

90 Tsuji M, Komatsu N, Kawamoto S *et al.* Preferential generation of follicular B helper T cells from $Foxp3^+$ T cells in gut Peyer's patches. *Science* 323, 1488–1492 (2009).

91 Beriou G, Costantino CM, Ashley CW *et al.* IL-17-producing human peripheral regulatory T cells retain suppressive function. *Blood* 113, 4240–4249 (2009).

92 Kryczek I, Banerjee M, Cheng P *et al.* Phenotype, distribution, generation, and functional and clinical relevance of Th17 cells in the human tumor environments. *Blood* 114, 1141–1149 (2009).

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

94 Wei G, Wei L, Zhu J *et al.* Global mapping of H3K4me3 and H3K27me3 reveals specificity and plasticity in lineage fate determination of differentiating $CD4^+$ T cells. *Immunity* 30, 155–167 (2009).

95 Oldenhove G, Bouladoux N, Wohlfert EA *et al.* Decrease of $Foxp3^+$ Treg cell number and acquisition of effector cell phenotype during lethal infection. *Immunity* 31, 772–786 (2009).

96 Zhou X, Bailey-Bucktrout SL, Jeker LT *et al.* Instability of the transcription factor Foxp3 leads to the generation of pathogenic memory T cells *in vivo*. *Nat. Immunol.* 10, 1000–1007 (2009).

97 Zheng Y, Chaudhry A, Kas A *et al.* Regulatory T-cell suppressor program co-opts transcription factor IRF4 to control Th2 responses. *Nature* 458, 351–356 (2009).

98 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* 106, 1903–1908 (2009).

99 Rubtsov YP, Niec RE, Josefowicz S *et al.* Stability of the regulatory T cell lineage *in vivo*. *Science* 329, 1667–1671 (2010).

100 Yi T, Zhao D, Lin CL *et al.* Absence of donor Th17 leads to augmented Th1 differentiation and exacerbated acute graft-versus-host disease. *Blood* 112, 2101–2110 (2008).

** **Along with [101–106], investigates the role of Th17 in graft-versus-host disease (GvHD) using IL-17-deficient mice.**

101 Kappel LW, Goldberg GL, King CG *et al.* IL-17 contributes to CD4-mediated graft-versus-host disease. *Blood* 113, 945–952 (2009).

** **Along with [100,102–106], investigates the role of Th17 in GvHD using IL-17-deficient mice.**

102 Oh I, Ozaki K, Meguro A *et al.* Altered effector CD4$^+$ T cell function in IL-21R$^{-/-}$ CD4$^+$ T cell-mediated graft-versus-host disease. *J. Immunol.* 185, 1920–1926 (2010).

** **Along with [100,101,103–106], investigates the role of Th17 in GvHD using IL-17-deficient mice.**

103 Iclozan C, Yu Y, Liu C *et al.* T helper17 cells are sufficient but not necessary to induce acute graft-versus-host disease. *Biol. Blood Marrow Transplant.* 16, 170–178 (2010).

** **Along with [100–102,104–106], investigates the role of Th17 in GvHD using IL-17-deficient mice.**

104 Chen X, Das R, Komorowski R, van Snick J, Uyttenhove C, Drobyski WR. Interleukin 17 is not required for autoimmune-mediated pathologic damage during chronic graft-versus-host disease. *Biol. Blood Marrow Transplant.* 16, 123–128 (2010).

** **Along with [100–103,105,106], investigates the role of Th17 in GvHD using IL-17-deficient mice.**

105 Yi T, Chen Y, Wang L *et al.* Reciprocal differentiation and tissue-specific pathogenesis of Th1, Th2, and Th17 cells in graft versus host disease. *Blood* 114, 3101–3112 (2009).

** **Along with [100–104,106], investigates the role of Th17 in GvHD using IL-17-deficient mice.**

106 Hill GR, Olver SD, Kuns RD *et al.* Stem cell mobilization with G-CSF induces type 17 differentiation and promotes scleroderma. *Blood* 116, 819–828 (2010).

** **Along with [100–105], investigates the role of Th17 in GvHD using IL-17-deficient mice.**

107 McCormick LL, Zhang Y, Tootell E, Gilliam AC. Anti-TGF-β treatment prevents skin and lung fibrosis in murine sclerodermatous graft-versus-host disease: a model for human scleroderma. *J. Immunol.* 163, 5693–5699 (1999).

108 Banovic T, MacDonald KP, Morris ES *et al.* TGF-β in allogeneic stem cell transplantation: friend or foe? *Blood* 106, 2206–2214 (2005).

109 Blaise D, Kuentz M, Fortanier C *et al.* Randomized trial of bone marrow versus lenograstim-primed blood cell allogeneic transplantation in patients with early-stage leukemia: a report from the Societe Francaise de Greffe de Moelle. *J. Clin. Oncol.* 18, 537–546 (2000).

110 Carlson MJ, West ML, Coghill JM, Panoskaltsis-Mortari A, Blazar BR, Serody JS. *In vitro*-differentiated TH17 cells mediate lethal acute graft-versus-host disease with severe cutaneous and pulmonary pathologic manifestations. *Blood* 113, 1365–1374 (2009).

* **Investigates the ability of *ex vivo* polarized Th17 cells to induce GvHD in each target organ after transfer.**

111 Mauermann N, Burian J, von Garnier C *et al.* Interferon-γ regulates idiopathic pneumonia syndrome, a Th17$^+$CD4$^+$ T-cell-mediated graft-versus-host disease. *Am. J. Respir. Crit. Care Med.* 178, 379–388 (2008).

112 Chen X, Das R, Komorowski R *et al.* Blockade of interleukin-6 signaling augments regulatory T cell reconstitution and attenuates the severity of graft versus host disease. *Blood* 114, 891–900 (2009).

113 Tawara I, Koyama M, Liu C *et al.* Interleukin-6 modulates graft-versus-host responses after experimental allogeneic bone marrow transplantation. *Clin. Cancer Res.* 17, 77–88 (2011).

