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Immunomodulatory Drugs: Immune Checkpoint Agents in Acute Leukemia

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Abstract

Intrinsic immune responses to acute leukemia are inhibited by a variety of mechanisms, such as aberrant antigen expression by leukemia cells, secretion of immunosuppressive cytokines and expression of inhibitory enzymes in the tumor microenvironment, expansion of immunoregulatory cells, and activation of immune checkpoint pathways, all leading to T cell dysfunction and/or exhaustion. Leukemic cells, similar to other tumor cells, hijack these inhibitory pathways to evade immune recognition and destruction by cytotoxic T lymphocytes. Thus, blockade of immune checkpoints has emerged as a highly promising approach to augment innate anti-tumor immunity in order to treat malignancies. Most evidence for the clinical efficacy of this immunotherapeutic strategy has been seen in patients with metastatic melanoma, where anti-CTLA-4 and anti-PD-1 antibodies have recently revolutionized treatment of this lethal disease with otherwise limited treatment options. To meet the high demand for new treatment strategies in acute leukemia, clinical testing of these promising therapies is commencing. Herein, we review the biology of multiple inhibitory checkpoints (including CTLA-4, PD-1, TIM-3, LAG-3, BTLA, and CD200R) and their contribution to immune evasion by acute leukemias. In addition, we discuss the current state of preclinical and clinical studies of immune checkpoint inhibition in acute leukemia, which seek to harness the body's own immune system to fight leukemic cells.

Keywords

Acute lymphoblastic leukemia; acute myeloid leukemia; co-inhibitory receptor; immune checkpoint pathway; immune evasion; immunotherapy; monoclonal antibody; T cells

1. INTRODUCTION

The functionality of T cells during immunity and tolerance is determined by the balance between co-stimulatory and co-inhibitory signals that fine-tune the amplitude, quality, and duration of T cell responses that are initiated upon antigen recognition by T cell receptors (TCRs) [1–3]. While engagement of a co-stimulatory receptor such as CD28 strongly amplifies antigen-specific TCR signaling with a resultant increase in lymphocyte

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CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

proliferation and activation, co-inhibitory receptors serve as immune checkpoints to prevent and limit uncontrolled T cell responses. In healthy individuals, immune checkpoints are crucial for maintaining self-tolerance, and thus preventing autoimmunity, and for limiting tissue damage when the immune system responds to infection. Tumor cells, however, can hijack these inhibitory pathways to evade immune recognition and consequent destruction by tumor antigen-specific cytotoxic T lymphocytes (CTLs; also known as $CD8^+$ effector T cells).

Under normal physiological conditions, the adaptive immune system is highly capable of recognizing and killing foreign cells, including aberrant tumor cells. However, differential expression of co-stimulatory and/or co-inhibitory molecules, among other immune escape mechanisms coopted by tumor cells, can dominantly diminish tumor-specific T cell responses [4, 5]. Sustained signaling via co-inhibitory molecules results in functional exhaustion of T cells, during which their ability to proliferate, secrete cytokines, and mediate lysis of tumor cells is sequentially lost [6]. Thus, these mechanisms ultimately contribute to immune escape by cancer cells [4, 7–9]. In this review, we will address the role of co-inhibitory molecules in immune evasion in acute leukemias and discuss the impact of blocking co-inhibitory molecule signaling in order to circumvent T cell inhibition.

2. ANTI-LEUKEMIC IMMUNE RESPONSES

The most convincing evidence that an intact immune system is highly potent in eliminating leukemia cells is seen in recipients of allogeneic hematopoietic stem cell transplantation (allo-HSCT) [10]. The anti-leukemic effect of allo-HSCT depends on the ability of immunocompetent donor T cells to recognize major histocompatibility antigens (MHAs) and/or minor histocompatibility antigens (MiHAs) present on residual leukemia cells and eliminate them, mediating the so-called graft-versus-leukemia (GVL) effect [11]. The best demonstration for the T cell-mediated GVL effect has been provided by the efficacy of donor lymphocyte infusion (DLI) in inducing disease remission in patients relapsing after allo-HSCT [12].

Besides this allogeneic anti-leukemic effect, there is also evidence for autologous anti-leukemic reactivity. Leukemia-specific cytolytic activity has been reported in patients after autologous HSCT [13], and T cell lines capable of lysing autologous acute myeloid leukemia (AML) cells have been generated *in vitro* [14]. Moreover, simple co-culture of monocyte-derived dendritic cells (DCs) with leukemic blasts, both derived from the peripheral blood (PB) of patients with AML, effectively activated leukemia-specific autologous T cells [15]. Nonetheless, leukemia cells exploit a variety of mechanisms to evade T cell-mediated immunity, leading to disease progression or relapse.

3. DYSREGULATION OF THE IMMUNE SYSTEM IN ACUTE LEUKEMIA

With the exception of immune checkpoint pathways, which will be discussed separately below, several innate and adaptive immune system aberrations encountered in patients with acute leukemia are summarized in Table 1. However, in any given leukemia patient, multiple

mechanisms likely cooperate to create an environment that supports the immune escape of leukemia cells.

Although a number of genetic and epigenetic changes within leukemia cells provide antigens that should easily be recognized by T cells [16, 17], leukemic blasts have been reported to downregulate human leukocyte antigen (HLA) molecules and to have altered antigen presentation properties that prevent T cell activation [18, 19]. Recently, AML cell loss of the non-shared HLA haplotype has been demonstrated in patients relapsing after HLA-haploidentical HSCT [20]. Furthermore, many leukemia-associated antigens (LAAs) are widely expressed on other tissues, thus generating only weak T cell responses [16, 21, 22].

Besides antigen presentation, other mechanisms contributing to the dysfunctional T cell responses in AML include expansion of suppressive immune cell populations, such as regulatory T cells (Tregs) (Box 1) [23–27] and myeloid-derived suppressor cells (MDSCs) [28, 29] as well as down-regulation of co-stimulatory receptors on the leukemia cells [30–34]. Other mechanisms of immune escape include production of immunosuppressive cytokines or reactive oxygen species (ROS) by leukemia cells themselves or within their microenvironment [35, 36] and impaired natural killer (NK) cell function [37, 38].

Box 1

Regulatory T cells (Tregs)

Tregs are a diverse population of $CD4^+$ T cells with immunosuppressive, rather than inflammatory properties [190]. A key characteristic of Tregs is the expression of the forkhead/winged-helix transcription factor FoxP3 [191, 192]. Tregs **suppress the activation and proliferation of other T cells, and therefore may hamper the activation** of anti-tumor effector cells. Mechanisms of suppression include production of inhibitory cytokines such as interleukin (IL)-10, transforming growth factor (TGF)-β, and IL-35 [193]. Tregs express high levels of several immune checkpoint molecules. Inhibitory antibodies targeting the many immune checkpoints are likely to enhance anti-tumor immunity by blocking Treg-mediated immunosuppression.

Furthermore, enzymes that degrade metabolically important amino acids (e.g. tryptophan, arginine) [39, 40] and ectonucleotidases like CD39 [41] may contribute to altered T cell immunity. In particular, expression of indoleamine 2,3 dioxygenase (IDO), an enzyme that catalyzes the rate-limiting step of tryptophan degradation along the kynurenine pathway, has been reported in AML blasts [42] and is associated with shortened relapse-free and overall survival (OS) [43]. Depletion of tryptophan and accumulation of its metabolites results in inhibition of effector T cell proliferation, increased T cell apoptosis, and peripheral induction of Tregs, which all lead to an impairment of the anti-leukemic cellular immune response [39, 44]. Several IDO inhibitors are under active clinical investigation in hematologic malignancies, including myelodysplastic syndrome (MDS) and B cell acute lymphoblastic leukemia (B-ALL) (NCT01822691 and NCT01595048).

