Increased regulatory T cells in acute lymphoblastic leukaemia patients

Siti-Zuleha Idris¹, Norfarazieda Hassan¹, Le-Jie Lee¹, Sabariah Md Noor¹, Raudhawati Osman², Marsith Abdul-Jalil¹, Abdul-Jalil Nordin¹, Maha Abdullah¹

¹Department of Pathology, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia, ²Haematology Unit, Department of Pathology, Hospital Kuala Lumpur, Jalan Pahang, Malaysia

Introduction: Regulation in adaptive immune response balances a fine line that prevents instigation of self-damage or fall into unresponsiveness permitting abnormal cell growth. Mechanisms that keep this balance in check include regulatory T cells (Tregs). Tregs consist of a small but heterogeneous population, which may be identified by the phenotype, CD3⁺⁴⁺CD25⁺CD127⁻. The role of Tregs in pathogenesis of cancers is thus far supported by evidence of increased Tregs in various cancers and may contribute to poorer prognosis. Tregs may also be important in acute leukaemias.

Objective: A review of the literature on Tregs in acute leukaemias was conducted and Tregs were determined in B-cell acute lymphoblastic leukaemias (ALLs).

Results: Studies on Tregs in B-cell ALL are few and controversial. We observed a significantly increased percentage of Tregs (mean ± SD, 9.72 ± 3.79% vs. 7.05 ± 1.74%; P = 0.047) in the bone marrow/peripheral blood of ALL (n = 17) compared to peripheral blood of normal controls (n = 35). A positive trend between Tregs and age (R = 0.474, P = 0.055, n = 17) implicates this factor of poor prognosis in B-cell ALL.

Discussion: Tregs in cancer are particularly significant in immunotherapy. The manipulation of the immune system to treat cancer has for a long time ignored regulatory mechanisms inducible or in place. In lymphoma studies, tumour-specific mechanisms that are unlike conventional methods in the induction of Tregs have been hypothesized. In addition, tumour-infiltrating Tregs may present different profiles from peripheral blood pictures. Tregs will continue to be dissected to reveal its mysteries and their impact on clinical significance.

Keywords: Regulatory T cells, B-cell acute lymphoblastic leukaemia, Age

Immunological tolerance

Cells of the adaptive immune system are unique in each carrying an antigen receptor of its own design. The high variability in antigen receptors in T cells (T-cell receptors) and B cells (B-cell receptor/immunoglobulin) is generated from gene rearrangement processes during cell development. These lead to their astounding ability to bind specifically to the myriad of foreign antigens on pathogens. Unfortunately, many of these receptors also find a match to self-antigens in the human body.

The immune system deals with the potential of self-damage by inducing self-tolerance, that is, the inability to recognize and thus the failure to mount an immune response to molecules of the body. There are various mechanisms that regulate and instil tolerance, which follows a temporal sequence, in general described as central and peripheral tolerance. Central tolerance occurs in the primary sites of lymphocyte development. The major processes that undertake this role is clonal deletion of autoreactive cells, aided by the AIRE gene. Other smaller inputs are from induction of cell anergy and receptor editing.¹ In peripheral tolerance, further clonal deletion of mature autoreactive cells may take place with the AIRE gene also playing a critical role² but unique to this area is the active suppression of cell activation by dendritic cells and regulatory T cells.³ B cells are dependent on T cells for activation and thus are additionally kept in check through regulation of T cells.