114 Karabon L, Wysoczanska B, Bogunia-Kubik K, Suchnicki K, Lange A. IL-6 and IL-10 promoter gene polymorphisms of patients and donors of allogeneic sibling hematopoietic stem cell transplants associate with the risk of acute graft-versus-host disease. *Hum. Immunol.* 66, 700–710 (2005).

115 Bucher C, Koch L, Vogtenhuber C *et al.* IL-21 blockade reduces graft-versus-host disease mortality by supporting inducible T regulatory cell generation. *Blood* 114, 5375–5384 (2009).

116 Meguro A, Ozaki K, Oh I *et al.* IL-21 is critical for graft-versus-host disease in a mouse model. *Bone Marrow Transplant.* 45, 723–729 (2010).

117 Das R, Chen X, Komorowski R, Hessner MJ, Drobyski WR. Interleukin-23 secretion by donor antigen-presenting cells is critical for organ-specific pathology in graft-versus-host disease. *Blood* 113, 2352–2362 (2009).

118 Thompson JS, Chu Y, Glass JF, Brown SA. Absence of IL-23p19 in donor allogeneic cells reduces mortality from acute GVHD. *Bone Marrow Transplant.* 45, 712–722 (2000).

119 Das R, Komorowski R, Hessner MJ *et al.* Blockade of interleukin-23 signaling results in targeted protection of the colon and allows for separation of graft-versus-host and graft-versus-leukemia responses. *Blood* 115, 5249–5258 (2010).

120 Taylor PA, Noelle RJ, Blazar BR. CD4$^+$CD25$^+$ immune regulatory cells are required for induction of tolerance to alloantigen via costimulatory blockade. *J. Exp. Med.* 193, 1311–1318 (2001).

** **Along with [121–125], established the suppressive role of Tregs on GvHD in mice.**

121 Cohen JL, Trenado A, Vasey D, Klatzmann D, Salomon BL. CD4$^+$CD25$^+$ immunoregulatory T cells: new therapeutics for graft-versus-host disease. *J. Exp. Med.* 196, 401–406 (2002).

** **Along with [120,122–125], established the suppressive role of Tregs on GvHD in mice.**

122 Hoffmann P, Ermann J, Edinger M, Fathman CG, Strober S. Donor-type CD4$^+$CD25$^+$ regulatory T cells suppress lethal acute graft-versus-host disease after allogeneic bone marrow transplantation. *J. Exp. Med.* 196, 389–399 (2002).

** **Along with [120,121,123–125], established the suppressive role of Tregs on GvHD in mice.**

123 Taylor PA, Lees CJ, Blazar BR. The infusion of *ex vivo* activated and expanded CD4$^+$CD25$^+$ immune regulatory cells inhibits graft-versus-host disease lethality. *Blood* 99, 3493–3499 (2002).

** **Along with [120–122,124,125], established the suppressive role of Tregs on GvHD in mice.**

124 Trenado A, Charlotte F, Fisson S *et al.* Recipient-type specific CD4$^+$CD25$^+$ regulatory T cells favor immune reconstitution and control graft-versus-host disease while maintaining graft-versus-leukemia. *J. Clin. Invest.* 112, 1688–1696 (2003).

** **Along with [120–123,125], established the suppressive role of Tregs on GvHD in mice.**

125 Edinger M, Hoffmann P, Ermann J *et al.* CD4$^+$CD25$^+$ regulatory T cells preserve graft-versus-tumor activity while inhibiting graft-versus-host disease after bone marrow transplantation. *Nat. Med.* 9, 1144–1150 (2003).

** **Along with [120–124], established the suppressive role of Tregs on GvHD in mice.**

126 Anderson BE, McNiff JM, Matte C, Athanasiadis I, Shlomchik WD, Shlomchik MJ. Recipient CD4$^+$ T cells that survive irradiation regulate chronic graft-versus-host disease. *Blood* 104, 1565–1573 (2004).

127 Zhao D, Zhang C, Yi T *et al. In vivo*-activated CD103$^+$CD4$^+$ regulatory T cells ameliorate ongoing chronic graft-versus-host disease. *Blood* 112, 2129–2138 (2008).

128 Taylor PA, Panoskaltsis-Mortari A, Swedin JM *et al.* L-Selectin^{hi} but not the L-selectin^{lo} CD4$^+$25$^+$ T-regulatory cells are potent inhibitors of GVHD and BM graft rejection. *Blood* 104, 3804–3812 (2004).

129 Nguyen VH, Shashidhar S, Chang DS *et al.* The impact of regulatory T cells on T-cell immunity following hematopoietic cell transplantation. *Blood* 111, 945–953 (2008).

130 Luo X, Tarbell KV, Yang H *et al.* Dendritic cells with TGF-β1 differentiate naive CD4$^+$CD25$^-$ T cells into islet-protective Foxp3$^+$ regulatory T cells. *Proc. Natl Acad. Sci. USA* 104, 2821–2826 (2007).

131 Pilat N, Baranyi U, Klaus C *et al.* Treg-therapy allows mixed chimerism and transplantation tolerance without cytoreductive conditioning. *Am. J. Transplant.* 10, 751–762 (2010).

132 Semple K, Yu Y, Wang D, Anasetti C, Yu XZ. Efficient and selective prevention of GVHD by antigen-specific induced tregs via linked-suppression in mice. *Biol. Blood Marrow Transplant.* 17, 309–318 (2011).

133 DiPaolo RJ, Brinster C, Davidson TS, Andersson J, Glass D, Shevach EM. Autoantigen-specific TGFβ-induced Foxp3$^+$ regulatory T cells prevent autoimmunity by inhibiting dendritic cells from activating autoreactive T cells. *J. Immunol.* 179, 4685–4693 (2007).

134 Zhang H, Podojil JR, Chang J, Luo X, Miller SD. TGF-β-induced myelin peptide-specific regulatory T cells mediate antigen-specific suppression of induction of experimental autoimmune encephalomyelitis. *J. Immunol.* 184, 6629–6636 (2010).

135 Koenecke C, Czeloth N, Bubke A *et al.* Alloantigen-specific *de novo*-induced Foxp3$^+$ Treg revert *in vivo* and do not protect from experimental GVHD. *Eur. J. Immunol.* 39, 3091–3096 (2009).

