Regulatory T Cells and Their Prognostic Relevance in Hematologic Malignancies

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Regulatory T cells (Tregs) have a fundamental function in monitoring the immune homeostasis in healthy individuals. In cancer and, in particular, in hematological malignancies, Tregs exert a major immunosuppressive activity, thus playing a critical role in tumor cell growth, proliferation, and survival. Here, we summarize published data on the prognostic significance of Tregs in hematological malignancies and show that they are highly conflicting. The heterogeneity of the experimental approaches that were used explains—at least in part—the discordant results reported by different groups that have investigated the role of Tregs in cancer. In fact, different tissues have been studied (i.e., peripheral blood, bone marrow, and lymph node), applying different methods (i.e., flow cytometry versus immunohistochemistry, whole blood versus isolated peripheral blood mononuclear cells versus depletion of CD25+ cells, various panels of monoclonal antibodies, techniques of fixation and permeabilization, and gating strategies). This is of relevance in order to stress the need to apply standardized approaches in the study of Tregs in hematological malignancies and in cancer in general.

1. Introduction

Regulatory T cells (Tregs) constitute a small-size subpopulation of CD4+ T cells, accounting for 1–4% of circulating CD4+ lymphocyte in humans, specialized in suppressive functions that control unwanted immune responses not only toward self-antigens but also toward foreign antigens in the context of the immune tolerance [1]. Gershon and Kondo from Yale University first proposed the existence of CD8+ T cells with suppressive activity more than 40 years ago [2]. However, after the initial great interest following this first report, due to the fact that a precise
definition of Tregs lacked for several years, no further advances in the study of this cell population were made for decades. In 1995, Sakaguchi and coworkers identified Tregs in mouse as CD4+ T cells expressing surface interleukin-2 (IL-2) receptor α-chain (CD25) [3]. Baecher-Allan and coworkers, using flow cytometry and analyzing sorted cells in vitro, identified a very small subset of T cells with high expression of CD25 and regulatory function in humans [4]. However, CD25 is not exclusively restricted to Tregs, and its surface expression is also seen on effector T lymphocytes after activation [5]. The intracytoplasmic Forkhead box P3 (FoxP3), a transcription factor required for the development, maintenance, and function of Tregs was subsequently identified [6, 7]. The central role of this transcription factor is confirmed by the fact that a FoxP3 single gene mutation on the X chromosome induces in Scurfy mice a severe autoimmune/inflammatory disease. In humans, the same mutation causes a disease called IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome), characterized by autoimmune manifestations in multiple endocrine organs, such as diabetes and thyroiditis, inflammatory bowel disease, and severe allergies [8]. Finally, the absence of the heterodimeric IL-7 receptor (CD127) combined with CD4, CD25, and FoxP3, has been shown to better identify Tregs, avoiding the contamination from other cell populations such as activated effector T cells [9, 10].

2. Regulatory T Cells and Prognostic Significance in Cancer

The role of Tregs in cancer appears to be relevant by promoting tumor progression and suppressing effective antitumor activity [11–13]. Overall, the large majority of studies report that the frequency and the suppressive function of Tregs are increased in cancer patients as compared to healthy subjects. However, some issues are still a matter of debate, in particular the prognostic significance of this cell subpopulation. In general, Tregs predict poor outcome in cancer patients [12], but some reports have shown that higher Treg numbers and preserved activity are associated with a better prognosis [14–16].

This review stems from the need to reassess the topic of prognostic relevance of Tregs in cancer, focusing on patients with hematologic malignancies. For this purpose, we reviewed a large body of published papers conducting a PubMed literature search (keywords: Regulatory T cells, Hodgkin lymphoma, non-Hodgkin lymphoma, chronic lymphocytic leukemia, chronic myeloid leukemia, acute lymphoblastic leukemia, acute myeloid leukemia, multiple myeloma, monoclonal gammapathies, myelofibrosis, essential thrombocytopenia, polycythemia vera, and Ph1-negative chronic myeloproliferative neoplasms).

3. Regulatory T Cells in Chronic Lymphocytic Leukemia

The accumulation of monoclonal B lymphocytes in the bone marrow, lymphoid organs, and peripheral blood is the hallmark of chronic lymphocytic leukemia (CLL), the most common form of leukemia in Western countries [17]. The importance of T cell dysregulation in the pathogenesis and development of CLL is now well established [18, 19], and in this setting, the role of Tregs has also been investigated [20, 21]. As shown in Table 1, several authors reported data on Tregs in CLL showing in the majority of cases an expansion of this population [22–31]. In addition, a correlation between higher Treg numbers and more aggressive clinical-biological features and adverse prognosis of CLL has been described.