A recent study has revealed yet another mechanism of immune escape in AML:deletional T cell tolerance, which is likely mediated by immature antigen-presenting cells (APCs) that

present LAAs in a context resulting in T cell deletion or anergy [45]. Lastly, and the subject of this review, upregulation of inhibitory ligands and their receptors (immune checkpoints) has been described on leukemia cells, serving as important mechanisms of immune evasion as well as potential therapeutic targets.

4. EMERGING ROLE OF IMMUNE CHECKPOINTS IN IMMUNE ESCAPE BY ACUTE LEUKEMIA

Multiple immune checkpoint molecules, described below, have been implicated in immune escape by tumors, including acute leukemia. A summary of the co-inhibitory receptors and their respective ligands discussed in this review is shown in Fig. (1).

4.1. CTLA-4

Biology—CTLA-4 (cytotoxic T-lymphocyte associated antigen 4, CD152) is one of the most well studied co-inhibitory receptors and the first one to be targeted in a clinical setting. CTLA-4 was discovered in 1987, when Brunet *et al.* [46] screened murine cytotoxic T cell-derived cDNA libraries and came across a 223-amino acid protein that clearly belonged to the B7 immunoglobulin superfamily. CTLA-4 is homologous to CD28, and they share identical ligands, CD80 and CD86. CTLA-4, however, binds CD86 and in particular CD80 with much higher avidity and affinity than does CD28 [47–49]. CTLA-4 is expressed predominantly on activated T cells [46] and on Tregs [50–52]. Engagement of CD80 or CD86 with CTLA-4, in contrast with that seen with the activating ligand CD28, results in inhibition of the early stages of T cell activation, thus dampening T cell responses (Box 2) [53]. Besides diminishing effector T cell activation, CTLA-4 signaling in Tregs controls autoreactive T cells and thereby promotes tolerance to self-antigens, further underscoring the diverse role of CTLA-4 in maintaining immune homeostasis. [52, 54] CTLA-4's major role as a central inhibitory checkpoint was demonstrated through the use of CTLA-4 knockout mice, which had hyperactivated immune systems, consequently resulting in lethal lymphoproliferative disease with massive multi-organ T cell infiltration [55, 56].

Box 2

The Biology of T cell Activation

Almost 30 years ago Jenkins and Schwartz [194] had shown that engagement of the T cell receptor (TCR) is not sufficient to fully activate T cells. T cell activation is dependent on a two step signaling. **Signal 1** involves the TCR recognizing a specific antigen peptide presented on the major histocompatibility complex (MHC complex) expressed on antigen-presenting cells (APCs) and is assisted by the presence of $CD4^+$ or $CD8^+$ on the T cell. Effective activation of naïve T cells, however, requires a **second co-stimulatory signal (signal 2)** that strongly amplifies TCR signaling to activate T cells. The best characterized co-stimulatory molecules that deliver signal 2 are the B7 molecules (CD80/B7.1 and CD86/B7.2). B7 molecules, expressed on APCs, are encountered by CD28, its receptor on the T cell surface. Ligation of CD28 by B7 molecules is necessary for the

optimal clonal expansion of naïve T cells. Importantly, CD28 does not interfere with T cell activation unless the TCR has firstly engaged its antigen through signal 1.

Mechanisms of Action—Multiple cell-intrinsic and cell-extrinsic mechanisms of action have been proposed for CTLA-4-mediated inhibition of T cell responses and are still being elucidated [57, 58]. Reports of cell-extrinsic mechanisms include increased secretion of cytokines such as TGF-β [59] (resulting in reduced antigen presentation, impaired effector T cell function, and expanded Treg activity), sequestration and active removal of CD80 and CD86 from the APCs surface through transendocytosis to prevent their engagement with CD28 [60, 61], activation of the tryptophan-degrading enzyme IDO, and enhancement of Treg function [62–64]. Cell-intrinsic pathways include the activation and recruitment of the inhibitory protein phosphatases PP2A and PTPN11 to the T cell synapse where they inhibit TCR and CD28-induced signaling, transcriptional inhibition of cell-cycle regulators, and competition with CD28 for their shared ligands [65–67]. Recent studies suggest that cell-intrinsic functions are not required for systemic control of effector T cell function as CTLA-4-deficient effector T cells did not demonstrate any apparent abnormality when mixed with wild-type cells *in vivo* [68, 69]. Further elucidation of CTLA-4's mechanisms of action is needed to fully understand its effects in clinical application.

CTLA-4 in Solid Tumor Malignancies—The concept of CTLA-4 blockade relies on reactivation of endogenous immune responses against antigens expressed on tumor cells. There was an initial concern that blocking CTLA-4 activity might lead to overt systemic hyperimmunity as seen in CTLA-4 knockout mice. Fortunately, antibody-mediated CTLA-4 blockade resulted in rejection of established immunogenic mouse tumors and provided long-lasting immunity without overt immune toxicities, thus encouraging subsequent clinical translation [70]. Two fully humanized anti-CTLA-4 monoclonal antibodies (mAbs) ipilimumab (MDX-101, Yervoy®; Bristol-Myers Squibb) and tremelimumab (CP-675,206; Pfizer) were brought to clinical testing [71–73]. In a phase III study of advanced melanoma patients comparing treatment with ipilimumab with or without the melanoma-specific vaccine gp100 versus the gp100 vaccine alone, ipilimumab treatment produced a 3.5 month survival benefit, and 18% of patients on the ipilumimab arms were still alive 2 years after treatment [74]. So-called immune-related adverse events (IRAEs) were observed in 10% to 15% of patients treated with ipilimumab. The success of ipilimumab in advanced melanoma resulted in its approval by the US Food and Drug Administration (FDA) in 2010. Not all melanoma patients, however, benefit from ipilumumab therapy, and no consistent predictive biomarkers of response have been validated [75–77]. A number of ongoing clinical trials are investigating the role of ipilimumab in various solid tumors and hematologic malignancies, either alone or in combination with other modalities such as cancer vaccines, other immunomodulatory agents, chemotherapy, or radiation [78].

CTLA-4 in Acute Leukemia—As described above, one immune escape mechanism involves the defective expression of ligands important for T cell co-stimulation by tumor cells. As CTLA-4 and CD28 share CD80 and CD86 as their common ligands, resulting in either T cell inhibition or stimulation, investigators have explored the expression of CD80

and CD86 by leukemic cells. Several groups have shown that leukemia cells (mainly M2, M4, and M5 AML by the French-American-British (FAB) classification) from both PB and bone marrow (BM) have a more consistent but heterogeneous expression of CD86 at baseline and generally lack or have a very low expression of CD80 [30–34]. Expression of CD80 and CD86 together on AML blasts was reported to positively correlate with prolonged remission post induction chemotherapy in one study [79], while other studies suggested that CD86 expression on leukemia blasts is associated with worse outcomes [32, 33]. LaBelle and colleagues directly examined the relative impact of CD80 and CD86 expression on anti-tumor immunity by challenging mice with C1498, a murine myelogenous leukemia cell line, expressing either CD80 or CD86 [80]. They demonstrated that C1498/CD80$^+$ AML tumors grew progressively, whereas expression of CD86 on AML cells resulted in tumor rejection implying effective induction of tumor-specific T cell immunity against wild-type C1498 leukemia. The escape of C1498/CD80$^+$ tumors from T cell-mediated immune elimination was found to be CTLA-4-dependent based on experiments showing that C1498/CD80$^+$ tumors regress after antibody blockade of CTLA-4 or after genetically deleting CTLA-4 from responding T cells [80].

