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PD-1 Checkpoint Blockade in Acute Myeloid Leukemia

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Abstract

Introduction—Immune checkpoints are regulatory pathways induced in activated T lymphocytes that regulate antigen responsiveness. These immune checkpoints are hijacked by tumors to promote dysfunction of anti-tumor effector cells and consequently of tumor escape from the host immune system.

Areas covered—PD1/PDL-1, a checkpoint pathway, has been extensively investigated in leukemia mouse models. Expression of PD-1 on the surface of activated immune cells and of its ligands, PD-L1 and PD-L2, on leukemic blasts has been documented. Clinical trials with PD-1 inhibitors in patients with hematological malignancies are ongoing with promising clinical responses.

Expert Opinion—Therapy of hematological cancers with antibodies blocking inhibitory receptors is expected to be highly clinically effective. Checkpoint inhibitory receptors and their ligands are co-expressed on hematopoietic cells found in the leukemic milieu. Several distinct immunological mechanisms are likely to be engaged by antibody-based checkpoint blockade. Co-expression of multiple inhibitory receptors on hematopoietic cells offers an opportunity for combining blocking antibodies to achieve more effective therapy. Up-regulation of receptor/ligand expression in the leukemic milieu may provide a blood marker predictive of response. Finally, chemotherapy-induced up-regulation of PD-1 on T cells after conventional leukemia therapy creates a solid rationale for application of checkpoint blockade as a follow-up therapy.

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1. Introduction

Human tumors, including hematological malignancies, have developed multiple strategies for escape from the host immune system. Mechanisms used by tumors for escape have been extensively investigated in the last decade,[1] and a better understanding of these mechanisms has facilitated the development of novel therapies aimed at arresting tumor immune evasion. One of the more recently discovered mechanisms of immune suppression operating in cancer involves immune cell intrinsic checkpoints that are induced on the surface of activated T cells.[2] Several such checkpoint molecules serving as negative regulators of activated T cells are known, including cytotoxic T-cell antigen-4 (CTLA-4), programmed death-1 (PD-1), T cell immunoglobulin mucin-3 (TIM-3), lymphocyte activation gene-3 (LAG-3), B and T cell lymphocyte attenuator (BTLA) and others. Surface expression and inhibitory functions of these receptors are up-regulated in T cells present in the tumor microenvironment.[3] While the presence of these inhibitory receptors on T cells is physiologically necessary to regulate cellular activation, their overexpression in disease leads to dysfunction of T cells and other immune effector cells.[4-7] In the setting of cancer, chronic overexpression of checkpoint molecules results in T-cell dysfunction and impairs anti-tumor immunity.[3]

It has been observed in animal models of tumor growth that blocking of checkpoint receptors with antibodies (Abs) can restore anti-tumor immunity and prevent tumor progression.[8, 9] One of the first checkpoint-blocking antibodies tested in preclinical studies and approved for therapy of patients with advanced melanoma in 2011 was ipilimumab, the anti-CTLA-4 Ab.[8, 10-12] Its administration to patients with advanced melanoma and blockade of CTLA-4 provided first evidence that this immune therapy results in durable responses and improved survival in 10-15% of patients.[12] The next anti-checkpoint Abs, pembrolizumab and nivolumab, approved for melanoma therapy, target PD-1. These antibodies are currently being actively investigated for the treatment of different cancers, including hematological malignancies. While more recent data for the blockade of the PD-1/PD-L1 pathway demonstrate durable responses in 30-35% of patients with advanced melanoma,[13] the factors underlying molecular, cellular and functional aspects of checkpoint inhibition in cancer patients are not yet understood and are being intensively investigated. Our current insights into early studies combining anti-CTLA-4 with anti-PD-Abs suggest that this combination shows impressive response rates and a relatively low toxicity profile. The mechanisms responsible for these clinical successes are not entirely worked out, and the evidence indicating that only subsets of patients respond to this immune therapy suggests that more extensive studies are required for improving its anti-tumor activity.

