

## CD4 (+)CD25 (+)Foxp3 (+) regulatory T cells and hematologic malignancies

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### TABLE OF CONTENTS

1. Abstract
2. Introduction
3. Regulatory T cells in non-hematopoietic disorders
4. Regulatory T cells in lymphoma
  - 4.1 Follicular lymphoma
  - 4.2 Hodgkin lymphoma.
  - 4.3 Diffuse large B cell lymphoma
  - 4.4 Cutaneous T cell lymphoma
  - 4.5 Summary of findings in lymphoma
5. Regulatory T cells in leukemia
  - 5.1 Acute leukemia
  - 5.2 Chronic lymphocytic leukemia
6. Regulatory T cells in myeloma
7. Regulatory T cells in myeloproliferative neoplasms, myelodysplastic disorders and aplastic anemia.
  - 7.1 Myeloproliferative neoplasms
  - 7.2 Myelodysplastic disorders
  - 7.3 Aplastic anemia
8. Summary and perspective
9. References

## 1. ABSTRACT

Regulatory T cells (Tregs) are an immunosuppressive T cell subset that functions to prevent autoimmunity and to regulate physiologic immune reactions. Tregs are also present in the tumor microenvironment and appear to play an important role in the pathophysiology of malignant processes. Available data suggests that this role is context-dependent, as a higher density of tumor infiltrating Tregs at diagnosis may be associated with either a positive or a negative clinical outcome. Negative prognostic associations are found primarily in solid tumors such as ovarian carcinoma, while positive associations have been reported in various lymphomas, most prominently in those of germinal center (GC) B cell derivation. Most of these observations are correlative, however, as mechanistic studies have lagged behind descriptive observations because of a lack of informative animal models. Nonetheless, the available data are intriguing and provide compelling support for the hypothesis that Tregs are pathobiologically relevant. This review focuses on studies of the role of CD4 (+)CD25 (+)FOXP3 (+) Tregs in hematopoietic malignancies and clonal myeloid neoplasms.

## 2. INTRODUCTION

Evidence for a distinct, functionally suppressive T cell subpopulation dates to more than twenty years ago, but the importance of this CD4 (+) subset to immune physiology and homeostasis has been realized only over the last decade. Initially, CD4 (+) regulatory T cells (Tregs) were identified phenotypically based on constitutive expression of the IL-2 receptor alpha-chain (CD25), resulting in the immunoprofile CD4 (+)CD25 (high). In a series of seminal experiments, depletion of CD25 expressing cells in host mice was shown to result in autoimmune disease and to enhance anti-tumor immunity (1-4). Subsequently, the biology and function of Tregs has been the subject of enormous scientific and medical interest. Both thymus-derived Tregs [called natural-Tregs (nTregs)] and induced Tregs (iTregs), that arise in peripheral tissues in the setting of high concentrations of TGF-beta, contribute to the Treg-pool and appear to have overlapping functional profiles (5-7). Tregs suppress the proliferation of bystander T cells, natural killer (NK) cells and B cells in a process that is mediated by CTLA-4, IL-10 or secreted or membrane-bound forms of TGF-beta1. Contact-dependent, granzyme B-mediated suppressive

mechanisms have also been identified that result in selective killing of bystander effector T cells (8); and in the tumor microenvironment, granzyme B is important for Treg-mediated suppression of tumor clearance (9). Moreover, available evidence supports a role for a Treg-specific, granzyme B-mediated process for the initiation and maintenance of allograft tolerance (10). Tregs also modulate the activity of antigen presenting cells, thereby indirectly suppressing T cell activation (11, 12). Furthermore, Tregs appear to play an important role in the suppression of B cell activation and proliferation. Patients with congenital deficiency of Tregs, an X-linked disorder termed immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome (13) have numerous circulating autoantibodies against gut, thyroid and blood cell antigens, resulting in enteropathy and peripheral blood cytopenias (14). Tregs render autoreactive B cell clones anergic to T cell help in secondary lymphoid tissue in mice (15). Tregs localize to secondary lymphoid follicles in human tonsils and suppress immunoglobulin production by follicle center B cells through a partially characterized cell contact-dependent mechanism that may involve TGF- $\beta$ 1 (16). Further, Tregs were reported to suppress autoreactive B cells in systemic lupus erythematosus patients through a cell contact dependent mechanism (17), and Tregs can preferentially kill activated B cells, over non-activated bystander B cells, through a cell contact-dependent mechanism involving granzyme B and perforin (18). Thus, aberrant B cell and T cell function are both consequences of absent or inadequate Tregs.