Most data on CTLA-4 expression are limited to T cells. However, an Italian group of investigators examined CTLA-4 expression at the protein and mRNA level in leukemia cell lines and primary leukemia samples (AML, ALL) and found that CTLA-4 is universally expressed in all types of leukemia [81]. Binding of CTLA-4 on leukemia cells with soluble recombinant ligands r-CD80 and r-CD86 led to apoptosis of AML cells [82]. Furthermore, leukemic cells may have the ability to interact with the CD80/CD86 ligands on APCs, potentially modulating immune responses and ultimately promoting the growth and persistence of leukemic cells [81].

The important role of CTLA-4 in modulating anti-leukemia immune responses has been demonstrated by the association between CTLA-4 polymorphisms and outcomes in AML patients [83]. In those patients who achieved a first complete remission (CR) after induction chemotherapy, the CTLA-4 CT60 A/A genotype was associated with a higher rate of leukemic relapse and decreased overall survival (OS) at three years [83]. In children with ALL, certain polymorphisms associated with higher CTLA-4 expression appear to be increased compared to healthy controls and were in fact highest within the high-risk group [84]. However, donor CTLA-4 genotypes were not found to affect the post-transplant outcomes (OS, relapse free survival, and incidence of acute and chronic graft-versus-host disease [GVHD]) among 780 patients with AML and/or MDS following HLA matched or one antigen mismatched unrelated donor HSCT [85]. Besides membrane-bound CTLA-4, a native soluble form of CTLA-4 (sCTLA-4), produced by alternative CTLA-4 mRNA splicing, has been described for a variety of autoimmune diseases and may play a role in regulating immune responses [86]. Significantly elevated levels of circulating sCTLA-4 also have been reported in pediatric patients with B-ALL and were associated with a poor prognosis [87, 88].

Several experimental studies have demonstrated that CTLA-4 can be targeted to enhance a clinical anti-leukemia immune response. CTLA-4 blockade significantly enhanced the capacity of AML-derived DCs to induce T cell responses against autologous AML cells *in*

vitro [89, 90]. This effect was manifested by increased frequency and number of AML-specific T cells, which produced more IFN-γ and were more cytotoxic towards autologous AML cells than those cultured without an anti-CTLA-4 mAb [90]. In the DA1-3b mouse model (tumor dormancy model) of AML, long-term persistent murine leukemia cells (minimal residual disease, MRD) were more resistant to CTL-mediated killing and had increased expression of PD-L1 and CD80 [89]. Upon CTLA-4 blockade (and PD-L1 blockade, described below), CTL-mediated lysis of these persistent AML cells was enhanced as was survival of naïve mice injected with AML cells at MRD levels.

The timing of CTLA-4 blockade may be important for its use in the allo-HSCT setting, as demonstrated in murine models of MHC- and/or MiHA-mismatched allo-HSCT [91, 92]. If administered immediately after transplant, anti-CTLA-4 mAb increased donor T cell expansion and GVHD, as well as host-mediated BM graft rejection due to the dominant effect of the CD28:B7 pathway. However, when administered later after allo-HSCT, CTLA-4 blockade potently augmented GVL immunity without substantially increasing GVHD-related side effects [91, 92].

In the clinical setting, a single infusion of ipilimumab (phase I study, NCT00060372) given to 29 patients with recurrent or progressive hematologic malignancies (including 2 AML patients) after a minimum of 90 days post-allo-HSCT or DLI (median 1 year, range 0.3–6.5 years) did not induce or exacerbate GVHD or promote graft rejection [93]. Organ-specific IRAEs occurred in 14% of patients, but importantly, two patients with Hodgkin lymphoma achieved CR and one patient with mantle cell lymphoma achieved a partial remission (PR) [93]. Analysis of the peripheral blood T lymphocytes before and after single-dose ipilimumab administration revealed that CTLA-4 blockade is associated with increased conventional $CD4^+$ T cell activation and intracellular CTLA-4 expression without resulting in changes in levels of Tregs [94]. Ipilimumab is now being evaluated in patients with relapsed MDS/AML (NCT01757639) and in patients with relapsed hematologic malignancies after allogeneic-HSCT (NCT01822509).

4.2. PD-1

Biology—Programmed death-1 (**PD-1**, CD279) is another inhibitory receptor that belongs to the B7/CD28 family. Almost two decades ago, PD-1 was identified on T cells undergoing apoptosis [95]. It was only later that its major role in inhibiting T cell activation and effector function in an inflammatory environment and controlling autoimmunity in peripheral tissues was elucidated [96, 97]. The importance of PD-1 as a peripheral inhibitory regulator was confirmed in PD-1 knockout mice that developed organ- and strain-specific autoimmune complications including cardiomyopathy, lupus-like arthritis, and glomerulonephritis [98, 99]. In contrast to CTLA-4 deficient mice, PD-1 deficient mice have milder phenotypes [100]. PD-1 functions during the effector phase of T cell activation and is expressed on a broad variety of cells including activated T cells, B cells, monocytes, dendritic cells, and NK cells [101]. Notably, naïve lymphocytes do not express PD-1 prior to activation [102].

PD-1 has two ligands, PD-L1 (B7-H1, CD274) and PD-L2 (B7-DC, CD273), that bind PD-1 with different affinity [96, 103–105]. Although **PD-L1** is expressed on different cell types, including hematopoietic and epithelial cells, its expression is generally low on normal

human tissues [106]. The inflammatory milieu, including that encountered in the tumor microenvironment, promotes PD-L1 upregulation on a variety of different tumor cells in response to proinflammatory cytokines such as IFN-γ [106]. Thus, PD-L1 plays a key role in mediating tumor cell immune resistance to endogenous immune responses (adaptive immune resistance) within the tumor microenvironment. Upregulation of PD-L1 by activation of oncogenic pathways may contribute to innate immune resistance [107]. The increased expression of PD-L1 on tumor cells has been associated with poor prognosis in certain solid tumors [108–112]. **PD-L2,** the second known ligand for PD-1, has both stimulatory and inhibitory functions on T cell activation. While it is mostly expressed on APCs, PD-L2 also is upregulated on certain tumors such as B cell lymphoma [104, 113, 114]. Moreover, $CD86^+$ HL-60 myeloid leukemia cells were shown to provoke T-helper cell responses and become immunosuppressive through the upregulation of both PD-L1 and PD-L2 [115].

Mechanisms of Action—Upon engagement by one of its ligands, PD-1 becomes phosphorylated at its cytoplasmic tail signaling motif, which then recruits protein phosphatases like SHP2 and results in dephosphorylation of the proximal TCR signaling molecules [29]. These changes also suppress CD28/TCR-mediated activation of phosphoinositide 3-kinase (PI3K) and Akt signaling, leading to inhibition of T cell activation [67]. In contrast to PD-1 signaling, CTLA-4 signaling inhibits Akt independently of PI3K [67]. Recent studies reported an interaction between PD-L1 and the CD80 co-stimulatory molecules, resulting in T cell inhibition [116, 117]. This finding has added an increased layer of complexity to the role of CD80 as a control nexus for mediating stimulatory and inhibitory signals from CD28, CTLA-4, and PD-1. Further investigation is needed to understand this additional dimension to the complex binding interactions of checkpoint proteins and their immunoregulatory functions. Furthermore, epigenetic regulation of the PD-1 gene after TCR stimulation regulates its expression and controls T cells responses. Demethylation of the PD-1 gene is observed upon activation of $CD8^+$ T cells, but becomes remethylated in functional memory T cells [118]. However, in chronic viral infections, exhausted effector memory T cells have upregulated PD-1 and maintained demethylation of the PD-1 locus [118, 119]. In addition, as PD-1 is expressed not only on effector T cells, but also on Tregs, NK cells, and B cells, inhibition of PD-1 may augment anti-tumor immunity through a variety of mechanisms including activation of T effector function, suppression of Treg inhibition, promotion of NK cell activity, and production of antibodies.