While patients with advanced melanoma were the first cohort to be successfully treated with checkpoint inhibitors, efforts are underway to extend this therapy to other solid tumors and, more recently, to hematological malignancies. This is an exceedingly important effort that aims at providing potentially beneficial immunotherapy to the cancer patient population at large. The purpose of this review is to discuss the rationale for and consider the potential impact of checkpoint inhibition on disease control in acute myeloid leukemia (AML). Although in comparison to solid cancers, the data on checkpoint inhibition in leukemia are limited, preclinical data overwhelmingly indicate that hematological malignancies, including

AML, which generally respond favorably to immune therapies, are also likely to benefit from checkpoint inhibition. As clinical trials with anti-PD-1 Ab checkpoint blockade in AML are being implemented, we anticipate that this immune therapy will rapidly move from the category of an experimental to an approved therapy for acute leukemias.

2. PD-1 Biology

Immune checkpoints are regulatory pathways that are induced in activated T cells and regulate the amplitude as well as the quality of T-cell antigen responses. These pathways are balanced by co-stimulatory and inhibitory signals and are critical in preventing autoimmunity and uncontrolled T-cell expansion that could facilitate oncogenic mutations. However, cancer has developed ways to exploit these immune cell-intrinsic checkpoints for escaping immune-mediated destruction.[1] In cancer, expression and functions of checkpoint molecules on T cells are up-regulated, leading to reduction or elimination of anti-tumor immune activity.[2] CTLA-4 and PD-1 are two of the most actively studied inhibitory receptors expressed by activated T-cells.

CTLA-4 receptor (CD 152) is present on T cells early in their activation stage, and it competes with CD28, a costimulatory receptor also expressed on T cells, for binding to CD80 (B7.1) and CD86 (B7.2) expressed on antigen-presenting cells (APC). Up-regulation of CTLA-4 activity interferes with co-stimulatory signals necessary for T-cell maturation and differentiation into effector cells. As discussed above, targeting of CTLA-4 with a blocking Ab, ipilimumab, in patients with advanced melanoma provided initial evidence that immune checkpoint blockade translates into clinical benefit in 10-15% of these patients by allowing restoration of more robust anti-cancer immune responses.[12]

The PD-1 receptor (CD279) is another inhibitory checkpoint also expressed by activated T-cells. (Figure 1) It is a type I transmembrane receptor member of the immunoglobulin superfamily and it binds two ligands: PD-L1 (B7-H1, CD274)[7, 14] and PD-L2 (B7-DC, CD273),[15, 16] both belonging to the B7 immunoglobulin superfamily. PD-1 differs from CTLA-4 in that its major role is to limit the inflammatory responses which occur in the periphery, when effector T cells recognize target antigens present on tissue cells. Uncontrolled T-cell response in this context leads to tissue damage. When the PD-1 receptor interacts with its ligands, PD-L1, which is expressed on most tissues and PD-L2, which is expressed on macrophages and dendritic cells, a signaling cascade is initiated that inhibits several of down-stream kinases involved in T-cell activation.[17] Inflammatory signals, such as interferon-γ (IFN-γ) secreted by T-helper 1 cells (Th1), induce PD-L1 and PD-L2 expression on tissue cells. [18]

In addition to activated effector T-cells, PD-1 is highly expressed on regulatory T cells (Treg), B-cells, and natural killer (NK) cells. When PD-1 expressed on Treg binds its ligand, induction of Treg and suppressor functions mediated by these cells are enhanced.[19] Therefore, the PD-1 pathway not only suppresses functions of effector T cells, lytic capacity of NK cells and B-cell antibody production, but it also promotes stability and functions of Treg, thus contributing to maintenance of immune suppression in the microenvironment.