The master controller of Treg activity is the transcription factor FOXP3 (19). The importance of FOXP3 is illustrated by the clinical features associated with the various *FOXP3* gene mutations that result in IPEX syndrome. In addition to the aforementioned problems due to autoantibody production, these patients suffer from other manifestations of systemic autoimmunity including T cell infiltration of the skin and organs resulting in enteropathies, eczema and endocrinopathies. For these patients, non-marrow ablative chemotherapy followed by allogeneic stem cell rescue is a viable treatment option (20). Mice deficient in *FOXP3* (called scurfy) have a spectrum of autoimmune pathologies, similar to that of patients with IPEX, including polyclonal hypergammaglobulinemia, Coombs-positive autoimmune hemolytic anemia and T cell hyperactivity (21). The human and murine pathologies observed in the setting of *FOXP3* gene abnormalities highlight its central importance for the maintenance and function of Tregs. *FOXP3* encodes a transcription factor in the *forkhead* family that, like other FOX transcription factors can have positive or negative effects on transcription of target sequences. Structurally, FOXP3 has the following three components: a forkhead (FKHR) domain for DNA binding and both zinc finger and leucine zipper motifs. IPEX patients may harbor mutations throughout the gene indicating that all regions are important for appropriate functional activity. FOXP3 appears to cooperate with the transcription factors NFAT and Runx1 to repress the *IL2* promoter and enhance expression of *Ctla4*, *Gitr* and *Cd25* (22). Forced overexpression of FOXP3 in conventional CD4 (+) T cells

induces the acquisition of a suppressive phenotype suggesting that it is both necessary and sufficient for Treg development (23). Thus, FOXP3 remains the most specific marker to identify Tregs among a heterogeneous T cell population.

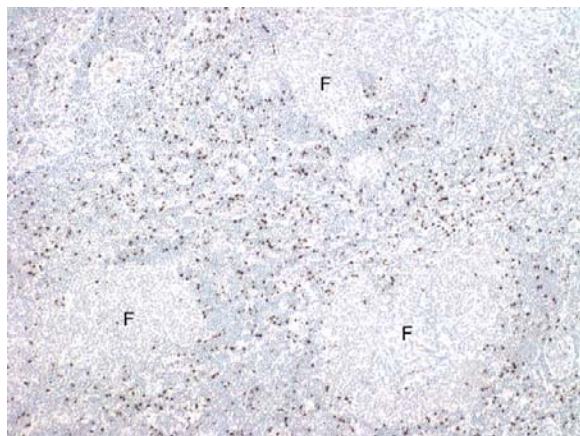
### 3. REGULATORY T CELLS IN NON-HEMATOPOIETIC TUMORS

Human malignancies of many types, including ovarian (24), prostate (25), colon (26) and breast cancer (27), have been shown to have a higher concentration of infiltrating Tregs compared to adjacent, morphologically benign tissue. Further, a substantial body of evidence suggests that the presence of high numbers of tumor infiltrating regulatory T cells in the inflammatory milieu of solid tumors is associated with an immunosuppressive microenvironment and poor outcome. Higher numbers of tumor infiltrating Tregs correlate with unfavorable clinical behavior in breast (27-30), ovarian (24), stomach (31) hepatocellular (32) and colon cancer (26). A potentially more informative evaluation of the tumor immune state may be performed on clinically resected specimens by determining the ratio of Tregs:cytotoxic T cells (CTCs). In this manner, tissues can be categorized as *suppressive* (high Treg:CTC state) or *cytotoxic* (high CTC:Treg state) using FOXP3 expression to quantify Tregs and CD8, granzyme B or T cell intracytoplasmic antigen-1 (TIA-1) to identify the CTC component. A higher Treg:CTC ratio (overall suppressive state) correlates with a less favorable clinical outcome in hepatocellular (33), ovarian (34) and colon cancer (35). These findings are consistent with the results of *in vitro* experiments cited above showing the suppressive effects of Tregs on bystander immune cells. According to the generally accepted model for Treg function in the tumor microenvironment, malignant cells may increase local Treg numbers through either recruitment or conversion of conventional T cells to regulatory T cells. Once present in the tumor milieu, Tregs aid in the maintenance of an immunosuppressive state and in turn inhibit the development of a significant host anti-tumor immune response allowing for tumor cell growth, proliferation and perhaps dissemination (36). This paradigm incorporates both *in vitro* and *in vivo* studies with clinical observations and has driven the development of new treatment strategies aimed at decreasing the intratumor Treg burden in an effort to increase the effectiveness and sustainability of host anti-tumor immunity. Some of these therapeutic approaches have shown efficacy in pre-clinical models (37).