PD-1 in Solid Tumor Malignancies—A critical milestone in the clinical development of PD1/PD-L1/-L2 pathway inhibitors was achieved in 2014 with the FDA approval of pembrolizumab (Keytruda®, Merck), and shortly after, nivolumab (Opdivo®, Bristol-Myers Squibb), two anti-PD-1 antibodies, for the treatment of patients with metastatic melanoma who have failed prior therapies with ipilumumab and, if BRAF V600 mutation positive, a BRAF inhibitor. Pembrolizumab produced a 26% response rate in this patient population, and, at most recent data analysis, 88% (36/41) of patients with objective responses had ongoing responses at follow-up of 1.4 to 8.5+ months [120]. Nivolumab produced responses

in 32% of patients compared to 11% response rate in chemotherapy arm, with 87% (33/38) of patients having ongoing responses at follow up of 2.6 to 10+ months [121].

A number of agents targeting PD-1 (nivolumab, pembrolizumab, and CT-011) or PD-L1 (MPDL3280A, BMS936559, and MEDI4736) are under clinical investigation. This fast-paced development of agents targeting PD-1 and PD-L1 was prompted by a favorable safety and efficacy profile observed in an initial Phase I study of repeat-dose nivolumab in patients with advanced solid tumors [122]. Nivolumab produced objective and durable responses in 28% of patients with melanoma, 18% of patients with non small cell lung cancer, and 27% of patients with renal cell carcinoma [122]. Notably, the expression of PD-L1 on tumor cells seemed to correlate with response. Furthermore, the frequency of IRAEs appeared less than with anti-CTLA-4 mAb treatment [122]. Updated survival data for advanced (but non-ipilimumab-treated) melanoma patients treated with nivolumab show 1-, 2-, and 3-year survival rates of 62%, 48%, and 41%, respectively [123]. Inhibiting both CTLA-4 and PD-1 in melanoma patients appeared to be more effective when given concurrently (objective response (OR) 53%) than when given sequentially (OR 20%) [124]. At the most promising dose level of ipilimumab 3 mg/kg and nivolumab 1 mg/kg, 2-year survival was 79% [124, 125]. While toxicities were increased with concurrent therapy, the side effects were qualitatively similar to that seen with monotherapy and dose-limiting in 21% of patients. Notably, IRAEs were generally manageable and reversible with treatment interruption, treatment discontinuation, or the administration of glucocorticoids [124]. Enthusiasm over the use of PD-1 inhibitors in hematologic malignancies comes from promising preclinical data and the success of early clinical trials in patients with Hodgkin lymphoma (HL). Nivolumab and pembrolizumab produced objective responses in 87% and 53% of relapsed or refractory HL patients, respectively, and 86% of patients treated with nivolumab maintained responses at 6 months [126–128]. Based on these results, the FDA granted nivolumab breakthrough status in relapsed HL.

PD-1 and PD-L1 in Acute Leukemia—Signaling through the PD-1/PD-L1 axis has been shown to impair anti-leukemic immunity. As described earlier in this review, PD-L1 and CD80 are upregulated on long-lived murine myeloid leukemia cells (MRD) that exhibit resistance to CTL-mediated killing [89]. Upon PD-L1 checkpoint blockade, CTL-mediated lysis of leukemic cells was enhanced *in vitro*, which was accompanied by increased levels of IFN-γ and TNF-α and decreased IL-10 production, and the survival of naïve mice injected MRD-derived leukemia cells was improved [89]. Zhang *et al.* [129] evaluated the role of PD-1/PD-L interactions in a murine model of AML and found that murine leukemia C1498 cells had low PD-L1 expression *in vitro*, but PD-L1 became upregulated *in vivo*. One could speculate that the leukemic microenvironment may stimulate upregulation of PD-L1, thus contributing to immune evasion. These investigators also demonstrated that leukemia-specific T cell immunity and survival upon AML challenge was increased in PD-1 knockout mice or in wild-type mice upon PD-L1 blockade using a mAb [129]. In another murine model of advanced AML, tumor progression was associated with upregulation of PD-1 expression on $CD8^+$ CTLs, but also correlated with an increased frequency of Tregs [130]. PD-1 knockout mice were less susceptible to C1498 challenge, despite having similar Treg percentages compared to wild-type mice. Interestingly, AML-associated Tregs impaired the

function of adoptively transferred CTLs *in vivo,* and Treg depletion followed by blockade of PD-L1 resulted in superior anti-leukemia responses than PD-L1 blockade alone [130].

Similar to what is seen in other malignancies, PD-L1 expression has been inconsistently found on human AML cells. A Japanese group reported low or absent PD-L1 expression on de novo AML cells, whereas PD-L2 was more frequently expressed [131]. High PD-L2 expression (>25% of leukemia cells) was associated with significantly shortened survival [131]. In contrast, several groups reported that PD-L1 is expressed on acute leukemia cells (AML and ALL) in PB and/or BM, but the frequency of PD-L1-positive leukemia varied across different studies from 18% to more than 50% [132–134]. A few studies also have reported that PD-L1 expression increased at the time of relapse [133, 134]. PD-L1 appeared to be expressed more frequently on acute monocytic leukemia (M5) and predicted for poor prognosis [133]. These different observations on PD-L1 expression on leukemic cells are likely due to multiple factors, including diverse patient populations studied (particularly given that AML is a very heterogenous disease), different assays and techniques used to detect PD-L1, different cell source (PB or BM) examined, and the use of different treatment regimens. Most recently, PD-L1 expression was measured on freshly isolated leukemic blasts from the PB and BM of 154 AML patients with or without *ex vivo* stimulation with IFN-γ [135]. IFN-γ exposure strongly increased PD-L1 expression on leukemia blasts, particularly in patients who had completed induction chemotherapy, suggesting that its upregulation is driven by an inflammatory tumor microenvironment [135]. Treatment with a hypomethylating agent (decitabine) appears to up-regulate PD-1, PD-L1, PD-L2, and CTLA-4 gene expression in PB mononuclear cells of patients with myeloid malignancies (MDS, AML, and chronic myelomonocytic leukemia) [136].