3. PD-1 Blockade

The critical role of PD-1 in immune suppression has been elucidated through a number of preclinical studies, many in the setting of chronic viral infections. CD8+ memory T cells seen in chronic viral infections such as human immunodeficiency virus (HIV), hepatitis C (HCV) or hepatitis B (HBV)[20-22] have impaired proliferative and cytokine responses and are commonly referred to as “exhausted T cells.” The role of PD-1 in inducing dysfunction of CD8+ memory T cells was first demonstrated in mice chronically infected with lymphocytic choriomeningitis virus (LCMV). PD-1 was found to be selectively and significantly upregulated on exhausted T-cells in mice with chronic LCMV.[23] Importantly, PD-1 blockade restored functions of these cells, resulting in a decreased viral load.[23] Similar findings have subsequently been made for HIV-1[24] as well as chronic hepatitis.[25]

The availability of PD-1 knockout (KO) mice has further helped in clarifying the role PD-1 plays in immune regulation. The PD-1 KO mice consistently developed late-onset autoimmunity, with variable sites of autoimmune tissue damage dependent on the expression levels and persistence of tissue antigens in the background mouse strain. For example, non-obese (NOD) diabetic mice rapidly develop diabetes when PD-1 is knocked out;[26] mice with the C57BL/6 background develop lupus-like autoimmunity,[27] and mice with the BALB/c background acquire autoimmune-mediated dilated cardiomyopathy.[28] The enhanced autoimmune phenomena in PD-1 KO mice appear to be primarily due to a higher frequency of tissue-invasive CD4+ and CD8+ T-cells polarized to the Th1 phenotype rather than increased autoantibody titers.[26]

The potential of the immune system for preventing cancer development has long been recognized.[29] In part, immune surveillance and immune elimination of tumors is accomplished through the recognition of tumor associated antigens (TAA) by the adaptive immune system.[1] Unfortunately, tumor cells frequently develop resistance to immune intervention and manage to escape from immune control. The responsible mechanisms may involve a loss or down-regulation in expression of TAA, alterations in the antigen-presenting machinery components in tumor cells or the development of resistance to cytotoxicity mediated by immune effector cells.[30] More established tumors develop abilities to produce a variety of immunoinhibitory factors which alter the tumor microenvironment and induce suppression of anti-tumor functions in immune effector cells.[31] The tumor microenvironment (TME) becomes enriched in Treg and myeloid-derived suppressor cells (MDSCs), which contribute to converting it into a highly immunosuppressive milieu. The PD-1/PD-L1 pathway participates in creating and maintaining tumor-associated immunosuppression.[32] Tumors effectively convert this normally protective pathway, which is responsible for guarding against inflammation-induced tissue injury, to one that now protects the tumor from immune intervention. Tumors corrupt the PD-1/PD-L1 pathway by bombarding activated PD-1+ T cells with the ligand, thus inducing functional T-cell paralysis.[33, 34]

Several lines of evidence indicate that the PD-1/PD-L1 pathway is exploited by tumors. First, PD-L1 is found to be overexpressed on tumor cells in many different solid and hematological cancers. PD-L1 overexpression in tumors is driven in part by chronic

exposure to the pro-inflammatory cytokine IFN-γ.[7, 32] Expression of PD-L1 on tumor cells has been reported to be associated with poor prognosis in many tumor types, demonstrating that immune tolerance mediated by the PD-1/PD-L1 pathway has clinical significance.[35-37] More recent studies indicate that levels of PD-L1 expression on tumor cells vary broadly, and it remains to be determined whether low expression or even absence of PD-L1 expression is associated with less effective therapy. Interestingly, clinical responses to anti-PD-1 Abs have been reported in patients whose tumors are negative for PD-L1.[38] Second, numerous preclinical studies have reported the efficacy of the PD-1 signaling blockade in cancer. When anti-PD-L1 Abs were used to block this signaling pathway, increases in the frequency of tumor infiltrating CD8+ T cells and decreases in the frequency of Treg were seen that correlated with the arrest of tumor growth in mouse models of cancer.[39, 40] Third, preclinical studies have also demonstrated that PD-1 blockade enhanced tumor responses to other forms of immunotherapy.[41, 42] Finally, first-in-man clinical trials of Abs specific for PD-1 or PD-L1 performed in patients with different advanced malignancies confirmed clinical benefits of this immune therapy, serving as proof of principle. In a phase I study of the PD-L1 Ab, MDX-1105, in patients with different advanced malignancies, an objective response rate of 6 to 17% was seen, with stable disease in up to 41% of patients.[43] In an additional dose-escalation trial of the anti-PD-1 Ab, MDX-1106, durable objective responses were seen in 18-28% of patients with non-small cell lung cancer, melanoma, and renal-cell cancer.[34] These early studies have paved the way for many other clinical trials targeting the PD-1 pathway that are now being implemented in numerous institutions worldwide. Hematological malignancies, which have historically been sensitive to immunotherapy, promise to be yet another sensitive target for therapy with anti-PD-1 antibody.