Regulatory T cells

The presence of an immunosuppressive population of cells was noted since the early 1970s and 1980s; however, it was the study by Sakaguchi et al.,⁴
working on autoimmune disease mice models that led to the confirmation of its existence. Realizing that two CD4+ populations were present in normal individuals, one potentially pathogenic, self-reactive T cells and the other controlling its activation or expansion, they sought to identify a T-cell-specific surface molecule that would differentiate these cells. Elimination of the IL-2 receptor α-chain (CD25) expressing T cells resulted in manifestation of autoimmune-related symptoms suggesting its self-tolerance maintaining role. This was to be called CD4+ CD25+ or suppressor T cells or regulatory T cells. In peripheral blood of normal individual, 5–10% of helper CD4+ cells consists of T regulators. In 2001, researchers identified FOXP3 mutation as the cause of scurfy in mice characterized by over-proliferation of CD4+ CD8– T lymphocytes, with extensive multi-organ infiltration and elevation of numerous cytokines. The same group characterized by over-proliferation of CD4+ CD8– T lymphocytes, with extensive multi-organ infiltration and elevation of numerous cytokines. The same group determined FOXP3 mutation in human immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome and declared it the human equivalent of scurfy. FOXP3 was confirmed expressed only in CD25+ CD4+ peripheral T cells and soon discovered as the transcription factor involved in development and function of Treg. FOXP3 suppresses IL-2, IL-4, and IFN-γ gene transcription through direct interaction with NF-kB and NF-AT and at the same time upregulates CD25, CTLA-4, and GITR. Subsequently, CD127 (IL-7 receptor) appeared as another potential Treg marker as an inverse correlation was observed between CD127 and Foxp3 expression in CD4+ CD25+ T cells. Weak or negative expression of CD127 T cells was highly suppressive in functional suppressor assays. CD127 has the intracellular origin of Foxp3. The majority of Foxp3 + Tregs are generated from progenitor cells in the thymus and are termed natural Tregs (nTreg). The use of the term thymus-derived Treg (tTreg) has also been recommended. Tregs can be generated from Foxp3-T-conventional cells (naïve cells) in peripheral sites and thus named induced Tregs (iTregs) or peripherally derived Tregs (pTregs). Furthermore, Tregs may also be generated in vitro with TGF-β from Foxp3-conventional T cells and are thus termed in vitro iTregs. The three main categories describe the inhibitory action of thymus-derived Tregs on target cells. First, cell–cell contact utilize surface bound molecules including TGF-β, cytolytic molecules (Fas and granzyme B), LAG3 and CTLA-4 (cytotoxic T-lymphocyte antigen 4), which are constitutively expressed in Tregs, and more recently elevation of cAMP (cyclic adenosine monophosphate) levels, which is associated with inhibition of cellular proliferation and differentiation of lymphocytes and associated markers CD73 and CD39. Secondly, secretion of inhibitory cytokines, TGF-β and IL-10 and more recently IL-35. Lastly, the competition for growth factors based on high expression of CD25 in Treg. Differentiation of tTreg in thymus is promoted through interactions with self-peptide on major histocompatibility complex (MHC) complexes. Differentiation of iTregs on the other hand is likely to occur in response to non-self antigens such as allergens, food and the commensal microbiota. Thus, iTreg play an essential role in maintaining feto-maternal tolerance, oral tolerance (to dietary antigens) and intestinal homeostasis.

Induced Tregs develop upon TCR triggering of naïve T cells by antigenic stimulation under supoptimal conditions unsuitable for effector T-cell generation. Factors mediating this conversion may include high levels of cytokines such as IL-2, IL-10, or TGF-β, low-dose antigens or immaturity of antigen-presenting cells. The two most common types of iTregs are T regulatory type 1 cells (Tr1) and T1/3. Tr1 cells secrete IL-10 and also have an IL-10-dependent induction process. T1/3 cells secrete large amounts of TGF-β and lower IL-4 and IL-10. They are induced by TGF-β. IL-2 is essential in TGF-β-induced Foxp3 expression. As most iTreg studies are experimental, it is not clear whether in vivo induced Tregs utilizes the same suppressor mechanisms. Through these various mechanisms, Tregs inhibit many innate and adaptive immune cells such as CD4+ T cells, CD8+ T cells, dendritic cells, macrophages, B cells as well as NK cells.

### Diseases of immune dysregulation
Foxp3 is synonymous with the IPEX syndrome where a defect in the transcription factor leads to severe autoimmunity including type 1 diabetes, autoimmune thyroiditis, eczema, bleeding abnormalities, and chronic wasting. Patients have increased susceptibility to infection and an elevated incidence of allergic-type symptoms such as severe eczema, increased serum immunoglobulin E, eosinophilia and food allergy. Thus, Tregs are essential for preventing autoimmunity by maintaining self-tolerance, suppressing allergy, asthma, and pathogen-induced immunopathology. Their role in chronically inflamed or transplanted tissues as well as tumours are clear from the presence of iTregs in these areas leading to impairment of protective immunity.