136 Vogtenhuber C, Bucher C, Highfill SL *et al.* Constitutively active Stat5b in CD4$^+$ T cells inhibits graft-versus-host disease lethality associated with increased regulatory T-cell potency and decreased T effector cell responses. *Blood* 116, 466–474 (2010).

137 Radojcic V, Pletneva MA, Yen HR *et al.* STAT3 signaling in CD4$^+$ T cells is critical for the pathogenesis of chronic sclerodermatous graft-versus-host disease in a murine model. *J. Immunol.* 184, 764–774 (2010).

138 Fujita S, Sato Y, Sato K *et al.* Regulatory dendritic cells protect against cutaneous chronic graft-versus- host disease mediated through CD4$^+$CD25$^+$Foxp3$^+$ regulatory T cells. *Blood* 110, 3793–3803 (2007).

139 Tawara I, Shlomchik WD, Jones A *et al.* A crucial role for host APCs in the induction of donor CD4$^+$CD25$^+$ regulatory T cell-mediated suppression of experimental graft-versus-host disease. *J. Immunol.* 185, 3866–3872 (2010).

140 Wang XN, Haniffa MA, Holtick U *et al.* Regulatory T-cell suppression of CD8$^+$ T-cell-mediated graft-versus-host reaction requires their presence during priming. *Transplantation* 88, 188–197 (2009).

* **Uses an interesting model system to evaluate the function of human Tregs.**

141 Takahashi T, Tagami T, Yamazaki S *et al.* Immunologic self-tolerance maintained by CD25$^+$CD4$^+$ regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. *J. Exp. Med.* 192, 303–310 (2000).

142 Zeiser R, Nguyen VH, Hou JZ *et al.* Early CD30 signaling is critical for adoptively transferred CD4$^+$CD25$^+$ regulatory T cells in prevention of acute graft-versus-host disease. *Blood* 109, 2225–2233 (2007).

143 Amarnath S, Costanzo CM, Mariotti J *et al.* Regulatory T cells and human myeloid dendritic cells promote tolerance via programmed death ligand-1. *PLoS Biol.* 8, E1000302 (2010).

144 Asakura S, Hashimoto D, Takashima S *et al.* Alloantigen expression on non-hematopoietic cells reduces graft-versus-leukemia effects in mice. *J. Clin. Invest.* 120, 2370–2378 (2010).

145 Nguyen VH, Zeiser R, Dasilva DL *et al.* *In vivo* dynamics of regulatory T-cell trafficking and survival predict effective strategies to control graft-versus-host disease following allogeneic transplantation. *Blood* 109, 2649–2656 (2007).

* **Along with** [146,147], **investigates trafficking of Tregs *in vivo* in mice.**

146 Ermann J, Hoffmann P, Edinger M *et al.* Only the CD62L$^+$ subpopulation of CD4$^+$CD25$^+$ regulatory T cells protects from lethal acute GVHD. *Blood* 105, 2220–2226 (2005).

* **Along with** [145,147], **investigates trafficking of Tregs *in vivo* in mice.**

147 Wysocki CA, Jiang Q, Panoskaltsis-Mortari A *et al.* Critical role for CCR5 in the function of donor CD4$^+$CD25$^+$ regulatory T cells during acute graft-versus-host disease. *Blood* 106, 3300–3307 (2005).

* **Along with** [145,146], **investigates trafficking of Tregs *in vivo* in mice.**

148 Chen X, Vodanovic-Jankovic S, Johnson B, Keller M, Komorowski R, Drobyski WR. Absence of regulatory T-cell control of Th1 and TH17 cells is responsible for the autoimmune-mediated pathology in chronic graft-versus-host disease. *Blood* 110, 3804–3813 (2007).

149 Sakoda Y, Hashimoto D, Asakura S *et al.* Donor-derived thymic-dependent T cells cause chronic graft-versus-host disease. *Blood* 109, 1756–1764 (2007).

150 Jones SC, Murphy GF, Korngold R. Post-hematopoietic cell transplantation control of graft-versus-host disease by donor CD425 T cells to allow an effective graft-versus-leukemia response. *Biol. Blood Marrow Transplant.* 9, 243–256 (2003).

151 Cai SF, Cao X, Hassan A, Fehniger TA, Ley TJ. Granzyme B is not required for regulatory T cell-mediated suppression of graft-versus-host disease. *Blood* 115, 1669–1677 (2010).

152 van Rood JJ, Loberiza FR Jr, Zhang MJ *et al.* Effect of tolerance to noninherited maternal antigens on the occurrence of graft-versus-host disease after bone marrow transplantation from a parent or an HLA-haploidentical sibling. *Blood* 99, 1572–1577 (2002).

153 Ichinohe T, Uchiyama T, Shimazaki C *et al.* Feasibility of HLA-haploidentical hematopoietic stem cell transplantation between noninherited maternal antigen (NIMA)-mismatched family members linked with long-term fetomaternal microchimerism. *Blood* 104, 3821–3828 (2004).

154 Teshima T, Matsuoka K, Ichinohe T. Impact of fetal–maternal tolerance in hematopoietic stem cell transplantation. *Arch. Immunol. Ther. Exp. (Warsz.)* 54, 165–172 (2006).

155 Matsuoka K, Ichinohe T, Hashimoto D, Asakura S, Tanimoto M, Teshima T. Fetal tolerance to maternal antigens improves the outcome of allogeneic bone marrow transplantation by a CD4$^+$CD25$^+$ T-cell-dependent mechanism. *Blood* 107, 404–409 (2006).

156 Aoyama K, Koyama M, Matsuoka K *et al.* Improved outcome of allogeneic bone marrow transplantation due to breastfeeding-induced tolerance to maternal antigens. *Blood* 113, 1829–1833 (2009).

157 Mold JE, Michaelsson J, Burt TD *et al.* Maternal alloantigens promote the development of tolerogenic fetal regulatory T cells *in utero*. *Science* 322, 1562–1565 (2008).

158 Jonuleit H, Schmitt E, Kakirman H, Stassen M, Knop J, Enk AH. Infectious tolerance: human CD25$^+$ regulatory T cells convey suppressor activity to conventional CD4$^+$ T helper cells. *J. Exp. Med.* 196, 255–260 (2002).