4. PD-1 Pathway in Acute Myeloid Leukemia

4.1 Sensitivity of AML to immunotherapy

Beginning with allogeneic stem cell transplant, immune-based therapies have been often used for treatment of AML. Graft-versus-leukemia (GVL) effect of allogeneic hematopoietic stem cell transplantation (HSCT) is a well-established, successful form of immunotherapy. AML is the most common indication for HSCT in North America.[44] Donor lymphocyte infusions (DLI) performed for relapsed AML, which rely solely on the GVL effect, lead to complete remission (CR) rates of 15-29%.[45, 46] The CRs attained through DLI are frequently durable.[47]

Cytotoxic T lymphocytes (CTL) are well-established mediators of cancer-directed immunotherapy. Successful T-cell activation requires at least 3 signals. First, a specific antigenic peptide bound to a major histocompatibility complex on the APC must be recognized by an antigen-specific T-cell receptor (TCR) present on the surface of the cognate T cell. Subsequently, an antigen-independent co-stimulatory signal resulting from the interaction of CD28 on the T cell with CD80 or CD86 expressed on the antigen-presenting cell (APC) has to be generated to promote T-cell response. Next, cytokine-mediated stimulation of clonal T-cell expansion and functional maturation of T cells takes place. In the setting of HSCT for AML, the specific antigens recognized by TCRs to induce

the GVL effect may include hematopoietic cell-restricted minor histocompatibility antigens on the recipient cells.[48] However, TAA, i.e., immunogenic antigens expressed by leukemic blasts, play a critical role in successful T-cell based immunotherapy in the autologous setting.

Since the discovery of TAA in melanoma[49], many AML blast-associated antigens have been identified.[50] Some of these antigens can be considered leukemia-specific antigens (LSA), including fusion proteins DEK-CAN,[51] and PML-RARα[52] as well as antigens generated by gene mutations such as internal tandem duplications (ITD) of the FMS-like tyrosine kinase 3 (*Flt3*) gene and mutations in the nucleophosmin 1 (*NPM1*) gene.[53, 54] Furthermore, many leukemia-associated antigens (LAA), i.e., antigens overexpressed by leukemia cells with limited expression by normal tissues, have also been characterized.[50] Most LSA and LAA antigens are restricted in their expression to specific subgroups of AML, which limits their usefulness in antigen-targeted therapies, such as anti-leukemia vaccines. However, this is not a problem with checkpoint inhibition blockade, which is expected to be able to reverse immune tolerance to any endogenous antigen expressed by each patient's AML blasts.

4.2 Suppressive microenvironment in AML

Despite the sensitivity of AML to immune attack, the microenvironment in AML is immunosuppressive, facilitating immune tolerance of leukemia cells. In vitro studies have demonstrated that factors secreted by primary AML cells, particularly arginase II, can prevent T-cell activation and proliferation.[55,56] HL-60 AML cells overexpress cyclooxygenase-2 (COX-2) and produce prostaglandin E_2 (PGE_2). They are also positive for indoleamine 2, 3-dioxygenase (IDO) upon induction with IFN-ɣ.[57] Recently, Human Leukocyte Antigen-G (HLA-G), known to contribute to cancer cell immune escape, was found to be present on the surface of human AML blasts.[58] Many other immunoinhibitiory soluble factors and cytokines produced by leukemic blasts or stromal cells in the leukemic bone marrow, including TGF-β1 and IL-10, may induce tolerance in hosts with AML.[59]