### Treg and cancers
Cancer or tumour is made up of malignant cells or tissues in the body. Tumour develops even in normal immune function but is kept in check by the concept of tumour surveillance. Nevertheless, the high prevalence of cancer suggests tumour escape mechanisms are in place. Maintenance of peripheral tolerance is mediated by immune homeostasis and promotion of
regulatory T cells (Tregs). In cancer, Tregs function become more complex and may contribute to tumour progression.\textsuperscript{18}

The vast majority of studies on Treg in cancer are performed on solid malignancies. Increased levels of CD4\(^+\) CD25\(^{hi}\) Tregs in the circulation as well as within the tumour-infiltrating lymphocytes (TIL) are hallmark indications of the function of Tregs. Co-expression of CTLA-4, IL-10 and TGF-\(\beta\) and also Foxp3 confirmed the suppressive phenotype. CD4\(^+\) CD25\(^{+}\) Foxp3\(^+\) Tregs were also observed in malignant ascites and draining lymph nodes. These convincing evidence of the mechanism of tumour escape are often supported by a reduced proportion of Tregs in patients after curative surgery, which then rebound upon recurrence of the disease.\textsuperscript{17}

Treg is an important biomarker in tumour burden and disease course. Higher frequencies of circulating Tregs were correlated with worse survival in gastric cancer\textsuperscript{19,20} and continue to be observed in other cancers such as melanoma\textsuperscript{21} and non-small cell lung cancer.\textsuperscript{22}

However, many other studies showed that Tregs numbers in peripheral blood may also correlate with better patient survival as seen in cancers such as glioblastoma,\textsuperscript{23} oral squamous cell carcinoma patients\textsuperscript{24} and HPV-positive oral and squamous cell carcinomas\textsuperscript{25} or had no correlation as observed in prostate cancer.\textsuperscript{26}

Examination of the tumour microenvironment in malignant tissue samples revealed similar contrasting results. It became evident that mere increased numbers in Tregs may not correlate with prognosis but is rather the pattern of localization.\textsuperscript{17} Evaluation results of TIL, CD8\(^+\) and Foxp3 lymphocytes and location of intratumoral subsite (front, centre, or within epithelium) revealed dependency on all three factors to determine clinical outcome in colorectal cancer.\textsuperscript{27}

Low peritumoural Treg infiltration was a significant predictor of unfavourable outcome in colorectal liver metastases.\textsuperscript{28} Diminished Teff/Treg ratio predicted poor outcome in glioblastoma suggesting absolute numbers may not be as informative for predicting clinical outcome as Teff/Treg ratio.\textsuperscript{29} Even subtypes of cancer cofactors as high levels of FOXP3 + TILs were significantly associated with poor survival in ER- breast cancers that lacked CD8\(^+\) T-cell infiltrates. On the other hand, in ER- breast cancers, FOXP3 + TILs were strongly associated with improved survival in the HER2+/ER- subgroup, particularly in those with co-existent CD8\(^+\) T-cell infiltrates.\textsuperscript{30}

Accumulation of Tregs in tissue may be regulated by chemokine/chemokine receptors such as CCL5, CCL22, CCL17, and CXCL12 expressed on cancer cells or other cells in the microenvironment. Tregs may be stimulated into proliferation by various means, generated de novo or increase in survival.\textsuperscript{17}