159 Waldmann H, Adams E, Fairchild P, Cobbold S. Infectious tolerance and the long-term acceptance of transplanted tissue. *Immunol. Rev.* 212, 301–313 (2006).

160 Carvalho A, Cunha C, Di Ianni M *et al.* Prognostic significance of genetic variants in the IL-23/Th17 pathway for the outcome of T cell-depleted allogeneic stem cell transplantation. *Bone Marrow Transplant.* 45, 1645–1652 (2010).

161 Dander E, Balduzzi A, Zappa G *et al.* Interleukin-17-producing T-helper cells as new potential player mediating graft-versus-host disease in patients undergoing allogeneic stem-cell transplantation. *Transplantation* 88, 1261–1272 (2009).

162 Broady R, Yu J, Chow V *et al.* Cutaneous GVHD is associated with the expansion of tissue-localized Th1 and not Th17 cells. *Blood* 116, 5748–5751 (2010).

163 Ratajczak P, Janin A, Peffault de Latour R *et al.* Th17/Treg ratio in human graft-versus-host disease. *Blood* 116, 1165–1171 (2010).

164 Leonardi CL, Kimball AB, Papp KA *et al.* Efficacy and safety of ustekinumab, a human interleukin-12/23 monoclonal antibody, in patients with psoriasis: 76-week results from a randomised, double-blind, placebo-controlled trial (PHOENIX 1). *Lancet* 371, 1665–1674 (2008).

165 Papp KA, Langley RG, Lebwohl M *et al.* Efficacy and safety of ustekinumab, a human interleukin-12/23 monoclonal antibody, in patients with psoriasis: 52-week results from a randomised, double-blind, placebo-controlled trial (PHOENIX 2). *Lancet* 371, 1675–1684 (2008).

166 Griffiths CE, Strober BE, van de Kerkhof P *et al.* Comparison of ustekinumab and etanercept for moderate-to-severe psoriasis. *N. Engl. J. Med.* 362, 118–128 (2010).

167 Segal BM, Constantinescu CS, Raychaudhuri A, Kim L, Fidelus-Gort R, Kasper LH. Repeated subcutaneous injections of IL12/23 p40 neutralising antibody, ustekinumab, in patients with relapsing-remitting multiple sclerosis: a Phase II, double-blind, placebo-controlled, randomised, dose-ranging study. *Lancet Neurol.* 7, 796–804 (2008).

168 Sandborn WJ, Feagan BG, Fedorak RN *et al.* A randomized trial of ustekinumab, a human interleukin-12/23 monoclonal antibody, in patients with moderate-to-severe Crohn's disease. *Gastroenterology* 135, 1130–1141 (2008).

169 Genovese MC, Van den Bosch F, Roberson SA *et al.* LY2439821, a humanized anti-interleukin-17 monoclonal antibody, in the treatment of patients with rheumatoid arthritis: A Phase I randomized, double-blind, placebo-controlled, proof-of-concept study. *Arthritis Rheum.* 62, 929–939 (2010).

170 Choy EH, Isenberg DA, Garrood T *et al.* Therapeutic benefit of blocking interleukin-6 activity with an anti-interleukin-6 receptor monoclonal antibody in rheumatoid arthritis: a randomized, double-blind, placebo-controlled, dose-escalation trial. *Arthritis Rheum.* 46, 3143–3150 (2002).

171 Yokota S, Imagawa T, Mori M *et al.* Efficacy and safety of tocilizumab in patients with systemic-onset juvenile idiopathic arthritis: a randomised, double-blind, placebo-controlled, withdrawal Phase III trial. *Lancet* 371, 998–1006 (2008).

172 Nishimoto N, Terao K, Mima T, Nakahara H, Takagi N, Kakehi T. Mechanisms and pathologic significances in increase in serum interleukin-6 (IL-6) and soluble IL-6 receptor after administration of an anti-IL-6 receptor antibody, tocilizumab, in patients with rheumatoid arthritis and Castleman disease. *Blood* 112, 3959–3964 (2008).

173 Gergis U, Arnason J, Yantiss R *et al.* Effectiveness and safety of tocilizumab, an anti-interleukin-6 receptor monoclonal antibody, in a patient with refractory GI graft-versus-host disease. *J. Clin. Oncol.* 28, 602–604 (2010).

174 Shima Y, Kuwahara Y, Murota H *et al.* The skin of patients with systemic sclerosis softened during the treatment with anti-IL-6 receptor antibody tocilizumab. *Rheumatology (Oxf.)* 49, 2408–2412 (2010).

175 Wolf D, Wolf AM, Fong D *et al.* Regulatory T-cells in the graft and the risk of acute graft-versus-host disease after allogeneic stem cell transplantation. *Transplantation* 83, 1107–1113 (2007).

176 Magenau JM, Qin X, Tawara I *et al.* Frequency of $CD4^+CD25^{hi}Foxp3^+$ regulatory T cells has diagnostic and prognostic value as a biomarker for acute graft-versus-host-disease. *Biol. Blood Marrow Transplant.* 16, 907–914 (2010).

177 Rosenzwajg M, Dhedin N, Maury S *et al.* Regulatory T cell content in the bone marrow graft does not predict the occurrence of acute GVHD. *Biol. Blood Marrow Transplant.* 17, 265–269 (2010).

178 Stanzani M, Martins SL, Saliba RM *et al.* CD25 expression on donor $CD4^+$ or $CD8^+$ T cells is associated with an increased risk for graft-versus-host disease after HLA-identical stem cell transplantation in humans. *Blood* 103, 1140–1146 (2004).

179 Clark FJ, Gregg R, Piper K *et al.* Chronic graft-versus-host disease is associated with increased numbers of peripheral blood $CD4^+CD25^{high}$ regulatory T cells. *Blood* 103, 2410–2416 (2004).

180 Lee SJ, Zahrieh D, Agura E *et al.* Effect of up-front daclizumab when combined with steroids for the treatment of acute graft-versus-host disease: results of a randomized trial. *Blood* 104, 1559–1564 (2004).