As previously discussed [20], the reported percentage of Tregs in CLL is highly variable. According to the majority of reports, the percentage of Tregs is higher in CLL patients than in normal controls, and when the absolute number is considered, Tregs are always found to be significantly greater in CLL as compared to healthy donors.

Interestingly, based on their experimental work, Jak et al. speculated that the accumulation of Tregs in CLL is due to an increased proliferation induced by CD27/CD70 interaction in the lymph node proliferation centers and to a decreased sensitivity to apoptosis [22].

Dasgupta et al. tried to establish an optimal threshold level for prognostic purpose [28]. The cut-off was assessed by receiver operating characteristic (ROC) analysis. A cut-off of 5.7% and 35 cells/μL for percentage and absolute Treg count, respectively, were determined as optimal in patients with CLL, along with a median Tregs percentage of 15.5% used to separate low- and high-risk patients. Using the same approach in the setting of Rai stage 0 CLL patients, our group found that the absolute number of Tregs was an independent predictor of time to the first treatment, with the best predictive cut-off being 41 cells/μL [24]. Overall, these data show that the absolute Treg number is able to identify Rai stage 0 CLL patients at higher risk of requiring therapy.

Rissiek et al., applying a multidimensional scaling analysis to assess the composition of the circulating T cell populations, generated T cell scores showing that suppressive T cell profiles emerge early during monoclonal B cell lymphocytosis (MBL), the well-recognized pre-CLL stage [31–33]. As the disease evolves from MBL to overt and advanced CLL, specific sequential changes in T cells appear, progressively compromising the effector T cells function and contributing to disease progression [30].

In our hands too, the absolute number of Tregs in MBL patients was lower compared to CLL patients, but slightly higher than healthy controls [30]. In addition, the absolute Treg cell number directly correlated with more advanced CLL clinical stages and higher circulating B cell numbers. Of note, the absolute number of Tregs was lower in MBL patients as compared to early-stage CLL patients (0/A according to Rai/Binet stage). In summary, Treg numbers increase gradually from normal subjects to “clinical” MBL patients and are significantly higher in CLL patients as compared to MBL patients.

Regarding the functional properties, some authors reported a reduced inhibitory function of Tregs in CLL [27, 34]. On the contrary, Piper et al. showed that in CLL patients Tregs retain their function and are not influenced by chemotherapy [35]. A correlation between a higher circulating Treg numbers and dysfunctional Vγ9Vδ2 T cells
| Reference          | Patients/controls evaluated | Samples tested | Marker panel used in Treg evaluation by flow cytometry | Treg frequency | Functional studies | Impact on prognosis |
|--------------------|----------------------------|----------------|--------------------------------------------------------|----------------|--------------------|---------------------|
| Beyer et al. [34]  | CLL/controls               | PB             | CD4/CD25                                               | Increased*    | Reduced inhibitory function | Extended disease (Binet stage) |
| Giannopoulos et al. [73] | CLL/controls | PB             | CD4/CD25/FoxP3                                         | Increased     | Not performed      | Binet stage         |
| Jak et al. [22]    | CLL/controls               | PB             | CD4/CD25/FoxP3                                         | Increased     | More resistant to drug-induced apoptosis than controls | Not evaluated        |
| D’Arena et al. [23, 24] | CLL/controls | PB             | CD4/CD25/CD127                                         | Increased with a gradual variation from normal subjects to clinical MBL to CLL | Not performed | Rai stage, lymphocytosis, LDH, first time to treatment |
| Weiss et al. [25]  | CLL/controls               | PB             | CD4/CD25/FoxP3                                         | Increased     | Not performed      | Unmutated IgVH, CD38, chromosomal aberrations Correlation with LDT (Tregs but not CD45RA+ Tregs and CD8+ Tregs were lower in CD38+ZAP70+ CLL group (with respect to CD38−ZAP70−)) |
| Lad et al. [26]    | CLL/controls               | PB and FNA     | CD4/CD25/CD127/IL-10                                   | Reduced both Treg and IL-10 expressing Treg; higher absolute number | Not performed | Not performed |
| Biancotto et al. [27] | CLL/controls | PB             | CD4/CD25/FoxP3                                         | Increased     | Slightly reduced suppressive activity | Correlation with ZAP-70 and CD38 expression |
| Dasgupta et al. [28] | CLL/controls | PB             | CD4/CD25/CD127/FOX3                                    | Increased     | Not performed      | Correlated with ZAP70 and CD38 expression |
| Mpakou et al. [29] | CLL/controls               | PB             | CD4/CD25/CD127                                         | Increased     | Suppression of T effector cells | Advanced stage     |
| D’Arena et al. [30] | Clinical MBL/CLL/controls | PB             | CD4/CD25/CD127                                         | Reduced as % but increased as absolute number with a gradual variation from normal subjects to clinical MBL to CLL | Not performed | Not performed |
| Rissiek et al. [31] | MBL/CLL/controls           | PB             | CD4/CD25/CD127/CD39                                    | Expansion, Highly suppressive CD39+ Treg subset increased in all disease stages | Increasingly suppressive regulatory function initiating at MBL stage effector function impairment after transition to CLL; partially recovered after chemo-immunotherapy | Shorter time to treatment |