In lymphoma, a heterogeneous group of serious and frequently fatal malignant disease of lymphocytes, similar to variation in unpredictability of Tregs infiltration has been observed. Wang and Ke hypothesize four types of Tregs function. Where increased Treg number is associated with poor survival, two types of Tregs are predicted (i) suppressor Tregs, that is, classical Tregs and (ii) malignant Tregs arising from a subset of T-cell lymphomas. Where reduced numbers of Tregs are associated with good prognosis, two other types of Tregs are postulated: (iii) direct tumour-killing Tregs shown by the ability of some B-cell lymphoma cells to be targets of Tregs cytotoxicity and (iv) incompetent Tregs showing reduced infiltration of Tregs potentially contributing to autoimmune symptoms. It remains to be seen whether similar concepts apply to other solid tumours.\textsuperscript{31}

Other associated immune markers may contribute to an overall better picture. In tumour-draining lymph nodes, reduced Treg levels were seen compared to control lymph nodes and tonsils, which decreased with increasing disease stage that may be explained by an alteration in the microenvironment including increased IL-6, IL-1b, IL-23 and lactic acid which converted Tregs from an anti-proinflammatory state to a pro-inflammatory state. A literature review to investigate the impact of immune factors within the tumour microenvironment of head and neck squamous cell carcinomas on clinical outcome concluded, if immunotherapy is to be introduced as an adjunct in the treatment of head and neck cancers, different immunological parameters will need to be selected for each distinct subsite.\textsuperscript{32}

### Acute leukaemias

Acute leukaemia is identified by the aggressiveness of the disease. Acute leukaemias are diseases involving progenitor cells and clonal malignancies of haematopoietic stem cells.\textsuperscript{33,34} Response to therapy varies in leukaemias\textsuperscript{35} and the most important prognostic factors in acute leukaemia are an early response to treatment.\textsuperscript{36}

There are two main groups of acute leukaemia, acute myeloid leukaemia (AML) and acute lymphoblastic leukaemia (ALL). There are more AML cases than ALL and more adults having AML. Nevertheless, ALL are the most common cancer in children.

ALL is a malignant disorder of lymphoid progenitor cells and affects both children and adults.\textsuperscript{37} Occurrence of ALL in childhood\textsuperscript{38} peaks at age 2–5 years old. ALL can be divided into two lineages, B-lineage and T-lineage ALL. Accurate diagnosis of
ALL helps in providing the best treatment to the patients. Although the outcome of ALL has improved globally, effective and good management will increase cure rates.\(^{39}\)

AML is associated with poor survival rates, which worsen with age. Early death was 19%. With intensive chemotherapy, 65% achieve complete remission and overall survival for age 16–55 increased to 7 years while for age 76–89 it was only 0.5 years.\(^{40}\)

**Tregs and acute leukaemia**

It is just as important to evaluate the role of Treg in acute leukaemias. Tregs could be recruited and exploited by leukaemic cells to evade immnosurveillance.\(^{41}\)

Wang et al. showed a higher proportion of CD4\(^{+}\)CD25\^{high}\) T cells in peripheral blood of AML patients and demonstrated inhibition of proliferation and IL-2 and IFN-\(\gamma\) production in CD4\(^{+}\)CD25\(^{-}\) T cells from patients.\(^{42}\) Circulatory CD4\(^{+}\)CD25\^{high}\)Foxp3\(^{+}\) was higher in AML compared to controls and predicted response to therapy.\(^{43}\) Zhang et al. also demonstrated elevated frequencies of CD4\(^{+}\)CD25\(^{+}\)CD127\(^{-}\)Tregs and was associated with poor prognosis.\(^{44}\) Evidence of Treg in AML is also provided by experiments involving treatment strategies. In a murine model of AML, removal of Treg with interleukin-2 dipheria toxin prevented AML-induced hypoproliferation of transplanted cytotoxic T cells thus increasing numbers at disease site, reducing AML disease burden resulting in long-term survival and anti-AML memory.\(^{45}\)

Furthermore, administration of azacitidine, a cancer drug that upregulates expression of tumour antigens in leukaemic blasts in vitro and expands Tregs in mouse model, may also have potential role in improving successful recovery in AML patients after allogeneic stem cell transplantation.\(^{46}\)