181 Vela-Ojeda J, Montiel-Cervantes L, Granados-Lara P *et al.* Role of $CD4^+CD25^{+high}Foxp3^+CD62L^+$ regulatory T cells and invariant NKT cells in human allogeneic hematopoietic stem cell transplantation. *Stem Cells Dev* 19, 333–340 (2010).

182 Blache C, Chauvin JM, Marie-Cardine A *et al.* Reduced frequency of regulatory T cells in peripheral blood stem cell compared to bone marrow transplantations. *Biol. Blood Marrow Transplant.* 16, 430–434 (2010).

183 Mold JE, Venkatasubrahmanyam S, Burt TD *et al.* Fetal and adult hematopoietic stem cells give rise to distinct T cell lineages in humans. *Science* 330, 1695–1699 (2010).

184 Miura Y, Thoburn CJ, Bright EC *et al.* Association of Foxp3 regulatory gene expression with graft-versus-host disease. *Blood* 104, 2187–2193 (2004).

185 Zorn E, Kim HT, Lee SJ *et al.* Reduced frequency of $Foxp3^+$ $CD4^+CD25^+$ regulatory T cells in patients with chronic graft-versus-host disease. *Blood* 106, 2903–2911 (2005).

186 Pastore D, Delia M, Mestice A *et al.* Recovery of CMV-specific $CD8^+$ T cells and T regs after allogeneic peripheral blood stem cell transplantation. *Biol. Blood Marrow Transplant.* 17, 550–557 (2011).

187 Rezvani K, Mielke S, Ahmadzadeh M *et al.* High donor FOXP3-positive regulatory T-cell (Treg) content is associated with a low risk of GVHD following HLA-matched allogeneic SCT. *Blood* 108, 1291–1297 (2006).

188 Mielke S, Rezvani K, Savani BN *et al.* Reconstitution of $Foxp3^+$ regulatory T cells (Tregs) after CD25-depleted allotransplantation in elderly patients and association with acute graft-versus-host disease. *Blood* 110, 1689–1697 (2007).

189 Engelhardt BG, Jagasia M, Savani BN *et al.* Regulatory T cell expression of CLA or $\alpha(4)\beta(7)$ and skin or gut acute GVHD outcomes. *Bone Marrow Transplant.* 46, 436–442 (2011).

190 Rieger K, Loddenkemper C, Maul J *et al.* Mucosal $Foxp3^+$ regulatory T cells are numerically deficient in acute and chronic GVHD. *Blood* 107, 1717–1723 (2006).

191 Landfried K, Bataille F, Rogler G *et al.* Recipient NOD2/CARD15 status affects cellular infiltrates in human intestinal graft-versus-host disease. *Clin. Exp. Immunol.* 159, 87–92 (2010).

192 Fondi C, Nozzoli C, Benemei S *et al.* Increase in $Foxp3^+$ regulatory T cells in GVHD skin biopsies is associated with lower disease severity and treatment response. *Biol. Blood Marrow Transplant.* 15, 938–947 (2009).

193 Schneider M, Munder M, Karakhanova S, Ho AD, Goerner M. The initial phase of graft-versus-host disease is associated with a decrease of $CD4^+CD25^+$ regulatory T cells in the peripheral blood of patients after allogeneic stem cell transplantation. *Clin. Lab. Haematol.* 28, 382–390 (2006).

194 Arimoto K, Kadowaki N, Ishikawa T, Ichinohe T, Uchiyama T. Foxp3 expression in peripheral blood rapidly recovers and lacks correlation with the occurrence of graft-versus-host disease after allogeneic stem cell transplantation. *Int. J. Hematol.* 85, 154–162 (2007).

195 Matsuoka K, Kim HT, McDonough S *et al.* Altered regulatory T cell homeostasis in patients with $CD4^+$ lymphopenia following allogeneic hematopoietic stem cell transplantation. *J. Clin. Invest.* 120, 1479–1493 (2010).

** **Comparative analysis of Treg and Tcon reconstitution after allogeneic stem cell transplantation and of association between Treg reconstitution and chronic GvHD.**

196 Fisson S, Darrasse-Jeze G, Litvinova E *et al.* Continuous activation of autoreactive $CD4^+$ $CD25^+$ regulatory T cells in the steady state. *J. Exp. Med.* 198, 737–746 (2003).

197 Vukmanovic-Stejic M, Zhang Y, Cook JE *et al.* Human $CD4^+$ $CD25^{hi}$ $Foxp3^+$ regulatory T cells are derived by rapid turnover of memory populations *in vivo*. *J. Clin. Invest.* 116, 2423–2433 (2006).

198 Brode S, Raine T, Zaccone P, Cooke A. Cyclophosphamide-induced type-1 diabetes in the NOD mouse is associated with a reduction of $CD4^+CD25^+Foxp3^+$ regulatory T cells. *J. Immunol.* 177, 6603–6612 (2006).

199 Curotto de Lafaille MA, Lino AC, Kutchukhidze N, Lafaille JJ. CD25- T cells generate $CD25^+Foxp3^+$ regulatory T cells by peripheral expansion. *J. Immunol.* 173, 7259–7268 (2004).

200 Matthews K, Lim Z, Afzali B *et al.* Imbalance of effector and regulatory CD4 T cells is associated with graft-versus-host disease after hematopoietic stem cell transplantation using a reduced intensity conditioning regimen and alemtuzumab. *Haematologica* 94, 956–966 (2009).

201 Palmer JM, Chen BJ, DeOliveira D, Le ND, Chao NJ. Novel mechanism of rapamycin in GVHD: increase in interstitial regulatory T cells. *Bone Marrow Transplant.* 45, 379–384 (2010).

202 Gatza E, Rogers CE, Clouthier SG *et al.* Extracorporeal photopheresis reverses experimental graft-versus-host disease through regulatory T cells. *Blood* 112, 1515–1521 (2008).

203 Di Biaso I, Di Maio L, Bugarin C *et al.* Regulatory T cells and extracorporeal photochemotherapy: correlation with clinical response and decreased frequency of proinflammatory T cells. *Transplantation* 87, 1422–1425 (2009).