The impact of the PD-1/PD-L1 inhibitory pathway on acute leukemias in the post-transplant setting has been evaluated in both mice and humans. In different murine models, heightened PD-1 expression on donor T cells post allo-HSCT was found to be associated with a lack of GVL reactivity [137, 138]. Functional exhaustion of donor T cells was fostered by the expression of allo-antigens on the recipient's non-hematopoietic cells [137, 138]. Similarly, adoptive transfer of allogeneic T cells, engineered to express TCRs against LAAs, provided a potent GVL effect without causing GVHD, but only if administered early post-transplant [139]. If administered later post-transplant, however, adoptive transfer is effective only with concurrent PD-L1 blockade due to PD-1 upregulation on T cells in leukemia-bearing mice [139]. In these three studies, donor T cell dysfunction could partially be restored by blockade of PD-L1; delayed blockade of PD-L1 after allo-HSCT improved the beneficial GVL effect without inducing GVHD [137–139]. In two patients relapsing after allo-HSCT, PD-L1 was found to be highly expressed on leukemic progenitor cells, whereas co-stimulatory molecules CD80 and CD86 were generally absent [34]. Furthermore, high PD-1 expression was noted on alloreactive MiHA-specific effector memory $CD8^+$ T cells. *Ex vivo* treatment with mAbs against PD-1 or PD-L1 restored proliferation and IFN-γ production of MiHA-specific effector memory T cells, thus enhancing alloreactive T cell responses, while stimulation with MiHA-loaded DCs alone was insufficient to activate these exhausted cells [34]. In addition, PD-1 blockade was more potent in activating MiHA-specific T cells from relapsed patients than those in remission [34]. Another study evaluated *ex vivo* vaccination with MiHA-loaded DCs that had undergone short interfering RNA (siRNA) silencing of PD-

L1 and PD-L2 [140]. DC vaccination augmented expansion and cytokine production of MiHA-specific effector memory $CD8^+$ T cells from leukemia patients early after DLI or later at relapse [140]. Furthermore, these expanded MiHA-specific $CD8^+$ T cells retained the capacity to degranulate and exert cytotoxic activity upon recognition of $MiHA^+$ target cells [140]. A phase I clinical study evaluated a single infusion of anti-PD-1 mAb CT-011 (CureTech Ltd, Yavne, Israel) in 17 patients with advanced hematologic malignancies, including 8 patients with AML [141]. Treatment with anti-PD-1 was well tolerated, and 6 patients benefited from treatment, including one patient with non-Hodgkin lymphoma (NHL) who achieved a CR. While one AML patient demonstrated disease stabilization and reduction in circulating blasts, another AML patient who received anti-PD-1 mAb 8 weeks post allo-HSCT, developed worsening of mild GVHD that was present at treatment, culminating in grade 4 acute GVHD [141]. As with CTLA-4 blockade, these studies underscore the importance of appropriate timing for the administration of checkpoint inhibitors in the post-transplant setting.

4.3. Other Immune Checkpoints

A number of other immune checkpoints are emerging as interesting targets for anti-cancer immunotherapy [4]. *In vitro* studies identified many of these receptors as important contributors to T cell dysfunction and immune evasion in a variety of hematologic malignancies [7]. Data on acute leukemias are limited but suggest that the key to enhancing anti-tumor immunity may lie in combinatorial approaches of checkpoint inhibition. Studies evaluating immune checkpoint blockade for the treatment of acute leukemia in mice or humans are summarized in Table 2.

TIM-3 (T cell immunoglobulin and mucin domain-containing protein 3), an inducible inhibitory receptor expressed on IFN-γ producing T helper 1 cells (T_H1), was recently shown to contribute to T cell co-inhibition in acute leukemias. TIM-3 inhibits T_H1 responses through interaction with its ligand galectin-9, which is expressed in a variety of tissues in mice and humans, including on tumor cells [142]. Increased frequency of TIM-3 expressing dysfunctional (exhausted) T cells was observed in the tumor microenvironment in murine models as well as in patients with melanoma or chronic viral infections and TIM-3 antibody blockade enhanced anti-tumor immunity [143–145]. Both mice and human AML cells express galectin-9, while co-expression of TIM-3 and PD-1 was observed on a subset of exhausted $CD8^+$ T cells in a systemic murine AML model: TIM-3 and PD-1 co-expression increased with AML progression [146]. Overexpression of TIM-3 on T cells also was shown to suppress immune responses through upregulation of MDSCs at the tumor site [147]. Higher expression of TIM-3 was observed on PB CD4+ and CD8+ T cells of newly diagnosed AML patients compared to healthy controls and appeared to correlate with the disease risk group and FLT3-ITD mutation status [148]. Furthermore, increased expression of TIM-3 on endothelial cells in human lymphoma contributes to impaired $CD4^+$ T cell responses and promotes lymphomagenesis [149]. In the murine AML model, only combined blockade of the TIM-3/galectin-9 and PD-1/PD-L1 pathways, using a TIM-3 fusion protein and a mAb against PD-L1, decreased tumor burden and lethality, whereas blockade of either pathway alone was insufficient to rescue mice from death from AML [146]. Besides its expression on T cells, TIM-3 has emerged as a specific marker for $CD34^+CD38^-$ myeloid

leukemia stem cells (LSC) and seems to be a promising candidate for LSC-targeted therapies [150–152].

LAG-3 (lymphocyte activation gene 3; CD223), another co-inhibitory receptor, is a transmembrane protein that is structurally similar to CD4 and binds the MHC II complex with high affinity [153]. LAG-3 is expressed on activated T cells, regulatory T cells, and NK cells [154, 155]. LAG-3 expression on effector T cells limits effective viral- or tumor-specific T cell responses [156]. Furthermore, LAG-3 is frequently co-expressed with PD-1 on exhausted T cells, and dual blockade of LAG-3 and PD-1 during T cell priming augmented proliferation and cytokine production by tumor-antigen-specific T cells [157]. However, activation of T cells under tolerizing conditions yielded distinct $CD8^+$ T cell populations expressing either or both of PD-1 and LAG3; these different populations had distinct functional characteristics, suggesting that, while both are inhibitory, PD-1 and LAG-3 do not have redundant effects on T cell function [158]. The synergistic potential of PD-1 and LAG-3 inhibition in activating T cells is further underscored by evidence that dual PD-1 and LAG-3 knockout mice reject even poorly immunogenic tumors and develop lethal autoimmunity albeit at a slower pace than seen in CTLA-4-deficient mice [159, 160]. In patients with Hodgkin lymphoma, the presence of both $CD4^+LAG3^+$ and $CD4^+FOXP3^+$ cells in the tumor environment coincided with defective tumor-specific (LMP1/2) T cell responses [161]. FoxP3 and LAG-3 were expressed on different regulatory T cell subsets, and deletion of $CD4^+LAG3^+$ T cells augmented tumor-specific responses *in vitro* [161]. Data on LAG-3 expression in acute leukemia are limited; one report found that only T cells isolated from CD30L- but not $CD30L^+$ AML patients expressed LAG-3 [162].

A soluble form of the LAG-3 receptor (IMP321) has been in clinical testing in patients with solid tumor malignancies and most recently an anti-LAG-3 mAb (BMS-986016) is being tested either alone or in combination with anti-PD1 in solid tumor malignancies as well as patients with lymphoma and multiple myeloma.

BTLA (B- and T-lymphocyte attenuator; CD272) is another inhibitory receptor belonging to the B7 family and has structural and functional similarities to CTLA-4 and PD-1 [163]. BTLA is mainly expressed on T cells, B cells, and DCs [164]. Unlike the other B7 family members, BTLA binds to HVEM (herpes virus entry mediator), a member of the TNF receptor superfamily [165]. HVEM itself has a complex signaling network with multiple other binding partners including CD160, LIGHT (homologous to lymphotoxin; TNFSF14), lymphotoxin-α, and herpes simplex glycoprotein D [166]. Furthermore, the signaling can be bi-directional, depending on the type and combination of interactions [164]. While engagement of HVEM with BTLA or CD160 leads to T cell inhibition, its ligation with LIGHT results in T cell co-stimulation [164]. Naïve T cells express both HVEM and BTLA, which form a heterodimeric complex and consequently make HVEM inaccessible to external ligands, thus inhibiting co-stimulation [167]. This mechanism appears to be important in self-tolerance as BTLA4-deficient mice developed autoimmune diseases [163, 168]. In melanoma patients, dysfunction of tumor antigen (NY-ESO-1)-specific effector $CD8^+$ T cells correlated with increased expression of BTLA together with co-expression of PD-1 and TIM-3 [169]. Importantly, T cell dysfunction could be reversed by triple blockade of BTLA/PD-1/TIM-3 [169].