Treg are often increased in frequency in AML and contribute to creating an immunosuppressive microenvironment.[60, 61] In mice, AML progression is associated with increased Treg infiltration at the site of disease. CTL adoptively transferred to leukemic mice have reduced proliferation and IFN-γ production at sites of Treg infiltration, with no effect on AML burden.[62] However, Treg depletion with IL-2 diphtheria toxin (IL-2DT) prior to adoptive transfer of CTL significantly decreases the tumor burden and improves survival of mice with AML compared to control mice or those treated with either agent (IL-2DT or CTL) alone.[62] Treg utilize a variety of mechanisms to mediate immunosuppression, including the production of inhibitory cytokines (IL-10, TGF-β) and suppressive factors such as adenosine or PGE_2, competition for IL-2 or for co-stimulatory factors on APCs, which skews dendritic cell (DC) differentiation toward an immature and tolerogenic phenotype, and transfer of cyclic AMP to effector T-cells upon direct contact.[31,61] Immature DC generated in the presence of Treg promote immunosuppression through expression of indoleamine 2,3-dioxygenase (IDO) which inhibits T cell proliferation by depleting tryptophan and promotes T-cell apoptosis by increasing levels of tryptophan metabolites.[63] In mice, IDO was also shown to promote conversion of conventional T cells

to Treg.[64] Interestingly, AML cells also express IDO, as indicated above, and IDO activity is higher in patients with AML compared to normal controls.[65, 66] In vitro, when IDO-expressing leukemic cells are co-incubated with T cells, the frequency of Treg and tryptophan catabolism increase, inhibiting naïve T-cell proliferation.[65]

Increases in the Treg frequency are observed in patients with AML at various stages of diagnosis and treatment. Compared to healthy controls, Treg percentages in the peripheral circulation are elevated at diagnosis in patients with AML,[60, 67, 68] and higher frequencies of Treg at diagnosis are associated with poor prognosis.[60, 67] In AML patients treated with cytotoxic chemotherapy or maintenance therapy, an increased frequency of strongly immunosuppressive Treg was observed, suggesting that therapy-resistant Treg contribute to leukemic relapse.[69, 70]

PD-1 is highly expressed on peripheral (inducible) Treg (pTreg),[19] and in solid malignancies, its level of expression increases in pTreg accumulating in the tumor microenvironment.[71] It has been reported that expression of PD-L1 in the tumor microenvironment controls the development of pTreg from human Th1 cells.[19, 72] This participation of the PD-1/PD-L1 pathway in maintaining CD4+T-cell plasticity and in the generation of Treg demonstrates the interplay existing between immunosuppressive pathways. Amplification of the PD-1/PD-L1 pathway in the tumor microenvironment translates into expansion of Treg and suggests that blockade of this pathway will also relieve Treg-mediated immunosuppression.

4.3 The PD-1/PD-L1 pathway and immune suppression in AML

In AML, the PD-1/PD-L1 pathway is hijacked by malignant cells to facilitate immune escape. Many preclinical studies have demonstrated up-regulation of the PD-1/PD-L1 pathway in AML and the negative impact of this amplification on disease control. In mice injected with an AML cell line (C1498), the percentage of CD8+ T-cells expressing PD-1 dramatically increased in the liver, a major site of C1498 dissemination.[73] Similarly, when C1498 cells were injected into mice and allowed to grow in vivo, PD-L1 expression on T cells increased compared to baseline.[74]

Functional consequences of the PD-1/PD-L1 pathway up-regulation in AML have been clearly demonstrated in PD-1 KO mice. When these mice are injected with C1498 AML cells, AML progression is slower than in wild-type (WT) mice, and the mice have significantly longer survival.[73, 74] This appears to be due to augmented antigen-specific CD8+T-cell responses, as both the number of tumor specific CD8+ T-cells and their effector function were increased in PD-1 KO compared to WT mice.[74] In addition to genetic PD-1 ablation, improved leukemic control has also been demonstrated with pharmacologic PD-1 inhibition. When a PD-1 blocking antibody was administered to WT mice with C1498 AML, the mice had lower AML burden, more CD8+ T cells infiltrating the liver and experienced longer survival than control mice.[74]