204 Bastien JP, Krosl G, Therien C *et al.* Photodepletion differentially affects $CD4^+$ Tregs versus $CD4^+$ effector T cells from patients with chronic graft-versus-host disease. *Blood* 116, 4859–4869 (2010).

205 Rao V, Saunes M, Jorstad S, Moen T. Cutaneous T cell lymphoma and graft-versus-host disease: a comparison of *in vivo* effects of extracorporeal photochemotherapy on $Foxp3^+$ regulatory T cells. *Clin. Immunol.* 133, 303–313 (2009).

206 Riley JL, June CH, Blazar BR. Human T regulatory cell therapy: take a billion or so and call me in the morning. *Immunity* 30, 656–665 (2009).

207 Di Ianni M, Del Papa B, Cecchini D *et al.* Immunomagnetic isolation of $CD4^+CD25^+Foxp3^+$ natural T regulatory lymphocytes for clinical applications. *Clin. Exp. Immunol.* 156, 246–253 (2009).

208 Seddiki N, Santner-Nanan B, Martinson J *et al.* Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. *J. Exp. Med.* 203, 1693–1700 (2006).

209 Kleinewietfeld M, Starke M, Di Mitri D *et al.* CD49d provides access to 'untouched' human $Foxp3^+$ Treg free of contaminating effector cells. *Blood* 113, 827–836 (2009).

210 Trzonkowski P, Bieniaszewska M, Juscinska J *et al.* 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.* 133, 22–26 (2009).

** **The first clinical results of the treatment of ongoing GvHD with Treg immunotherapy.**

211 Hoffmann P, Eder R, Edinger M. Polyclonal expansion of human $CD4^+CD25^+$ regulatory T cells. *Methods Mol. Biol.* 677, 15–30 (2011).

** **Along with** [212], **discusses practical issues of methodology for isolation and expansion of Tregs.**

212 Hoffmann P, Boeld TJ, Eder R *et al.* Loss of FOXP3 expression in natural human $CD4^+CD25^+$ regulatory T cells upon repetitive *in vitro* stimulation. *Eur. J. Immunol.* 39, 1088–1097 (2009).

** **Along with** [211], **discusses practical issues of methodology for isolation and expansion of Tregs.**

213 Liu G, Yang K, Burns S, Shrestha S, Chi H. The S1P(1)–mTOR axis directs the reciprocal differentiation of Th1 and Treg cells. *Nat. Immunol.* 11, 1047–1056 (2010).

214 Coenen JJ, Koenen HJ, van Rijssen E *et al.* Rapamycin, not cyclosporine, permits thymic generation and peripheral preservation of $CD4^+$ $CD25^+$ $Foxp3^+$ T cells. *Bone Marrow Transplant.* 39, 537–545 (2007).

215 Qu Y, Zhang B, Zhao L *et al.* The effect of immunosuppressive drug rapamycin on regulatory $CD4^+CD25^+Foxp3^+$T cells in mice. *Transpl. Immunol.* 17, 153–161 (2007).

216 Kopf H, de la Rosa GM, Howard OM, Chen X. Rapamycin inhibits differentiation of Th17 cells and promotes generation of $Foxp3^+$ T regulatory cells. *Int. Immunopharmacol.* 7, 1819–1824 (2007).

217 Kim YM, Sachs T, Asavaroengchai W, Bronson R, Sykes M. Graft-versus-host disease can be separated from graft-versus-lymphoma effects by control of lymphocyte trafficking with FTY720. *J. Clin. Invest.* 111, 659–669 (2003).

218 Hashimoto D, Asakura S, Matsuoka K *et al.* FTY720 enhances the activation-induced apoptosis of donor T cells and modulates graft-versus-host disease. *Eur. J. Immunol.* 37, 271–281 (2007).

219 Taylor PA, Ehrhardt MJ, Lees CJ *et al.* Insights into the mechanism of FTY720 and compatibility with regulatory T cells for the inhibition of graft-versus-host disease (GVHD). *Blood* 110, 3480–3488 (2007).

220 Samanta A, Li B, Song X *et al.* TGF-β and IL-6 signals modulate chromatin binding and promoter occupancy by acetylated Foxp3. *Proc. Natl Acad. Sci. USA* 105, 14023–14027 (2008).

221 Li B, Samanta A, Song X *et al.* FOXP3 interactions with histone acetyltransferase and class II histone deacetylases are required for repression. *Proc. Natl Acad. Sci. USA* 104, 4571–4576 (2007).

* **Along with** [222,223], **demonstrates that histone deacetylase inhibitors promote Treg generation and function.**

222 Tao R, de Zoeten EF, Ozkaynak E *et al.* Deacetylase inhibition promotes the generation and function of regulatory T cells. *Nat. Med.* 13, 1299–1307 (2007).

* **Along with** [221,223], **demonstrates that histone deacetylase inhibitors promote Treg generation and function.**

223 Xiao Y, Li B, Zhou Z, Hancock WW, Zhang H, Greene MI. Histone acetyltransferase mediated regulation of Foxp3 acetylation and Treg function. *Curr. Opin. Immunol.* 22, 583–591 (2010).

* **Along with** [221,222], **demonstrates that histone deacetylase inhibitors promote Treg generation and function.**

224 Reddy P, Maeda Y, Hotary K *et al.* Histone deacetylase inhibitor suberoylanilide hydroxamic acid reduces acute graft-versus-host disease and preserves graft-versus-leukemia effect. *Proc. Natl Acad. Sci. USA* 101, 3921–3926 (2004).

225 Reddy P, Sun Y, Toubai T *et al.* Histone deacetylase inhibition modulates indoleamine 2,3-dioxygenase-dependent DC functions and regulates experimental graft-versus-host disease in mice. *J. Clin. Invest.* 118, 2562–2573 (2008).

226 Polansky JK, Kretschmer K, Freyer J *et al.* DNA methylation controls Foxp3 gene expression. *Eur. J. Immunol.* 38, 1654–1663 (2008).

227 Baron U, Floess S, Wieczorek G *et al.* DNA demethylation in the human FOXP3 locus discriminates regulatory T cells from activated FOXP3$^+$ conventional T cells. *Eur. J. Immunol.* 37, 2378–2389 (2007).