*Increased at diagnosis; significantly reduced after fludarabine therapy. CLL: chronic lymphocytic leukemia; MBL: monoclonal B cell lymphocytosis; PB: peripheral blood; FNA: fine needle aspiration; LDT: lymphocyte doubling time.
in untreated CLL patients was also shown, thus corroborating the hypothesis that Tregs may not be only bystanders but have a functional role in this setting [36].

A normalization in Treg number was observed after fludarabine therapy [34], and also in CLL patients treated with lenalidomide, suggesting that such drugs are able to modulate cell-mediated immunity in CLL [37].

Finally, we also tested the ability of green tea, a popular beverage in China, Japan, and increasingly used in Western countries, to modulate Treg number in peripheral blood of CLL patients in the early phases of the disease, for which at the present time there is no effective intervention and a “wait and see” policy is generally adopted [38, 39]. We showed that the B cell lymphocyte count and the absolute circulating Treg number were reduced after 6-month consumption of oral green tea extract, suggesting that this compound can modulate circulating Tregs in CLL patients with early stage of disease and delay disease progression.

4. Regulatory T Cells in Lymphomas and Monoclonal Gammopathies

The neoplastic lymph nodes in Hodgkin lymphoma (HL) and non-Hodgkin lymphoma (NHL) contain not only neoplastic B cells but also nontumoral T cells, macrophages, and dendritic cells, constituting the so-called tumor microenvironment. The importance of the microenvironment in the pathogenesis and progression of lymphomas is still a matter of debate and many studies have focused on the role of its different components, including Tregs. Tregs are increased in lymphoma tissues and are able to inhibit cytotoxic CD8+ T cells exposed to lymphoma B cells [40].

Marshall et al. showed that HL-infiltrating lymphocytes are highly enriched in Tregs, which induce a profoundly immunosuppressive environment [41]. This was confirmed by Schreck et al. who demonstrated that in classical HL the microenvironment is dominated by Th2 cells and Tregs [42]. Moreover, a high ratio of Tregs over Th2 cells resulted in a significantly shortened disease-free survival.

However, conflicting results have been reported regarding the prognostic significance of Tregs infiltration in both HL and NHL. In fact, whereas in follicular lymphoma (FL), the most common form of low-grade NHL, germinal center (GC) diffuse large B cell lymphomas (DLBCL), and HL, an intrafollicular infiltration of Tregs, has a positive prognostic significance; this is not true in the case of non-GC-type DLBCL [43]. Moreover, as shown in Table 2, in some reports, a higher number of Tregs correlates with a good prognosis, while in other, it does not [43–49]. Of interest, Kim et al. evaluating Tregs on node biopsy of extranodal natural killer/T cell lymphomas showed that patients with poor performance status and with non-upper aerodigestive tract had a decreased number of Tregs (<50/0.40 mm<sup>3</sup>), while an increased number (>50/0.40 mm<sup>3</sup>) was associated with prolonged overall survival and progression-free survival [48]. Finally, Carreras et al. reported that the median Treg number in patients with FL at diagnosis had a median cell percentage of 10.5% [49]. Furthermore, patients were classified as having Tregs >10%, 5–10%, and <5% with a 5-year overall survival of 80%, 74%, and 50%, respectively. Patients with transformed DLBCL showed lower Treg number with respect to patients with grades 1–3 FL.