Limited studies are available on Tregs and ALL. Increased CD4\(^{+}\) CD25\(^{+}\) Tregs in peripheral blood was observed in both B-cell ALL and T-cell ALL together with higher levels of IL-10 and TGF-\(\beta\) compared to normal individuals.\(^{47}\) Another study, however, showed decreased CD4\(^{+}\) CD25\(^{+}\) cells levels in B-ALL patients at diagnosis although populations of CD4\(^{+}\) CD25\(^{+}\) Foxp3\(^{+}\) Tregs and CD4\(^{+}\) CD25\(^{+}\) IL-10\(^{-}\) Tregs were high. Tregs after successful treatment returned to similar levels as those in normal individuals. CD4\(^{+}\) CD25\(^{+}\) cells from B-ALL patients also exerted higher suppressive activity on CD4\(^{+}\) CD25\(^{-}\) T responder cells.\(^{48}\) Combined immunotherapy using dendritic cells as well as a method to disrupt the immunoregulatory pathways (CD25\(^{+}\) and CTLA-4 suppressive T cells) in a T-ALL murine model resulted in cure and in vivo generation of immune memory.\(^{49}\)

We examined the presence of Tregs (CD3\(^{+}\) CD4\(^{+}\) CD25\(^{+}\) CD127\(^{-}\) ) in the mononuclear cells of bone marrow or peripheral blood of a cohort of B-cell ALL and normal controls from subjects of Malay ethnicity. Table 1 summarizes the characteristics of these subjects. Significantly increased levels (mean \(=\) SD, 9.53 \(\pm\) 3.82% vs. 7.05 \(\pm\) 1.74%) of Tregs were observed in B-cell ALL patients (n = 17) compared to normal controls (n = 35), using the non-parametric Mann–Whitney test (\(P = 0.047\)), shown in Fig. 1. We also observed a positive trend in correlation between percentages of Tregs and age of patients (\(R = 0.474\), \(P = 0.055\)) which was not observed in normal controls (\(R = 0.070\), \(P = 0.690\)) as determined by Spearman’s rho test.

Thus, percentage of regulatory T cells in normal individuals was comparable to reported range of 1–13%. Increased Tregs in B-cell ALL were supportive of similar observation by Wu et al.\(^{47}\) The other study on B-ALL by Bhattacharya et al.\(^{48}\) did not observe this difference; however, the subjects in this study were of median 8 years and age range 1.5–17 years lower than our median age of 13 and range of 2–60 years. A positive trend between age and Treg percentages was observed in our samples, which may explain the significantly increased percentage in Treg seen here, which is known to be associated with poorer prognosis. In general, B-cell ALL patients aged 10 years or older have an inferior outcome to patients aged 1 to younger than 10 years.\(^{50,51}\)

**Targeting regulatory T cells in cancer therapy**

Thus, many studies confirm the importance of Tregs in the pathogenesis of cancers as well as acute leukaemias and their significance as targets for alternative therapy and utility as prognostic factors and strategy for therapies.\(^{41}\) Controlling the function of these suppressive cells in the tumour environment without

| Table 1 Characteristics of B-cell ALL patients and normal controls |
|-----------------------------|-----------------|-----------------|
| **Baseline characteristics** | **B-cell ALL patients** | **Normal controls** |
| Subjects, n | 18 | 35 |
| Sex, n (%) | | |
| Female | 7 (38.9%) | 9 (25.7%) |
| Male | 11 (61.1%) | 26 (74.3%) |
| Age (years), median (range) | 13 (2–60) | 41 (17–75) |

Diagnosis was determined by hematologists based on numerous blasts seen in blood smear and by morphological, cytochemical, and immunophenotyping examination of the bone marrow.