228 Wieczorek G, Asemissen A, Model F *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.* 69, 599–608 (2009).

229 Takahashi T, Kuniyasu Y, Toda M *et al.* Immunologic self-tolerance maintained by CD25$^+$CD4$^+$ naturally anergic and suppressive T cells: induction of autoimmune disease by breaking their anergic/suppressive state. *Int. Immunol.* 10, 1969–1980 (1998).

230 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.* 174, 3290–3297 (2005).

231 Zeiser R, Nguyen VH, Beilhack A *et al.* Inhibition of CD4$^+$CD25$^+$ regulatory T-cell function by calcineurin-dependent interleukin-2 production. *Blood* 108, 390–399 (2006).

232 Zorn E, Mohseni M, Kim H *et al.* Combined CD4$^+$ donor lymphocyte infusion and low-dose recombinant IL-2 expand Foxp3$^+$ regulatory T cells following allogeneic hematopoietic stem cell transplantation. *Biol. Blood Marrow Transplant.* 15, 382–388 (2009).

233 Brunstein CG, Miller JS, Cao Q *et al.* Infusion of *ex vivo* expanded T regulatory cells in adults transplanted with umbilical cord blood: safety profile and detection kinetics. *Blood* 117, 1061–1070 (2011).

** **The first prospective study of immunotherapy using cord blood-derived Tregs in cord blood transplantation as a prophylaxis of GvHD.**

234 Di Ianni M, Falzetti F, Carotti A *et al.* Tregs prevent GVHD and promote immune reconstitution in HLA-haploidentical transplantation. *Blood* 117, 3921–3928 (2011).

235 Maury S, Lemoine FM, Hicheri Y *et al.* CD4$^+$CD25$^+$ regulatory T cell depletion improves the graft-versus-tumor effect of donor lymphocytes after allogeneic hematopoietic stem cell transplantation. *Sci. Transl. Med.* 2, 41–52 (2010).

236 Lovett-Racke AE, Rocchini AE, Choy J *et al.* Silencing T-bet defines a critical role in the differentiation of autoreactive T lymphocytes. *Immunity* 21, 719–731 (2004).

237 O'Connell RM, Kahn D, Gibson WS *et al.* MicroRNA-155 promotes autoimmune inflammation by enhancing inflammatory T cell development. *Immunity* 33, 607–619 (2010).

original article

Annals of Oncology 23: 1537–1541, 2012
doi:10.1093/annonc/mdr487
Published online 29 October 2011

Postprogression survival for first-line chemotherapy of patients with advanced non-small-cell lung cancer

H. Hayashi[1], I. Okamoto[1]*, S. Morita[2], M. Taguri[2] & K. Nakagawa[1]

[1]*Department of Medical Oncology, Kinki University Faculty of Medicine, Osaka-Sayama;* [2]*Department of Biostatistics and Epidemiology, Yokohama City University Medical Center, Yokohama, Japan*

Received 12 July 2011; revised 5 September 2011 & revised 13 September 2011; accepted 14 September 2011

Background: Given the growing number of drugs available for non-small-cell lung cancer (NSCLC), an effect of first-line chemotherapy on overall survival (OS) might be confounded by subsequent therapies. We examined the relation between postprogression survival (PPS) and OS in phase III trials of first-line chemotherapy for advanced NSCLC.
Patients and methods: A literature search identified 69 trials that were published during the past decade. We partitioned OS into progression-free survival (PFS) and PPS and evaluated the relation between OS and either PFS or PPS. We also examined whether any association might be affected by the year of completion of trial enrollment.
Results: The average PPS was longer in recent trials than in older trials (6.5 versus 4.4 months, $P < 0.0001$). For all trials, PPS was strongly associated with OS ($r = 0.82$), whereas PFS was moderately associated with OS ($r = 0.43$). The correlation between OS and PPS in recent trials was stronger than that in older trials ($r = 0.89$ and 0.66).
Conclusions: Our findings indicate that, especially for recent trials, PPS is highly associated with OS in first-line chemotherapy for advanced NSCLC, whereas PFS is only moderately associated with OS.
Key words: chemotherapy, non-small-cell lung cancer, overall survival, phase III trial, progression-free survival

introduction

Lung cancer remains the leading cause of cancer death worldwide [1, 2], with non-small-cell lung cancer (NSCLC) accounting for ~85% of lung cancer cases. Most individuals with NSCLC have metastatic disease at the time of diagnosis and therefore have a poor prognosis. The standard treatment of advanced NSCLC over the past decade has been platinum-based chemotherapy because of the moderate improvement in survival it confers [3–6]. Although many patients initially achieve clinical remission or disease stabilization with first-line chemotherapy, nearly all subsequently experience disease progression and eventually die of advanced NSCLC.

Overall survival (OS) has been traditionally recognized as the most important therapeutic objective for NSCLC patients. However, in view of the growing number of drugs and combinations thereof that are available for the treatment of such patients, any effect of first-line chemotherapy on OS might be confounded by subsequent therapies [7]. Indeed, an improvement in progression-free survival (PFS) has not necessarily resulted in an improved OS in recent randomized trials in patients with NSCLC [8, 9].

The effect of therapies instituted after disease progression on survival in clinical trials is thus of interest. However, little is known about postprogression survival (PPS) in NSCLC. In the present study, we partitioned OS of phase III trials for chemotherapy-naive patients with NSCLC into PFS and PPS and assessed the association of each with OS.

methods

search strategy and selection of trials

An independent review of PubMed citations from 1 January 2000 to 31 October 2010 was carried out. Key words included in the search were 'non–small cell lung cancer', 'clinical trial', 'advanced', and 'chemotherapy'. The search was limited to randomized controlled phase III trials and articles published in English. We reviewed each publication, and phase III studies that compared two or more first-line systemic chemotherapies (including treatment with molecularly targeted agents) for advanced or metastatic NSCLC were selected. To find any additional trials, we searched the reference lists of included trials as well as of large systematic reviews. We also checked articles that were in press at leading journals and searched websites listing abstracts from conferences (organized by the American Society of Clinical Oncology or the Federation of European Cancer Societies). We included trials that provided data for both OS and either PFS or time to progression (TTP), whether or not these parameters were explicitly defined. Trials were excluded if they investigated only immunotherapy regimens or hormonal therapies. Trials that were designed to assess combined modality treatments, including radiation therapy and surgery, were also excluded. To avoid bias, two observers (HH and IO) independently abstracted the data from the trials.