Regarding the frequency and prognostic significance of Tregs, conflicting results have also been obtained in the field of monoclonal gammopathies (Table 3). In some reports, Tregs were found to be increased in frequency, while in others they were reduced or comparable with respect to healthy subjects [50]. Again, some authors reported a correlation with tumour burden and with worse prognosis, but this was not consistent among different publications [50–57]. We recently published our data on the flow cytometric evaluation of Tregs in multiple myeloma (MM) and monoclonal gammopathies of undetermined significance (MGUS) [51]. We found no differences in Treg frequency in MM and MGUS with respect to normal controls, and no correlations with main clinical and laboratory features in this disease setting were observed.

5. Regulatory T Cells in Acute Leukemias, Chronic Myeloid Leukemia, and Ph1-Negative Chronic Myeloproliferative Neoplasms

Few studies have been published regarding the role of Tregs in acute myeloid and lymphoid leukemias (Table 4) [58–61]. In a study by Bhattacharya et al., an increased number of Tregs was found in patients with B cell acute lymphoblastic leukemia (B-ALL), and a correlation with disease progression was highlighted [58].

Regarding chronic myeloid leukemia (CML), an interesting paper has been published by Zahran and Badrawy, in which Tregs were found increased in the peripheral blood of affected individuals as compared to controls. Moreover, Tregs frequency correlated with the level of BCR/ABL, basophil number, blast cell count, and Sokal score, and Treg number was higher in accelerated and blastic phase with respect to chronic phase [62]. Of note, Treg frequency declined after therapy with imatinib. Rojas et al. found a lower Treg number in patients who achieved a complete cytogenetic response [63], while higher Treg frequencies were found after stem cell transplant compared to normal controls and newly diagnosed patients [64]. Finally, the correlations with Sokal score and basophil number were validated by other studies [65, 66], whereas the impact of treatment has not been confirmed, since no changes in Treg frequency were observed after 6 months of tyrosine kinase inhibitors therapy [65]. Table 5 summarizes the results of studies analyzing Tregs in CML.

Hassellbalch et al. studied patients with Ph1-negative chronic myeloproliferative neoplasms and found that circulating Tregs were significantly expanded in patients treated with IFN-α2 with respect to healthy donors and in patients treated with hydroxyurea [66]. Kovacsovics-Bankowski et al. analyzed patients with polycythemia vera (PV) and essential thrombocytemia (ET) and found increased numbers of circulating Tregs and an enrichment in highly suppressive subsets (defined as CD39<sup>+</sup>/HLA-DR<sup>+</sup>) in patients treated with PegIFN-α with respect to those treated with hydroxyurea [67].
Table 2: Most relevant published studies investigating the frequency and the prognostic significance of Tregs in lymphomas.

| Reference          | Patients/controls evaluated | Samples tested | Marker panel used in Treg evaluation by flow cytometry | Treg frequency | Functional studies | Impact on prognosis                                                                 |
|--------------------|------------------------------|----------------|--------------------------------------------------------|----------------|-------------------|-----------------------------------------------------------------------------------|
| Tzankov et al. [43]| Lymphomas                   | Node biopsy    | FoxP3 (IHC)                                           | Increased      | Not performed     | Correlation with disease-specific and failure-free survival in FL and disease-specific survival in germinal center-like DLBCL and OS and failure-free survival in classical HD, but negative prognostic effect in nongerminai center DLBCL. Independent prognostic significance for failure-free survival in classical HD and of borderline significance for OS in classical HD and disease-specific survival in germinal center-like and nongerminai center DLBCL. |
| Alvaro et al. [44] | Classical HL                | Node biopsy    | FoxP3 (IHC)                                           | Not reported   | Not performed     | Small number influenced negatively EFS and DFS                                     |
| Schreck et al. [42]| Classical HL/hyperplastic tonsils | Node biopsy    | FoxP3 (IHC)                                           | Increased      | Not performed     | Increased DFS and EFS; high Tregs/Th2 ratio correlated with shortened DFS         |
| Garcia et al. [45]| Gastric MALT lymphoma       | Gastric biopsy | FoxP3 (IHC)                                           | Increased with respect to DLBCL but similar to chronic gastritis | Not performed | Higher number correlated with response to antibacterial eradication therapy       |
| Koreishi et al. [47]| Relapsed/refractory HL     | Node biopsy    | FoxP3 (tissue microarray)                             | Not reported   | Not performed     | Lower Tregs correlated with poor OS                                               |
| Chang et al. [46]  | DLBCL/normal                | Node biopsy    | CD4+ CD25+                                            | Increased      | Not performed     | Higher with poor survival and IPI                                                   |
| Kim et al. [48]    | Extranodal natural killer/T cell lymphoma | Node biopsy    | FoxP3 (IHC)                                           | Heterogenous expression | Not performed | The decreased number of Tregs was more common in patients with poor performance status or in those presented in nonupper aerodigestive tract. Patients with increased numbers of Tregs showed prolonged OS and PFS. Inversely correlated with OS. Patients with very low numbers of Tregs (<5%) presented more frequently with refractory disease. No correlation with FLIP. Patients with transformed DLBCL had lower Treg percentages than FL grades 1, 2, or 3. |
| Carreras et al. [49]| FL at diagnosis and relapse | Node biopsy    | FoxP3 (IHC)                                           | Not reported   | Not performed     |                                                                                   |