### 4. REGULATORY T CELLS IN LYMPHOMA

#### 4.1. Follicular lymphoma

Patients with follicular lymphoma (FL) have a heterogeneous clinical outcome. Therefore, identification and validation of informative prognostic markers, especially those derived from the immune infiltrate, has attracted considerable research interest with the goal of more accurately stratifying patients into prognostic groups at the time of diagnosis. An association between the composition of the immune microenvironment and clinical outcome in FL was suggested by a frequently cited 2004



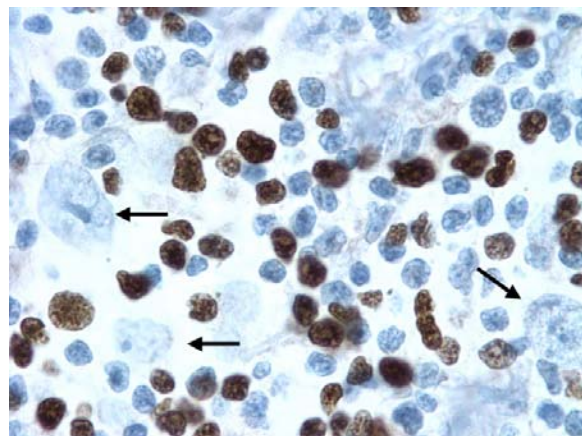
**Figure 1.** An interfollicular pattern of FOXP3 (+) infiltrating regulatory T cells in a case of follicular lymphoma (100x magnification). A formalin fixed paraffin embedded tissue section from a case of follicular lymphoma was subjected to immunohistochemical staining with antibodies to FOXP3. Positive cells have a nuclear pattern of staining and are present in a predominantly interfollicular localization (follicles marked with “F”). This pattern has been associated with a more favorable clinical outcome.

study (38) in which survival of patients was correlated with gene expression signatures derived from the tumor immune infiltrate. A favorable signature was characterized by high expression of T cell-associated genes, while the unfavorable signature was characterized by high expression of macrophage and dendritic cell-associated genes (38). More recent studies, involving a spectrum of lymphomas, suggest that quantification of specific immune cell subsets including T cells (cytotoxic T cells, follicular helper T cells and Tregs), mast cells, dendritic cells and macrophages may reveal additional associations between immune effectors and clinical outcome, thereby providing an informative set of biomarker that can be assessed at diagnosis using immunohistochemistry, gene expression analysis and flow cytometry. In FL, the identification of greater numbers of tumor-associated macrophages (TAMs) by histochemical staining is one immune characteristic that correlates consistently and reproducibly with an unfavorable outcome (39-41) with the association being further validated by gene expression studies (38, 42). Conversely, high numbers of infiltrating Tregs in the FL microenvironment have been correlated with a favorable prognosis (43-46). When evaluating clinical parameters, FL patients who present with low numbers of tumor infiltrating Tregs are more likely to have refractory disease (44), and patients who present without B symptoms and with a low follicular lymphoma international prognostic index (FLIPI) score are characterized by increased intratumoral Tregs (47). In addition, specific, recurrent, non-random architectural patterns of both Treg and TAM infiltration have been noted in FL and may relate closely with prognosis, especially in the context of particular treatment protocols (39, 44, 47, 48). For Tregs, these patterns are generally summarized as diffuse, follicular or

interfollicular. In the diffuse pattern, no discernible architectural distribution is noted. A follicular pattern denotes predominant localization of FOXP3 (+) cells to the malignant follicular structures while the interfollicular pattern, as the name suggests, is associated with predominant localization between malignant follicles (Figure 1). These characteristic patterns may be associated with other, as yet unidentified, immune parameters or perhaps they signify the overall immunosuppressive state of the lymphoma microenvironment. Functional studies are needed to illuminate these relationships. In one clinical study, FL patients with long survival were more likely to have an interfollicular distribution of FOXP3 (+) cells (46). Another study showed that an interfollicular distribution of Tregs was associated with more favorable outcome regardless of treatment (49). A third study showed that a predominantly follicular distribution of Tregs was associated with shorter overall survival in a group of high risk FL patients who required treatment at the time of presentation (39). In support of this last observation, another series of 102 uniformly treated FL patients demonstrated that a follicular pattern of Treg infiltration was associated with shorter overall survival (OS) and higher risk of transformation (50). Cases with follicular FOXP3 positivity were also more likely to have higher numbers of TAMs (50). Together, available evidence in FL patients suggests an association between favorable outcome and higher overall numbers of intratumoral Tregs or an interfollicular pattern while a predominantly follicular Treg distribution is associated with unfavorable outcome. These findings are descriptive, however, as mechanistic data is lacking. For example, the functional relationship, if any, between Tregs and TAMs remains largely unknown in spite of their prognostic associations.