data abstraction

We analyzed in detail the primary and secondary efficacy end points, following the definitions of the authors of each trial. When not specifically

Correspondence to: Dr I. Okamoto, Department of Medical Oncology, Kinki University Faculty of Medicine, 377-2 Ohno-higashi, Osaka-Sayama, Osaka 589-8511, Japan.
Tel: +81-72-366-0221; Fax: +81-72-360-5000;
E-mail: ichi-okamoto@dotd.med.kindai.ac.jp

stated by the authors, we considered the primary end point to be that used for calculation of sample size. For the sake of simplicity, two end points (PFS and TTP) based on tumor assessment are collectively referred to as PFS in the present study, similar to the approach adopted in a recent report [10]. Median OS and median PFS were extracted from all trials that provided data for each treatment group. Median PPS was defined as median OS minus median PFS for each trial. We also obtained the following information from each report: year of completion of trial enrollment, number of patients randomized, number of patients in each treatment arm, number of treatment arms in each trial, proportion of patients who were male or had adenocarcinoma, and median age of the patients.

data analysis

We summarized the survival data (median OS, median PFS, median PPS, and median PFS/median OS) as the average and standard error (SE) for trial arms. SE was calculated on the basis of previously described models [11]. We also calculated the percentage of OS accounted for by PPS for each trial arm as: 100 − (100 × median PFS/median OS). To assess the relation between median OS and either median PFS or median PPS, we used Spearman's rank correlation coefficient. To account for differences in sample size among trial arms, we weighted all analyses by the number of patients in each arm. In addition, all trials were divided into two groups on the basis of the year in which trial enrollment was completed. Given that the median year for completion of enrollment in the 69 analyzed trials was 2002, we dichotomized at year 2002 (older trials, up to and including 2002; recent trials, 2003 and later) in order to evaluate a possible change in PPS, and we assessed whether the evaluated relations might be dependent on the year of completion of trial enrollment. We examined differences in the survival data between older and recent trials by normal approximation of the average survival data (z test). All reported P-values correspond to two-sided tests, and those of P-values <0.05 were considered statistically significant. Analyses were carried out with SAS for Windows release 9.2 (SAS Institute, Cary, NC).

results

characteristics of the trials

Our search yielded a total of 467 potentially relevant publications. Initially, 366 studies were excluded for at least one of the following reasons: they examined other malignancies or combined modality treatments, they were not randomized, they were phase I or II trials, they were review articles, they represented subgroup analyses, or they were duplicates. The selection process for the randomized controlled trials is shown in Figure 1. Review of the remaining 101 publications yielded 69 trials that were considered to be highly relevant for the present study. The main characteristics of the 69 phase III trials included in the analysis are listed in Table 1. A total of 37 986 patients with advanced NSCLC were enrolled, with a median number of patients per study of 433 (range 153–1725). Most of the trials had a high proportion of male patients and of patients with adenocarcinoma. The average median age of the patients was 62.3 years. Ten trials used an end point based on tumor assessment (PFS or TTP) as the primary end point, whereas OS was assessed as the primary end point in 53 trials. The other six trials used response rate or quality of life as the primary end point.

median OS, PFS, and PPS in all trials and in subgroups based on year of completion of trial enrollment

The survival data for trial arms according to the year in which trial enrollment was completed are shown in Table 2. Although the average median PFS in older (up to and including 2002) trials was the same (4.9 months) as that in recent (2003 and later) trials, the average median PPS was ~50% longer in the

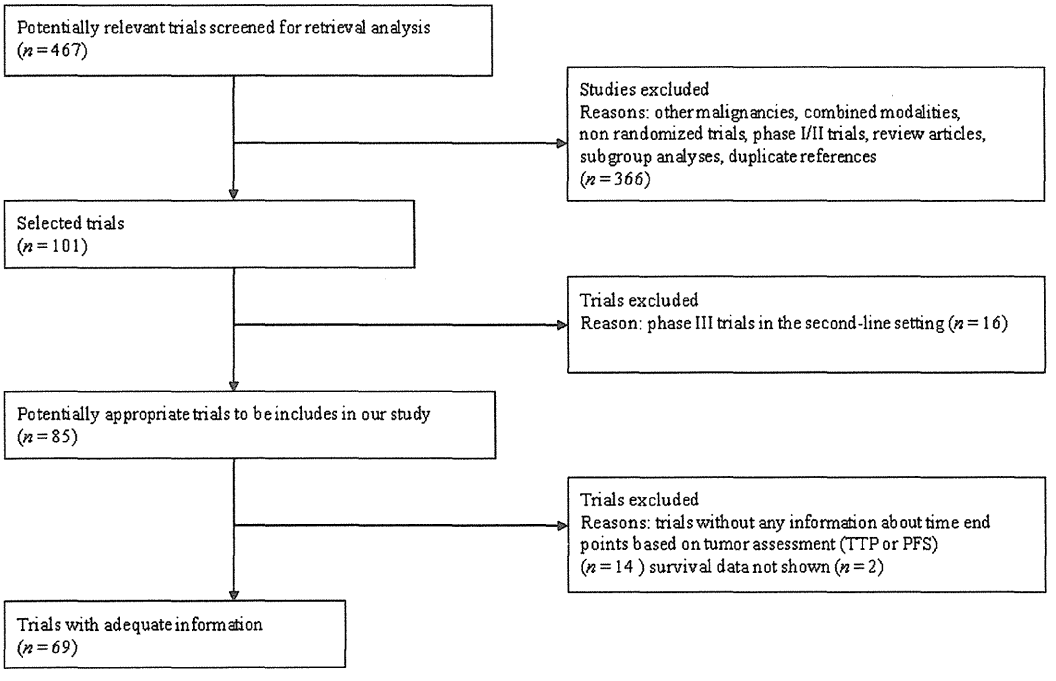

Figure 1. Flow chart showing the progress of trials through the selection process.