The role of BTLA in suppressing T cell immunity against MiHA post allo-HSCT was evaluated in patients with hematologic malignancies, including AML. HVEM was found to be highly expressed on primary leukemic cells, whereas BTLA was moderately and more heterogeneously expressed [170]. The expression of LIGHT and CD160 was generally low or absent. MiHA specific $CD8^+$ effector memory T cells were found to express high levels of BTLA and PD-1. *Ex vivo* blockade of the BTLA-HVEM pathway using an anti-BTLA mAb and/or inhibition of PD-1 by an anti-PD1 mAb restored proliferation of highly functional MiHA-specific $CD8^+$ T cells and augmented alloreactive T cell responses. In several patients, the effect of BTLA blockade was more pronounced compared to PD-1 blockade, underscoring its importance in allogeneic tumor-specific T cell immunity [170].

CD200R is an inhibitory receptor expressed by cells of either the myeloid or lymphoid lineage, including NK and T cells [171, 172]. CD200R exclusively binds CD200 (OX2), a type I membrane glycoprotein. CD200 is expressed by a variety of cells and tissues and has been shown to be expressed by AML blasts in both mice and humans [173–175]. In mice, CD200 expression by leukemia cells was associated with enhanced tumor growth that could be inhibited by CD200 Fc administration [173]. In humans, an association between CD200 expression by AML blasts (43% of AMLs; most commonly seen on core binding factor AMLs) and poor prognosis was reported [176]. Signaling through the CD200/CD200R was shown to promote immune evasion in AML patients by reducing the function and frequency of activated NK cells and memory T cells, as well as by promoting the expansion of $FoxP3^+$ Tregs [174, 177–179]. Notably, *in vitro* mAb blockade of the CD200R/CD200 ligation restored the cytolytic activity of NK cells [174].

Novel Checkpoint Receptors—A number of other inhibitory receptors are emerging as promising new targets for augmenting anti-tumor immunity and have been reviewed elsewhere [4, 7, 180]. Among them are A2aR, an adenosine receptor that promotes peripheral tolerance by inducing T cell anergy and generation of Tregs, specifically $LAG\text{-}3^+$ Tregs [181], and PD-1H/VISTA, a PD-1 homolog expressed on hematopoietic cells and T cells that when inhibited prevented acute GVHD in a murine transplant model [182, 183].

Although inhibitory receptors associated with NK cell functionality are technically not immune checkpoints, significant defects in NK cell function have been shown to contribute to immune escape by acute leukemias [38]. The two common AML fusion proteins PML-RARA and AML1-ETO were recently shown to specifically downregulate CD48, the ligand of the NK cell receptor 2B4 (CD244), thus leading to impaired cytolytic activity of NK cells [184]. Therefore, receptors on NK cells, such as 2B4 (CD244) and the broad category of killer cell immune globulin-like receptors (KIR), may also be attractive targets to enhance anti-tumor immunity of acute leukemias [38, 185].

5. INTEGRATION OF CHECKPOINT BLOCKADE INTO CLINICAL MANAGEMENT OF ACUTE LEUKEMIAS - FUTURE PROSPECTS

Harnessing the body’s own immune system to fight cancer through immune checkpoint inhibition represents a breakthrough in cancer treatment. The recent FDA approval of the three checkpoint inhibitor mAbs targeting CTLA-4 or PD-1 for the treatment of metastatic

melanoma has paved the way for the evaluation of this highly promising strategy in other malignancies, including acute leukemias. However, the clinical translation of checkpoint inhibitors faces some critical challenges. Firstly, only a limited number of patients respond to this therapeutic intervention and predictive bio-markers of response have not thus far been identified. Such reliable biomarkers are urgently needed both for evaluating a tumor's immunogenicity and for assessing the likelihood of achieving a response to treatment. Second of all, not all tumors are equally immunogenic, and these tumors frequently employ a variety of different mechanisms to promote immune escape and survival. For example, tumor cells including leukemia blasts may aberrantly express antigens or fail to express co-stimulatory molecules, which are necessary for T cell activation. Also, certain tumors have been shown to poorly express MHC molecules or recruit Tregs or MDSCs to suppress other T cell subsets including CTLs. Another layer of complexity in AML stems from the different immune milieu of the BM and PB wherein dissimilar immune escape mechanisms may be operational. Thorough evaluation of a tumor's potential to be immunologically targeted seems to be crucial for a successful implementation of such novel therapeutic strategies. Acute leukemias, due to ease of obtaining samples before and after treatment, may provide an ideal setting to address such questions.

Given that acute leukemias, in particular AML, are a very heterogeneous disease, one has to address the question whether all - or if not all - which of the many distinct subgroups are susceptible to checkpoint blockade. For instance, expression of CD200 and PD-L1 has only been found to be of prognostic significance for core binding factor leukemias and monocytic leukemia (FAB M5), respectively [133, 176]. Secondly, clinical responses to checkpoint blockade in hematologic malignancies may be difficult to measure. It is unlikely that checkpoint blockade alone will be sufficient to eliminate the bulk of leukemic blasts present at initial diagnosis. If agents that inhibit cellular checkpoints must be integrated with cytotoxic chemotherapeutic agents, the optimal timing of each therapy must be explored. Additionally, the expression of certain inhibitory receptors such as PD-L1 on leukemia cells may be promoted by the inflammatory milieu created by chemotherapy. Furthermore, one must not only consider therapeutic efficacy but also tolerance in such studies since IRAEs may not be easily tolerable in patients undergoing intensive chemotherapeutic treatment. Thus, administration of checkpoint inhibitors may be appealing at the time of consolidation chemotherapy when it is also likely to be better tolerated. Beyond its potential in the upfront setting, checkpoint inhibition is an attractive strategy for preventing disease relapse once patients have achieved complete remission or at the very least in the presence of only minimal residual disease. Even in this setting, however, timing is critical as evidenced by tumor-specific changes and differing potential for GVL versus GVHD seen in several murine and human studies that have evaluated CTLA-4 and PD-1 blockade in the post-transplant setting [91, 93, 138, 141].

As preclinical and recent clinical data suggest, the key to success may lie in combinatorial approaches to checkpoint inhibition [120, 124, 125]. Despite these promising results we are still left with the question of which and how many of the multiple checkpoint receptors to target. Which combinations make sense and which do not? Is dual, triple, or even several receptor blockades associated with a higher risk of toxicities or should we aim to target common downstream targets like SHP2 or Akt? Given that some checkpoint receptors signal

bi-directionally or have both inhibitory and stimulatory ligands, it is clear that antibodies targeting these checkpoint receptors have to be carefully designed.