**Table 1 Characteristics of B-cell ALL patients and normal controls**

| **Baseline characteristics** | **B-cell ALL patients** | **Normal controls** |
|-----------------------------|-----------------|-----------------|
| Subjects, n | 18 | 35 |
| Blast cells | | |
| Morphology | >20% | None |
| Immuno-phenotyping | CD19\(^{+}\) CD20\(^{+}\) CD45dim | |
| Cytochemistry | PAS\(^{+}\) | |
| Sex, n (%) | | |
| Female | 7 (38.9%) | 9 (25.7%) |
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Diagnosis was determined by haematologists based on numerous blasts seen in blood smear and by morphological, cytochemical, and immunophenotyping examination of the bone marrow.
compromising peripheral tolerance represents a significant challenge for cancer therapies as increased Tregs in the peripheral blood of cancer patients presents a potent immunosuppressive features.52

Cancer vaccines are used either to treat existing tumours (therapeutic vaccines) or prevent development of cancer (prophylactic vaccine). As a biological response modifier, cancer vaccines are designed to stimulate or restore the body’s natural ability to mount an immune response against foreign peptides. Much effort has been put into identifying tumour-specific or tumour-associated antigens for the purpose of developing vaccines. Vaccines against cancer-causing viruses, HPV and HBV, have been commercialized to prevent cervical and liver cancer, respectively.

Producing effective therapeutic vaccines has proven much more difficult and challenging than developing cancer preventive vaccines, as cancers put up strong barriers to prevent removal by B cells and cytotoxic cells.53 Dendritic cell-based vaccine was developed to further boost immune response and today has achieved major breakthrough.54 Nevertheless, only one, Sipuleucel-T (Provenge®) has so far been approved by the US Food and Drug Administration (FDA) to treat advanced prostate cancer.

The role of Treg in impeding progress of these methods of immunotherapy has been demonstrated. Cancer vaccination in cancers such as melanoma and B-CLL patients showed positive induction of CD4+ CD25+ Fox3+ CD127− and TGF-β-producing TH3 cell Tregs. Thus, many immunotherapeutic regimens now include strategies that target Tregs to restore function of T cells and NK cells. These include depletion of Tregs with the use of cyclophosphamide, a chemotherapeutic drug which may function by inducing apoptosis and decrease proliferation and attenuation of suppressive function. Various anti-human CD25 antibodies are being evaluated to target the high-affinity IL-2Rα receptor that is constitutively expressed on major Tregs. Targeting function of Tregs may be conducted through inhibition of molecules such as CTLA-4, GITR, and TLR. Disrupting intratumoural homing of Tregs through blockade of chemokine/chemokine receptors such as CCL22/CCR4 and CCL5/CCR5 is also in progress. Modulation of Treg proliferation/conversion is also attempted by targeting dendritic cells, which is involved in the activation and induction of Tregs.17

Many examples have also shown that Tregs in the tumour microenvironment are tumour antigen specific. Thus, Tregs are specific not only to self but also to foreign peptides. In colorectal cancers, Tregs specific for telomerase, CEA, EGFR, Mucin-1, and HER2/neu have been detected indicating Tregs control of tumour-associated antigen-specific effector cells is in an antigen-selective manner adding another perspective to immunotherapeutic development.

**Conclusion**

Tregs is an essential component in immune homeostasis maintaining self-tolerance by suppressing the action of both cells of the innate and adaptive system against self-molecules. Without Tregs, the body is
potentiated towards attacks from its own immune system resulting in autoimmune and allergy diseases. Increased Tregs is a feature in the cancer microenvironment. Nevertheless, its association with treatment outcome is more complex and dependent on substrate as well as subtype of tumour and other factors in the environment. Unravelling these mysteries will greatly advance progress in strategies to target Tregs and overcome many of the difficulties encountered in immunotherapies.

Disclaimer statement
Contributors S.-Z.I., N.H., L.-J.L., M.A.-J.: collected data and performed experiments. S.Md.N., R.O., M.A.-J., A.-J.N., M.A.: contributed to conception and design of study. S.-Z.I. and M.A.: drafted and revised manuscript: All authors approved the final version of the manuscript.

Funding
Work carried out in this study was funded by research grants from Ministry of Science, Technology and Innovation (Project No.: 02-01-04-SF008) and Ministry of Higher Education, Malaysia (Project No.: 04-01-11-1333RU). We would like to acknowledge the Ministry of Health, Malaysia, for supporting this work as well as providing financial support to student.

Conflict of interest
There is no conflict-of-interests in the publication of this manuscript.

Ethics approval
None.

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