#### 4.2. Hodgkin lymphoma

Classical Hodgkin lymphoma (cHL) is a tumor derived from germinal center B cells that have essentially extinguished the B cell transcription program and, therefore, do not express lineage specific markers such as CD20 (51). The bulk of the tumor consists of a benign inflammatory infiltrate consisting of lymphocytes (predominantly T cells), neutrophils, eosinophils, plasma cells and histiocytes with variable amounts of sclerosis (Figure 2). The tumor cells are interspersed and typically represent only 1-5% of the total tumor cellularity. With modern treatment strategies, most patients are cured. However, as cHL tends to affect young patients, late effects of treatment such as secondary malignancies and pulmonary and cardiac pathology that are a consequence of involved field radiation, arising years to decades treatment is complete, remain important clinical problems. Therefore, an opportunity to decrease treatment intensity without compromising efficacy in patients with a good prognosis is desirable. As with follicular lymphoma, characterization of the immune infiltrate in cHL is one strategy for predicting outcome. Multiple research groups have found that a greater number of intratumoral Tregs or a comparatively high Treg:CTC ratio correlates with a more favorable progression free survival, OS or both (45, 52, 53). Alvaro *et al.* studied 257 newly diagnosed cases of cHL and found that comparatively low numbers of FOXP3



**Figure 2.** Numerous FOXP3 (+) infiltrating regulatory T cells in the inflammatory background of classical Hodgkin lymphoma (1000x magnification). A formalin fixed paraffin embedded tissue section from a case of classical Hodgkin lymphoma was subjected to immunohistochemical staining with antibodies to FOXP3. Positive cells have a nuclear pattern of staining. Hodgkin/Reed-Sternberg cells are shown at the arrows. Increased FOXP3 (+) T cells in the tumor microenvironment have been associated with better outcome in classical Hodgkin lymphoma.

(+) cells in combination with high numbers of TIA-1 (+) CTCs was an independent negative prognostic factor (53). We found, in a series of 98 newly diagnosed cHL patients, a marginal association between low numbers of intratumor FOXP3+ cells and poorer failure free survival (FFS) (52). However, when the ratio of FOXP3 (+) cells to granzyme B (+) CTCs was assessed, a much stronger association was found. A low FOXP3:granzyme B ratio in the tumor microenvironment (cytotoxic tumor state) was independently associated with poor FFS and OS. In an assessment of intratumoral CTCs alone, increased CD8 (+) CTCs co-expressing granzyme B was by itself associated with unfavorable outcome in a series of cHL patients (54). A study by Tzankov *et al.* of 280 cHL patients found that higher numbers of FOXP3 (+) cells in the tumor was independently associated with better FFS and marginally associated with better OS (45). Muenst and colleagues found that higher numbers of granzyme B (+) infiltrating cells and lower numbers of FOXP3 (+) cells (cytotoxic tumor state) were associated with worse survival (55), however, a study by Schreck and colleagues of 87 cases of cHL found no association between FOXP3 (+) cells and outcome (56). Interestingly, they did find that higher numbers of TH2 cells, identified by expression of the transcription factor c-maf, were associated with better survival (56). Therefore, the majority of the studies cited have produced similar findings and suggest that overall higher numbers of intratumoral Tregs are a favorable prognostic factor while identification of intratumoral CTCs is prognostically unfavorable. Furthermore, the ratio of Tregs:CTCs (where higher ratios predict better survival) may be a superior biomarker relative to the assessment of either T cell subtype alone, a conclusion that is

counterintuitive and begs for explanation. The tumor cells of cHL (Hodgkin or Reed-Sternberg cells) may express programmed death-1-ligand (PD-L1), a negative regulator of T cell receptor signaling. This observation suggests that the functional state of infiltrating T cells may be compromised based on expression of the ligand's cognate receptor, PD-1 (57). In fact, PD-1 (+) T cells are found in the tumors and are associated with reduced overall survival (55). Interestingly, PD-1 positivity correlated with higher numbers of granzyme B (+) cells and lower numbers of FOXP3 (+) cells suggesting that PD-1 expression was on the CTCs and not on the Tregs and that the Tregs were likely functional.

#### 4.3. Diffuse Large B cell lymphoma

Diffuse large B cell lymphoma (DLBCL) is an aggressive lymphoma of larger-sized B cells that can be subtyped into GC-type and non GC-type based on gene expression profiling (58) or by an algorithm based on immunohistochemical staining patterns (59). Prior to the availability of immunomodulatory treatments with rituximab, subtyping in this way demonstrated a more favorable outcome in GC-type DLBCL. However, now that rituximab has become standard therapy for DLBCL patients, the prognostic significance of this subtyping scheme has become controversial (60). New markers based on the immune microenvironment, however, hold promise for predicting outcome in rituximab-treated patients although only a relatively small number of studies have addressed the significance of intratumoral Tregs in DLBCL patients. A study of 195 patient tumor samples, in which Tregs were quantified by FOXP3 immunohistochemistry and CTCs were quantified by TIA-1 immunostaining, demonstrated that patients with fewer infiltrating CTCs had a better outcome while the number of Tregs was not associated with survival (61). Another study showed a significantly better survival in cases with higher numbers of tumor Tregs at diagnosis (62) while a third showed a positive correlation between intratumoral FOXP3 (+) cells and disease specific survival in the GC subtype of DLBCL but a negative prognostic correlation in non-GC DLBCL (45). Still another study showed that increased tumor Tregs at diagnosis was independently associated with adverse outcome in 50 "high clinical risk" DLBCL patients (63). Together, these studies indicate that a comparatively higher number of tumor Tregs may be associated with a more favorable outcome in certain subsets of DLBCL, perhaps related to GC or non-GC differentiation. The finding that increased CTCs are associated with adverse outcome is, again, counterintuitive from an immunologic standpoint and is similar to observations in cHL cited above.