Based on the promising preclinical and clinical data discussed in this review, two checkpoint agents have made the long way from murine leukemic models to their evaluation in phase I clinical studies of acute leukemias. Anti-CTLA-4 (ipilimumab) is currently being tested in a US multicenter study enrolling patients with relapsed or refractory high-risk myelodysplastic syndrome (MDS) or acute myeloid leukemia (NCT01757639). Two other trials are evaluating ipilimumab in patients with relapsed hematologic malignancies (including acute leukemias) after allo-HSCT (NCT01822509 and NCT00060372). Blockade of PD-1 (nivolumab) is being tested in two phase I trials of AML patients in complete remission post induction chemotherapy (NCT02275533 and NCT01096602 (anti PD-1 together with a dendritic cell vaccine). Taken together, data from a number of preclinical and phase I trials show clearly that manipulation of inhibitory networks has emerged as an attractive strategy to increase anti-tumor immunity in patients with acute leukemia and that dual blockade of immune checkpoints can produce additive and perhaps even synergistic anti-leukemic responses. Ideally, these emerging therapies will meet the high demand for new treatment strategies to prevent relapses in the post-remission stage of acute leukemia, particularly for patients who are ineligible for allo-HSCT.

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ABBREVIATIONS

ALL	Acute lymphoblastic leukemia
allo-HSCT	Allogeneic hematopoietic stem cell transplantation
AML	Acute myeloid leukemia
APC	Antigen presenting cell
BM	Bone marrow
BTLA	B- and T-lymphocyte attenuator, CD272
CD	Cluster of differentiation
CR	Complete remission
CTL	Cytotoxic T lymphocyte (also known as $CD8^+$ effector T cell)
CTLA-4	Cytotoxic T-lymphocyte associated antigen 4, CD152
DC	Dendritic cell
DLI	Donor lymphocyte infusion

FAB	French-American-British classification of acute leukemias
FDA	United States Food and Drug Administration
GVHD	Graft-versus-host disease
GVL	Graft-versus-leukemia
HL	Hodgkin's Lymphoma
HLA	Human leukocyte antigen
HVEM	Herpes virus entry mediator
IDO	Indoleamine 2,3 dioxygenase
IFN	Interferon
IL	Interleukin
IRAE	Immune-related adverse event
KIR	Killer inhibitory receptor
LAA	Leukemia associated antigen
LAG-3	Lymphocyte activation gene 3, CD233
mAb	Monoclonal antibody
MCL	Mantle cell lymphoma
MDS	Myelodysplastic syndrome
MDSC	Myeloid-derived suppressor cell
MHA	Major histocompability antigen
MiHA	Minor histocompability antigen
MLR	Mixed lymphocyte reaction
MRD	Minimal residual disease
NHL	Non-Hodgkin lymphoma
NK cell	Natural killer cell
OR	Overall response
OS	Overall survival
PB	Peripheral blood
PBMC	Peripheral blood mononuclear cell
PD-1	Programmed death-1; CD279

PI3K	Phosphoinositide 3-kinase
PR	Partial remission
ROS	Reactive oxygen species
siRNA	Short interfering RNA
TCR	T cell receptor
T_H1	T helper 1 cells
TIM-3	T cell immunoglobulin and mucin domain-containing protein 3
TNF	Tumor necrosis factor
Treg	Regulatory T cell

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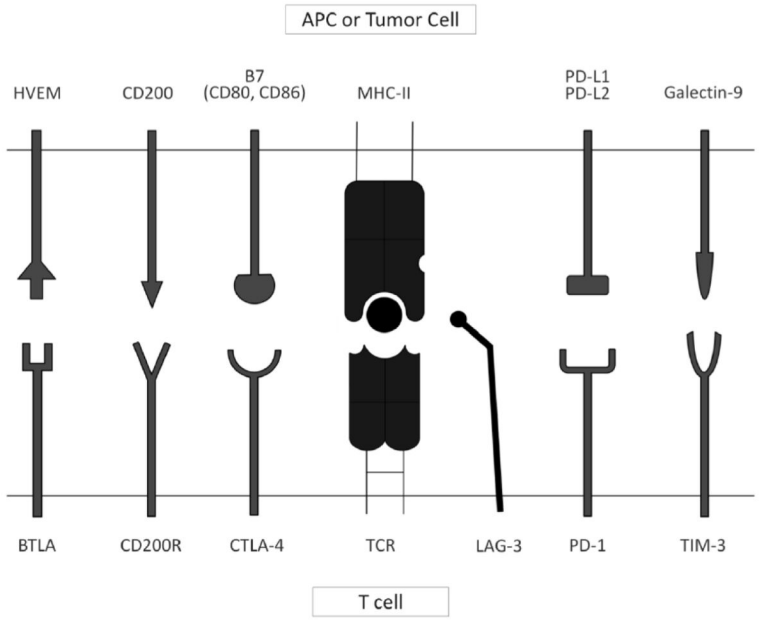

Fig. (1). Selected Immune Checkpoint Receptors and their Respective Ligands

Tumors co-opt immune checkpoint pathways as a critical mechanism of immune evasion. Immune checkpoints are initiated by ligand-receptor interactions and inhibit T cell activation and proliferation. Fig. (**1**) shows selected immune checkpoint receptors including BTLA (B- and T-lymphocyte attenuator) that binds to its ligand HVEM (herpes virus entry mediator), CD200R that engages with CD200, CTLA-4 (cytotoxic T-lymphocyte associated antigen 4) that binds the B7 molecules (CD80 and CD86), LAG-3 (lymphocyte activation gene 3) that binds to the MHC complex, PD-1 (programmed cell death-1) that engages with PD-L1 or PD-L2, and TIM-3 (T cell immunoglobulin and mucin domain-containing protein 3) that binds galectin 9 on the antigen presenting cell or tumor cell.

Table 1

Immunologic Changes in Patients with Acute Leukemia *.

References	No. of Patients	Major Findings	Condition	Specimen
LeDieu *et al.* [186]	36	↑ absolute number of $CD8^+$ T cells. ↑ numbers of $CD3^+CD56^+$ NK-T cells. Aberrant T cell activation pattern. Impaired immune synapse formation between T cells and AML blasts.	Newly diagnosed AML	PB
Kanakry *et al.* [25]	20	Tregs represent an expanded T cell population in early lymphocyte recovery after intensive induction chemotherapy. Tregs are oligoclonally skewed and primarily peripherally derived.	Newly diagnosed AML	PB
Shenghui *et al.* [26]	182	↑ Tregs compared to healthy control. ↑ Treg frequency in BM > PBMC of the same patient. ↓ Treg frequency in patients who achieved CR compared to patients with persistent AML. Higher Tregs at diagnosis associated with poor response to therapy.	Newly diagnosed AML	BM and PB
Szczepanski *et al.* [24]	31	↑ Tregs and suppressive activity in AML patients compared to normal controls. ↑ pretreatment Treg frequency associated with poor response to chemotherapy.	Newly diagnosed AML	PB
Wang *et al.* [23]	36	↑Tregs in PBMC > BM. ↑ apoptotic fraction of Tregs in AML > control Tregs suggesting a rapid turnover of Tregs.	AML	BM and PB
Curti *et al.* [42, 44]	76	AML blasts constitutively express IDO. Co-culture of $CD4^+CD25^-$ T cells with IDO^+AML cells results in conversion into $CD4^+CD25^+$ Tregs.	Newly diagnosed AML	BM and PB
Chamuleau *et al.* [43]	286	High IDO gene expression levels in leukemic blasts correlates with significantly shortened overall and relapse-free survival.	AML	BM and PB
Aurelius *et al.* [36]	26	Monocytic AML cells produce reactive oxygen species (ROS) that kill T cells and NK cells by triggering PARP-1 dependent apoptosis.	Newly diagnosed AML	PB and BM
Luczynski *et al.* [187]	20	ALL blasts express low levels of co-stimulatory molecules.	Pre-B ALL	PB
Kebelmann *et al.* [188]	10	BM blasts lack expression of CD80. ↓ expression of the adhesion molecule ICAM-1. ↑ secretion of IL-10 by leukemic cells.	Pediatric Relapsed Pre-B ALL	BM
Zheng *et al.* [189]	48	Leukemic cells lack expression of CD80. 50% of AML samples express CD86.	ALL (n=6), AML(n=30), other hem. malignancies (n=12)	PB
Costello *et al.* [37]	18	↓ NK cell cytolytic activity. Leukemic blasts resistant to lysis by NK cells.	AML	PB

* Abbreviations: NK cells - natural killer cells; PB - peripheral blood, BM - bone marrow; PBMC - peripheral blood mononuclear cells; hem. - hematologic

Table 2

Immune Checkpoint Blockade for Treating Acute Myeloid Leukemia in Mice or Humans*.