HL: Hodgkin’s lymphoma; FL: follicular lymphoma; DLBCL: diffuse large B cell lymphoma; MALT: mucosa-associated lymphoid tissue; IHC: immunohistochemistry; OS: overall survival; DFS: disease-free survival, EFS: event-free survival.
Table 3: Most relevant published studies investigating the frequency and the prognostic significance of Tregs in monoclonal gammopathies.

| Reference                  | Patients/controls evaluated | Samples tested | Marker panel used in Treg evaluation by flow cytometry | Treg frequency | Functional studies                                                                 | Impact on prognosis                                                                 |
|----------------------------|-----------------------------|----------------|---------------------------------------------------------|----------------|-----------------------------------------------------------------------------------|-----------------------------------------------------------------------------------|
| Prabhala et al. [50]       | MGUS/MM/controls            | PBMC           | CD4/FoxP3                                               | Decreased      | Unable to suppress anti-CD3-mediated T cell proliferation                         | Not evaluated                                                                     |
| Beyer et al. [52]          | MGUS/MM/controls            | PBMC           | CD4/CD25/FoxP3 (% of CD4⁺ cells)                        | Increased in MM versus MGUS (trend without statistical significance) | Strong inhibitory function                                                      | Not evaluated                                                                     |
| Feyler et al. [53]         | MGUS/MM/controls            | PBMC and BM    | CD4/CD25/FoxP3                                         | Increased in PBMC but not in BM | Not evaluated                                                      | Correlation with disease burden (paraprotein)                                    |
| Gupta et al. [54]          | MM                          | PBMC           | CD4/CD25/CD127/FoxP3 (% of CD4⁺ cells)                  | Reduced in untreated which increased after treatment with lenalidomide | Able to inhibit proliferation of CD4⁺CD25⁻ T cells | Increase of Tregs in responding patients to therapy; decrease correlation with ISS I + II |
| Muthu Raja et al. [55]     | MGUS/SMM/MM                 | PB/BM whole    | CD4/CD25/CD127/CD45RA (% of CD4⁺ cells)                | Increased in MM but not in SMM and MGUS | Able to inhibit proliferation of CD4⁺ T cells and the secretion of IFN-γ   | Correlation with adverse clinical features (hypercalcemia, lower normal PC, and IgA subtype; no correlation with ISS; predict time to progression; MM patients with ≥5% of Tregs had inferior time to progression |
| Giannopulos et al. [56]   | MM/controls                 | PBMC           | CD4/CD25/FoxP3                                         | Increased      | Not evaluated                                                      | Correlation with shorter overall survival                                       |
| Foglietta et al. [57]      | MM/MGUS/controls            | Fresh PB and frozen BM | CD4/CD25/FoxP3                                         | Similar        | Effective suppressor function                                                     | No correlation with the pattern of BM infiltration                                 |
| D’Arena et al. [51]        | MM/MGUS/controls            | PB whole       | CD4/CD25/CD127 (% and absolute number)                 | Similar        | Effective suppressor function                                                     | No correlation with laboratory and clinical variables; no correlation with outcome |