#### 4.4. Cutaneous T cell lymphoma

Studies performed in cutaneous T cell lymphoma (mycosis fungoides) (64) and in extranodal NK/T cell lymphoma (65) also demonstrated a positive correlation between the number of tumor-associated Tregs and a more favorable clinical outcome. An additional study in cutaneous T cell lymphoma found a functional defect in circulating Tregs from patients who had progressed to advanced stages of cutaneous T cell lymphoma, and this finding correlated with tumor burden (66), suggesting that

disease progression occurs in the setting of loss of suppressive function in circulating Tregs.

### 4.5 Summary of findings in lymphoma

Although there are minor disparities, the preponderance of evidence in lymphoma, whether cHL, FL, GC-type DLBCL, T-cell lymphoma or NK-cell lymphoma, sharply contrast with those in solid tumors and show that Tregs in the lymphoma microenvironment are, generally, a favorable prognostic marker. In further contrast to observations in solid tumors, intratumoral CTCs appear to be an unfavorable marker in cHL and DLBCL. The most strikingly positive correlation between Tregs and favorable outcome in lymphoma patients is in those lymphomas with a GC B-cell derivation. This subset includes cHL, FL and GC-type DLBCL. Given these findings, caution is warranted in extrapolating treatment strategies that may well prove effective in solid tumors, such as Treg depletion, to the setting of lymphomas. The question remains, how might Treg infiltration lead to a more favorable outcome in lymphoma? Tregs could exert a directly suppressive affect on lymphoma cells in much the same way as they do in the regulation of normal B and T cell activation. Tregs could also act indirectly and suppress another immune cell subset, perhaps dendritic cells or tumor infiltrating macrophages, that themselves facilitate lymphoma growth and survival. Alternatively, Tregs could simply be a surrogate marker for some unidentified characteristic of the malignant cells or the tumor microenvironment that leads to better outcome. Functional and mechanistic studies are needed to assess further the role of Tregs in the lymphoma microenvironment.

## 5. REGULATORY T CELLS IN LEUKEMIA

The functional impact of Tregs in the setting of tissue based malignancies such as solid tumors and lymphoma and those based primarily in the blood and bone marrow such as acute leukemia and plasma cell myeloma (PCM) may be quite different. However, relatively few studies have addressed either the role of Tregs in primarily leukemic diseases or the utility of Treg quantification in predicting clinical outcome. The majority of studies available for review focus on two relatively common forms of leukemia, acute myelogenous leukemia (AML) and chronic lymphocytic leukemia (CLL).

### 5.1. Acute leukemia

Acute leukemias are bone marrow-based processes in which immature hematopoietic precursor cells, either myeloblasts, in AML or lymphoblasts, in acute lymphoblastic leukemia (ALL) accumulate and constitute 20% or more of the total bone marrow cellularity. The process is usually, but not always, accompanied by leukemia cells in the peripheral blood. Current prognostic markers in AML and ALL are derived predominantly from genetic characteristics of the malignant cells including non-random translocations, recurrent somatic mutations or both. The relationships between these genetic abnormalities and clinical outcome have been extensively characterized with findings implemented into established treatment protocols. Nonetheless, studying biomarkers derived from non-

malignant cells that are part of the bone marrow microenvironment may provide additional insights into the pathobiology of acute leukemia.

In some cases, AML blasts have been reported to express indoleamine 2,3-dioxygenase (IDO) (67), an enzyme involved in tryptophan degradation, and IDO expression has been implicated in localized immunosuppression due to tryptophan depletion. Derivatives of tryptophan degradation, including kynurenine, that accumulate as a consequence IDO activity also have immunosuppressive effects. *In vivo*, IDO expression in AML correlates with increased circulating Tregs and, in an *in vitro* system, co-culture of conventional T cells with IDO-expressing AML cells results in expression of FOXP3 and acquisition of suppressive activity by the FOXP3 (+) T cells (68). IDO activity in the sera of AML patients is abnormally high and can be evaluated by assessing the kynurenine:tryptophan ratio (69). Furthermore, survival is decreased in the setting of a high ratio, suggesting that supranormal IDO expression is a marker of poor prognosis (69). In support of this interpretation, high IDO mRNA levels correlate with poor outcome in AML (70). Overall, the role of IDO in myeloid blasts of AML may be similar to that of normal monocyte-derived dendritic cells that also express high levels of IDO. In this context, the enzymatic activity of IDO drives generation of Tregs (71) that are capable of suppressing tumor antigen specific CTCs through contact-dependent and non-contact dependent mechanisms (71). In other malignant processes, such as colon and ovarian cancer, IDO overexpression also correlates with an unfavorable clinical outcome (72, 73). These data suggest that IDO-induced Tregs have an overall negative effect on immune regulation in AML and that their presence predicts an inferior outcome. Additional studies focusing directly on Tregs are needed to define more definitively their role in AML.