Checkpoint Molecule	PRECLINICAL STUDIES		CLINICAL STUDIES
	Non Transplant	**Transplant**	
CTLA-4	**LaBelle *et al.*, 2002** [80]; **mouse, *in vivo*** • Treatment with anti CTLA-4 mAb • Regression of C1498/CD80+ leukemia • Prolonged survival	**Blazar *et al.*, 1999** [91]; **mouse, *in vivo*** • Murine model of MHC mismatched allo- SCT • Treatment with anti CTLA-4 mAb early post allo-SCT: increased GvHD • Treatment with anti CTLA-4 mAb delayed post allo-SCT: Augmentation of GVL effect without increasing GvHD	**NCT00060372**, **Bashey *et al.*, 2009** [93] • Ipilimumab after allo-HSCT • No GvHD • IRAEs in 14% of patients • 2CR in HL and 1PR in MCL
	Saudemont & Quesnel, 2004 [89]; **mouse, *in vitro*** • Treatment with anti CTLA-4 mAb • Enhanced CTL-mediated lysis of persistent AML cells • Increased survival of naïve mice challenged with persistent AML cells	**Fevery *et al.*, 2007** [92]; **mouse, *in vivo*** • Murine model of MiHA mismatched allo-SCT • Treatment with anti CTLA-4 mAb early post allo-SCT: acute GvHD • Treatment with anti CTLA-4 mAb delayed post allo-SCT: – Augmentation of GVL effect without increasing GvHD – Exacerbation of autoimmunity	**NCT01757639** • Ipilimumab in relapsed MDS/AML • Recruiting patients
	Zhong *et al.*, 2006 [90]; **human, *in vitro*** • Enhanced cytolytic activity and ↑ numbers of AML-reactive T cells after stimulation by AML-derived DCs in the presence of CTLA-4 blockade		**NCT01822509** • Ipilimumab in relapsed hematologic malignancies after allo-HSCT • Recruiting patients
PD-1	**Saudemont & Quesnel, 2004** [89]; **mouse, *in vitro*** • PD-L1 upregulated on persistent murine leukemia cells • Treatment with Anti-PD-L1 mAb: • Enhanced CTL-mediated lysis of persistent AML cells • Increased survival of naïve mice challenged with	**Flutter *et al.*, 2010** [138]; **mouse, *in vivo*** **Koestner *et al.*, 2011** [139]; **mouse, *in vivo*** • Increased PD-1 expression on T cells post allo-SCT correlates with lack of GVL effect • Treatment with anti PD-L1 delayed post allo-SCT enhanced alloreactive T cell responses without exacerbating GvHD	**Berger *et al.*, 2008** [141] • Phase I clinical study of anti-PD1 mAb (CT-011) in patients with advanced hematologic malignancies • IRAEs in 61% of patients • 6 of 17 patients benefited from treatment; 1CR in NHL

Checkpoint Molecule	PRECLINICAL STUDIES		CLINICAL STUDIES
	Non Transplant	**Transplant**	
	persistent AML cells		
	Zhang *et al.*, 2009 [129]; **mouse, *in vivo*** • Murine C1498 AML cells upregulate PD-L1 *in vivo* • Treatment with Anti-PD-L1 mAb or PD1 KO mice: Enhanced leukemia- specific T cell responses • Increased survival	**Hobo *et al.*, 2010** [140]; **human, *in vitro*** • *Ex vivo* vaccination with PD-L1/-L2 silenced MiHA-expressing DCs post allo HSCT • Enhanced alloreactive MiHA-specific activity of $CD8^+$ effector memory T cells	**NCT01096602** • Anti PD-1 (CT-011) together with DC/AML vaccine for patients in CR post chemotherapy • Recruiting patients
	Zhou *et al.*, 2010 [130]; **mouse, *in vivo*** • Leukemia progression associated with Treg expansion and upregulation of PD- 1 on CD8+ T cells • PD-1 KO mice: • Increased CTL proliferation and function • Decreased AML tumor burden • Long-term survivors • Superior anti-tumor responses with PD- L1 blockade coupled with Treg depletion	**Norde *et al.*, 2011** [34]; **human, *in vitro*** • Increased PD-L1 expression on leukemia progenitors in patients at relapse post allo-HSCT • Tretament with anti-PD-1 or PD-L1 mAb: Enhanced alloreactive CTL responses *ex vivo* • PD-1 blockade was more potent in activating MiHA-specific T cells from relapsed patients than from those in remission	**NCT02275533** • Anti PD-1 (nivolumab) for AML patients in CR post chemotherapy • Recruiting patients
TIM-3	**Zhou *et al.*, 2011** [146]; **mouse,*in vivo*** • Treatment with anti-PD-L1 and/or mTIM-3 hFc • Combined blockade of TIM-3 and PD- • 1 increased tumor rejection and improved survival • mTIM-3 hFc alone: No ↓ tumor burden, no improved survival		

Checkpoint Molecule	PRECLINICAL STUDIES		CLINICAL STUDIES
	Non Transplant	**Transplant**	
	• anti-PD-L1 alone: Delayed tumor growth		
BTLA		**Hobo *et al.*, 2012** [170]; **human, *in vitro*** • Treatment with anti-BTLA mAb: • Restored proliferation and functionality of MiHA-specific CD8+ T cells *ex vivo* in patients post allo-HSCT • Augmented allo-reactive T cell responses • Effect of BTLA blockade was more pronounced than PD-1 blockade	
CD200R	**Coles *et al.*, 2011** [174]; **human, *in vitro*** • Treatment with anti-CD200 mAb: • Enhanced NK cytotoxicity against AML cells **Memarian *et al.*, 2013** [179] **human, *in vitro*** • CD200 expression by leukemia cells is associated with Treg expansion and AML progression • Treatment with anti-CD200 mAb or anti-CD200R mAb decreased TGF-β and IL-10 expression and increased IL- 12 and IFN-γ expression in autologous MLRs	**Gorczynski *et al.*, 2001** [173]; **mouse, *in vivo*** • CD200 expression by leukemia cells associated with enhanced tumor growth • Treatment with CD200Fc inhibited tumor growth post allo-HSCT	

*Abbreviations: allo-HSCT - allogeneic hematopoietic stem cell transplantation; BMT - bone marrow transplant; CTL - cytotoxic T lymphocyte; GVHD - graft versus host disease; GVL - graft versus leukemia; HL - Hodgkin's Lymphoma; MCL - Mantle Cell Lymphoma; MLR - mixed lymphocyte reaction; NHL - Non Hodgkin Lymphoma; Tregs - regulatory T cells