MM: multiple myeloma; MGUS: monoclonal gammopathy of uncertain significance; SMM: smoldering multiple myeloma; ISS: international staging system; PB: peripheral blood; PBMC: peripheral blood mononuclear cells; BM: bone marrow.
Table 4: Most relevant published studies investigating the frequency and the prognostic significance of Tregs in acute leukemias.

| Reference                | Patients/controls evaluated | Samples tested | Marker panel used in Treg evaluation by flow cytometry | Treg frequency | Functional studies                                                                 | Impact on prognosis                  |
|--------------------------|-----------------------------|----------------|---------------------------------------------------------|----------------|------------------------------------------------------------------------------------|--------------------------------------|
| Bhattacharya et al. [58]  | B-ALL                       | PBMC /BM       | CD4/CD25/CD127/FoxP3                                    | Decreased      | Higher suppressive capability on CD4\(^+\)CD25\(^-\) regulatory T cells than controls | Increased frequency with disease progression |
| Wu et al. [59]           | B-ALL/T-ALL/controls        | PB             | CD4/CD25                                                 | Higher         | Not performed                                                                       | Not evaluated                        |
| Wang et al. [60]         | AML/controls                | PBMC /BM       | CD4/CD25                                                 | Higher         | Inhibition of proliferation and cytokine production (IL2, IFN-\(\gamma\)) of CD4\(^+\)CD25\(^-\) T cells; improved IL-10 production under coculture of both subsets with stimulation | Not evaluated                        |
| Idris et al. [61]        | B-ALL/controls              | PB and BM      | CD4/CD25/CD127                                          | Increased      | Not performed                                                                       | Correlation with age                 |

ALL: acute lymphoblastic leukemia; AML: acute myeloid leukemia; PB: peripheral blood; BM: bone marrow; PBMC: peripheral blood mononuclear cells; IL: interleukin; IFN: interferon.
Table 5: Most relevant published studies investigating the frequency and the prognostic significance of Tregs in chronic myeloid leukemia.

| Reference          | Patients/controls evaluated | Samples tested | Marker panel used in Treg evaluation by flow cytometry | Treg frequency | Functional studies | Impact on prognosis                                                                 |
|--------------------|-----------------------------|---------------|------------------------------------------------------|----------------|-------------------|-------------------------------------------------------------------------------------|
| Zahran and Badrawy [62] | CML/controls                | PB            | CD4/CD25/FoxP3                                       | Increased      | Not performed     | Correlations with the level of BCR/ABL, basophils and blast cells. Significantly higher in accelerated phase and blastic phase than in chronic phase and with high Sokal score. Reduction of Tregs after therapy with IM |
| Hus et al. [65]    | CP CML/controls              | PB            | CD4/CD25/FoxP3                                       | Increased      | Not performed     | Correlation with higher basophiles. No change in frequency after 6 months of TKI inhibitors |
| Bachy et al. [74]  | CP CML/controls              | CD4+ enriched PBMC cells | CD4/CD25/CD127/FoxP3                                  | Increased in PB. Increased in BM of patients on IM compared to healthy volunteers. | No difference in inhibition | Correlation with Sokal risk score |
| Rojas et al. [63]  | CP CML/controls              | PBMC          | CD4/CD25/CD127/CD62L/FoxP3                          | Lower in patients in complete cytogenetic response | Enhanced proliferative response to purified protein derivative | Not evaluated |
| Nadal et al. [64]  | CP CML/controls              | PBMC          | CD4/CD25/CD127/FoxP3/CTLA-4                         | Higher frequencies after transplant than normal controls and newly diagnosed patients | Purified Tregs from SCT patients had a more potent suppressive activity than those from healthy volunteers | Not evaluated |