### 5.2. Chronic lymphocytic leukemia

CLL is a relatively common neoplasm that arises as a consequence of clonal expansion of mature B-lymphocytes. In addition to the leukemic component of the disease, many patients with CLL have lymphatic or tissue involvement of varying degrees. The proliferative/survival advantage of CLL cells appears to be dependent on interactions with stromal cells in the bone marrow and lymph nodes and on other microenvironmental cues (74). Deficient T cell function is also a hallmark of the disease (75, 76) but the mechanisms that underlie this process have not been identified. The frequency of CD4 (+)CD25 (high) T cells, a phenotype consistent with but not entirely specific for Tregs, is increased in CLL patients. This subset of T cells has immunosuppressive activity that appears to be abrogated by fludarabine, a therapeutic agent commonly used to treat patients with CLL (77). A recent study also demonstrated increased Tregs in CLL patients using a more specific immunophenotypic definition [i. e., CD4 (+)CD25 (high)FOXP3 (+)] (78). This study reported a positive correlation between a higher number of circulating Tregs and disease progression but no association between Treg frequency and CLL cell



expression of the established adverse prognostic markers CD38 and ZAP-70 (78). CLL cells can directly induce Treg expansion via a CD70-dependent process (79). Moreover, interaction of CLL cells with stromal cells results in upregulation of CD70 expression on the leukemic cells (80), suggesting that the same cues that result in CLL cell survival lead to an increase in the number of Tregs. The direct effect, if any, of Tregs on CLL cells is unknown. Indirectly, Tregs may suppress the anti-leukemia immune response. In support of this hypothesis, early stage clinical studies using allogeneic dendritic cells pulsed with CLL cell lysates stimulated a tumor specific cytotoxic T cell response in a subset of patients that correlated with decreased CD4 (+)CD25 (+) T cells (81). This study implies an inverse correlation between Tregs and anti-leukemia immunity. Further, it suggests that immunomodulatory agents such as thalidomide (82), that decrease Treg frequencies, may have a beneficial effect in patients with CLL.

### 6. REGULATORY T CELLS IN MYELOMA

Like CLL, plasma cell myeloma (PCM) is a process that is dependent on stromal cell signals for tumor cell survival and proliferation. Manifestations of PCM include an increase in clonal bone marrow plasma cells typically accompanied by an IgG or IgA paraprotein. When the tumor burden is low, such that clinical and pathologic criteria for a diagnosis of PCM are unmet, the process is classified as monoclonal gammopathy of undetermined significance (MGUS). For patients with MGUS, the risk of developing PCM is approximately 1% per year (83). As with the other hematopoietic processes discussed, the role of Tregs in the pathophysiology of the disease is largely speculative, and conflicting data exists. Dysfunctional T cell-mediated immunity is a hallmark of advanced stages of PCM and significant anti-tumor cytotoxicity appears to be lacking *in vivo* in the tumor microenvironment (84). However, myeloma antigen reactive T cells may be generated *in vitro* by exposure to tumor-loaded dendritic cells (DCs) (84) and, in a murine model, injection of tumor loaded DCs along with chemotherapy leads to regression of myeloma tumors (85). These findings suggest that there is no intrinsic defect in T cells in PCM but that a microenvironmental factor, or perhaps a defect in DCs themselves, prevents development of immunity. Treg-mediated inhibition has been postulated as one potential mediator of this effect. One study reported that the frequency of CD4 (+)FOXP3 (+) Tregs in the peripheral blood of MGUS and PCM patients is decreased as compared to normal controls and that the Tregs fail to suppress T-cell proliferation (86). Another group found essentially the opposite result in that CD4 (+)CD25 (high)FOXP3 (+) T cells with intact inhibitory activity were *increased* in the peripheral blood of patients with MGUS and PCM as compared to healthy controls (87). The latter findings were corroborated by a study that that found increased Tregs in the peripheral blood but not bone marrow in a cohort of PCM patients along with a positive relationship between Treg frequency and paraprotein level but not disease stage (88). Tregs also quickly reconstitute in the bone marrow of PCM patients following allogeneic

stem cell transplantation and may do so by preferential homing, but their presence does not correlate with disease recurrence (89). Additional studies are required to illuminate more fully the impact of Tregs on the microenvironment in PCM.

## 7. REGULATORY T CELLS IN MYELOPROLIFERATIVE NEOPLASMS, MYELOYDYSPLASTIC DISORDERS AND APLASTIC ANEMIA

### 7.1. Myeloproliferative neoplasms

The immune system plays an incompletely defined role in the pathobiology of both myeloproliferative neoplasms (MPNs) and myelodysplastic syndromes (MDS). In the case of MPNs [a grouping that includes chronic myelogenous leukemia (CML), polycythemia vera, essential thrombocytosis and primary myelofibrosis] a role for immune regulation of the malignant clone in CML is suggested by both *in vitro* and *in vivo* studies. CML is a consequence of somatic mutation t (9;22) (q34;q11) (the Philadelphia chromosome) arising in a hematopoietic stem cell or primitive progenitor. The translocation generates the fusion gene *BCR-ABL* whose product is a chimeric protein with constitutive tyrosine kinase activity. Expression of BCR-ABL in the mutant stem cell results in clonal expansion with a selective proliferative advantage in late myeloid differentiation. CML is characterized clinically by granulocytosis and splenomegaly. The disease can be cured by myeloablative therapy in combination with allogeneic stem cell transplant with graft vs. leukemia effect being essential for eradication of the malignant clone. Moreover, the observation, that infusion of donor lymphocytes can induce durable remissions in patients who fail initial allotransplant, highlights the importance of cellular immunity in eliminating the malignant clone. Further support for a role for immune regulation in CML is based on identification of expanded cytotoxic T cell clones that are specific for leukemia antigens including BCR-ABL and proteins that are over expressed by CML cells including proteinase-3 and Wilms tumor 1 protein (90). Prior to development of molecularly targeted therapy (imatinib mesylate, Gleevec) for CML, interferon based therapy was the standard of care, with 10-25% of patients treated with interferon-based therapy achieving a durable complete cytogenetic remission (91). In the case of CML patients treated with interferon, there is evidence that immune mechanisms contribute to destruction of the mutant clone. For example, interferon has been shown to induce upregulation of expression of major histocompatibility antigens in antigen presenting cells and to augment the cytotoxic activity of lymphocytes against tumor cells (92). More compelling evidence for an immunomodulatory effect induced by interferon against the CML clone was reported by Fujii (92) who showed expansion of T cell clones expressing V-beta9 and V-beta20 in complementarity-determining region 3 (CDR3) of the beta variable (V-beta) chain of the T-cell receptor in patients responsive to interferon but not in patients unresponsive to interferon. These findings suggest that patients responsive to interferon have a specific immune signature that is a consequence of clonal expansion of T

cells reactive to a discrete set of antigens expressed by the mutant clone. Moreover, by using serological analysis of tumor antigens through screening an expression cDNA library (SEREX), Yang and colleagues (93, 94) identified two tumor antigens (CML66L and CML28) that elicit immune responses in CML patients who achieved remission after treatment with interferon.

Recent experiments in a murine model of CML suggest that the PD-1/PD-1 ligand system may also contribute to immune surveillance of the leukemic cells. In those studies, leukemia-specific CTL cells became exhausted, maintaining only limited cytotoxic activity and failing to produce interferon-gamma or tumor necrosis factor-alpha (95). The CTLs were found to express PD-1 at high density while CML cells expressed PD-1 ligand, thereby providing a plausible explanation for the suppression of T cell proliferation. Additional support for this hypothesis was provided by experiments that showed that the effects of PD-1 over expression were abrogated in PD-1 deficient mice or in mice treated with anti-PD-1 ligand antibody, and PD-1 was found to be upregulated on CD8 (+) T cells in patients with CML (95).

Currently, imatinib mesylate (Gleevec) is the standard of care for CML. This drug targets the ATP-binding and solvent pocket of the Abelson tyrosine kinase (ABL) of the BCR-ABL fusion protein. However, imatinib is not entirely specific for the Abelson kinase, having activity against two other tyrosine kinases, platelet derived growth factor receptor and c-kit receptor. Further, Larmonier and colleagues (96) recently presented evidence that imatinib impairs the immunosuppressive function of Tregs. *In vitro* experiments showed that the drug inhibited FOXP3 expression and STAT3 and STAT5 phosphorylation, and *in vivo* studies indicated that imatinib enhanced antitumor immune responses to dendritic cell-based immunization against an imatinib-resistant BCR-ABL negative lymphoma (96). These experiments support the concept that some of the anti-leukemia activity of imatinib may be a consequence of immune modulation and suggest that the drug may have activity in cancer immunotherapy where Treg expansion contributes to impaired tumor immunity.