CP: chronic phase; BM: bone marrow; IM: imatinib; PB: peripheral blood; PBMC: peripheral blood mononuclear cells; BM: bone marrow; SCT: stem cell transplant; IM: imatinib; TKI: tyrosine kinase inhibitor.
| Reference                      | Patients/controls evaluated | Samples tested | Marker panel used in Treg evaluation by flow cytometry | Treg frequency | Functional studies                  | Impact on prognosis                                                                 |
|-------------------------------|-----------------------------|----------------|--------------------------------------------------------|----------------|-------------------------------------|--------------------------------------------------------------------------------------|
| Hasselbalch et al. [66]        | PV                          | PBMC           | CD4/CD25/CD127                                         | Not increased  | Inhibitory activity preserved       | Marked expansion of Tregs in patients treated with IFN-α2 with than treated with hydroxyurea |
|                               | ET                          |                 |                                                        |                |                                     |                                                                                      |
|                               | PMF                         |                 |                                                        |                |                                     |                                                                                      |
|                               | Controls                    |                 |                                                        |                |                                     |                                                                                      |
| Kovacsovics-Bankowski et al.  | PV                          | PB             | CD4/CD25/FoxP3/Ki-67                                   | Not reported   | Not performed                       | Tregs (including highly suppressive CD39 HLA-DR) increase in patients treated with PeglIFNα |
| [67]                          | ET                          |                 |                                                        |                |                                     |                                                                                      |
|                               |                             |                 |                                                        |                |                                     | In patients with CALR mutation genotype association with longer disease duration and hemoglobin concentration |
| Massa et al. [68, 69]         | PMF                         | PB             | CD4/CD25/CD127/FoxP3                                   | Reduced        | Not performed                       |                                                                                      |

PV: polycythemia vera; ET: essential thrombocythemia; PMF: primary myelofibrosis; PB: peripheral blood; PBMC: peripheral blood mononuclear cells; CALR: calreticulin; IFN: interferon.
Moreover, molecular nonresponder patients showed a trend towards increased frequency of Tregs compared to responder patients, but no changes were observed in terms of absolute numbers of Tregs. Overall, a positive correlation between proliferating Tregs (Ki-67'), highly suppressive Tregs (CD39+/HLA-DR'), and JAK2V617F allelic burden was found, thus suggesting that the lack of ability of PegIFN-α treatment to decrease circulating Tregs predicts a poor molecular response.

Primary myelofibrosis (PMF) is a clonal disease of the hematopoietic stem cell characterized by a variable degree of bone marrow fibrosis, splenomegaly, and an increased risk of leukemic transformation. Contradictory data about Tregs in PMF have been published (Table 6). Massa et al. reported a reduced frequency and absolute number of Tregs in PMF than in normal controls [68]. No association with clinical-biological features of the disease was found, but a correlation between reduced Treg frequency and longer disease duration in patients with CALR mutation genotype was described. In these patients, a higher Treg frequency is significantly associated with advanced disease, higher IPSS/DIPSS score, and lower hemoglobin concentration. The same authors later documented the effect of ruxolitinib on Treg frequency, showing that the treatment with this small-molecule JAK1/2 inhibitor leads to a profound and long-lasting reduction in the frequency of circulating Tregs [69]. Wang et al. found no significant differences in the number of Tregs in patients with primary or post-ET myelofibrosis [70]. However, they reported that ruxolitinib significantly inhibits the release of sIL2-Rα, an inflammatory cytokine produced by Tregs, contributing to the clinical improvement of constitutional symptoms induced by the drug. These data have been further confirmed by an in vitro study in which the JAK1/2 inhibition by ruxolitinib was able to prevent Treg differentiation [71]. Table 6 summarizes the results of studies analyzing Tregs in Ph1-negative chronic myeloproliferative neoplasms.

6. Conclusions

Tregs have a fundamental function in maintaining the immune homeostasis in healthy individuals. In cancer and in particular in hematological malignancies, Tregs exert a major immunosuppressive activity, thus playing a critical role in tumor cell growth, proliferation, and survival. Published data on the prognostic significance of the Treg number in hematological malignancies show conflicting results. In our opinion, this variability reported by different groups is most likely explained by the heterogeneity of the experimental approaches that are used. In fact, different tissues have been studied (i.e., peripheral blood, bone marrow, and lymph node) and different analytic methodologies have been applied (i.e., flow cytometry versus immunohistochemistry). Moreover, while some authors studied the whole blood compartment, others evaluated the Treg population in isolated peripheral blood mononuclear cells or in a CD25-depleted subpopulation. Finally, various panels of markers, different techniques of fixation and permeabilization, and several gating strategies have been applied. This is of relevance to stress the need to apply standardized approaches in the study of Tregs in hematological malignancies and in cancer in general.

In perspective, in light of the increasing evidence of the important role of Tregs in immune evasion mechanism exerted by tumor cells, therapeutic interventions targeting intratumoral Treg infiltrates may be conceived in order to fight cancer. Treg inhibition or depletion, the latter uses monoclonal antibodies targeting surface antigens on Tregs such as CD25, is currently under investigation [72].

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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