Somatic mutation of *Janus Kinase 2* (*JAK2G1849T* resulting in *JAK2V617F*) in a hematopoietic stem cell or primitive myeloid progenitor is observed in approximately 90% of patients with polycythemia vera and in ~50% of patients with essential thrombocytosis and primary myelofibrosis. In the case of these Philadelphia chromosome negative MPNs, a role for immunomodulation in general and Tregs in particular in disease pathobiology is less completely developed than in the case of CML, although, like CML, both polycythemia vera and essential thrombocytosis are responsive to treatment with interferon (97, 98). As with CML, treatment of polycythemia vera with interferon is associated with development of tumor specific antigen in a subset of patients, but whether Treg-mediated immune tolerance contributes to the pathobiology of polycythemia vera or other Philadelphia chromosome negative MPNs and whether treatment with interferons

enhances immune recognition of neoplastic cells by suppressing Treg function in MPN patients remains to be elucidated.

### 7.2. Myelodysplastic disorders

MDS is a heterogeneous group of diseases characterized clinically by varying degrees of anemia, leukopenia and thrombocytopenia. The bone marrow of patients with MDS is typically hypercellular with the peripheral blood cytopenia being a consequence of intramedullary apoptosis (also termed ineffective erythropoiesis). Approximately 50% of patients have non-random karyotypic abnormalities. An International Prognostic Scoring System (IPSS) stratifies patients with MDS into low-, intermediate- and high-risk groups based on the number of peripheral blood cytopenias, percentage of myeloblasts in the bone marrow and karyotypic features. Compelling evidence suggests that immune mechanisms play a role in disease pathogenesis. Support for this hypothesis was provided by studies of Kordasti and colleagues (99) who reported a higher number of Tregs in MDS patients with 5% or more blast, a high IPSS score and disease progression. The results of these studies implicate Treg expansion in suppression of host anti-tumor response.

### 7.3. Aplastic anemia

Tregs have also been implicated in the pathophysiology of aplastic anemia, a non-malignant, polyclonal immune-mediated bone marrow failure syndrome. Unlike MDS, aplastic anemia is characterized by a hypocellular marrow, and non-random karyotypic abnormalities are uncommon. Solomou and colleagues (100) reported that essentially all patients with aplastic anemia have decreased Tregs at presentation, a finding that supports the hypothesis that the marrow failure of aplastic anemia is a consequence of autoimmune destruction of hematopoietic stem cells.

## 8. SUMMARY AND PERSPECTIVE

Given the diversity of findings, the difficulty in extrapolating the results of relatively simplistic *in vitro* experiments to the complexity of the *in vivo* tumor microenvironment and the limitations of murine models of lymphoma and leukemia, it is difficult to draw definitive conclusions about the role of Tregs in malignancies, especially those of hematopoietic origin. However, an apparent theme is the contrast between solid tumors, where tumor-infiltrating Tregs are correlated with a more aggressive clinical outcome, and lymphomas and leukemias where both positive and negative associations have been reported. Thus, the role of Tregs in tumors appears to be context dependent. These observations suggest that the therapeutic strategy of Treg-depletion may be effective in treating solid tumors but could be detrimental for certain hematopoietic malignancies, especially GC-derived B cell lymphomas. In these cases, increased tumor infiltrating Tregs appear to be associated with a better clinical outcome. Little experimental data exists to explain why Tregs may be negatively associated with survival in one circumstance and positively correlated in another. Because T and B cells represent physiologic targets for Treg-

mediated suppression in normal immune regulation, it is conceivable that certain T and B cell tumors could also be targeted by Tregs. Another important point to consider is the functional status of immune subsets within the tumor microenvironment. Simply quantifying markers associated with Tregs or CTCs may lead to erroneous assumptions as the expression of such markers does not define function. One conclusion that may be drawn from these studies is that there is a complex interplay between the competing interests of the host immune system and the immune evasion mechanisms of a tumor. Clearly, much additional work, both clinical/translational and mechanistic, is needed to unravel the role that Tregs play in tumor biology.

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**Abbreviations:** Treg: regulatory T cell, NK: natural killer, TIA: T cell intracytoplasmic antigen, TGF: transforming growth factor, CTC: cytotoxic T cell, DC: dendritic cell, IPEX: immune dysregulation, polyendocrinopathy,

enteropathy, X-linked syndrome, FL: follicular lymphoma, FLIPI: follicular lymphoma international prognostic index, cHL: classical Hodgkin lymphoma, DLBCL: diffuse large B cell lymphoma, GC: germinal center, CLL: chronic lymphocytic leukemia, AML: acute myeloid leukemia, ALL: acute lymphoblastic leukemia, IDO: indoleamine 2,3-dioxygenase, PCM: plasma cell myeloma, MGUS: monoclonal gammopathy of undetermined significance, PD: programmed death, OS: overall survival, FFS: failure free survival, CML: chronic myelogenous leukemia, JAK: Janus kinase, MPN: myeloproliferative neoplasm, MDS: myelodysplastic syndrome, IPSS: international prognostic scoring system, PV: polycythemia vera.

**Key Words:** Tregs, FOXP3, Lymphoma, Leukemia, Myeloproliferative Neoplasm, Myelodysplastic Syndrome, Biomarkers, Review

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