Expression of PD-1 and its ligands is also increased in hematopoietic cells of patients with AML. One study of 124 patients with myeloid malignancies, including 69 with myelodysplastic syndrome (MDS) and 9 with AML, sampled at various stages of treatment

found that the PD-L1 mRNA expression level was upregulated by ≥2 fold in 36% and 25% of CD34+ cells in MDS and AML, respectively, compared to CD34+ normal control cells.[75] PD-L2 was also upregulated in a smaller proportion of CD34+ cells, i.e., 12% in MDS and 33% in AML.[75] In a smaller subset of patients, mRNA expression correlated perfectly with PD-L1 expression on CD34+ cells by immunohistochemistry. Expression levels of PD-L1, PD-L2, and PD-1 were also increased in peripheral blood mononuclear cells (PBMCs). In fact, expression levels of PD-L2 and PD-1 were higher in PBMCs than in CD34+ cells.[75] Another cohort of 154 patients with AML demonstrated no significant increase in surface PD-L1 expression on leukemia cells at initial diagnosis compared to healthy controls. However, stimulation with IFN-γ significantly increased PD-L1 expression on AML blasts but not in normal controls.[76] Interestingly, PD-L1 expression on myeloid precursor cells increased more dramatically with IFN-γ stimulation in samples of patients in complete remission or at relapse than in myeloid precursor cells of newly-diagnosed AML patients.[76] These findings demonstrate that PD-L1 expression on myeloid precursor cells and leukemic blasts occurs in a substantial portion of patients with AML. At this time, it is not clear whether the frequency of positive cells or levels of expression can be related to disease progression or relapse. It would be important to establish how expression levels of PD-L1 on AML blasts and PD-1 on activated T cells or vice versa are regulated. Also, a better understanding of the timing required for up-regulation of expression levels requires studies in larger patient cohorts, where PD-1 and its ligands can be measured longitudinally. Nevertheless, the existing data suggest that activity of the PD-1/PD-L1 pathway, similar to Treg-mediated suppression,[70] may be particularly increased upon recovery from cytotoxic chemotherapy.[76] Consistent with these observations, PD-L1 expression in CD34+ cells prior to treatment did not appear to have prognostic significance in small cohorts of patients with AML. In 72 patients with MDS or AML tested prior to any treatment, PD-L1 expression in CD34+ cells was not associated with worse survival.[75] However, in a smaller cohort of those 72 patients enrolled in a clinical trial and treated with hypomethylating agents and vorinostat, upregulation (≥ 2 fold) of PD-L1 or PD-L2 in peripheral blood mononuclear cells during therapy was associated with a significantly worse median survival: 6.6 months compared to 11.7 months in patients without demonstrable upregulation of PD-1 ligands. Similarly, Norde and colleagues found that in patients who relapsed late after allogeneic transplant, despite the presence of circulating alloreactive T-cells to hematopoietic cell-restricted minor histocompatibility antigens, PD-L1 was highly expressed on the leukemic cells at baseline or upon stimulation with IFN-γ.[77] Furthermore, stimulation of allogeneic CD3+ T-cells with the PD-L1-expressing AML cells led to significantly enhanced T-cell proliferation and cytokine production when performed in presence of anti-PD-1 antibody compared to isotype controls.[77] In aggregate, these findings suggest that the development of functionally- impaired T-cells during therapy through up-regulation of the PD-1 checkpoint leads to impaired control of leukemia and that PD-1 blockade restores anti-leukemia T-cell functions and thus is likely to offer therapeutic advantages.

4.5 Therapeutic potential of PD-1/PD-L1 Blockade in AML

In considering molecular mechanisms responsible for clinical success of the PD-1/PD-L1 pathway blockade with Abs, it is important to remember the broad cellular and tissue distribution of the receptor and its ligands as well as distinct effects these Abs are likely to

exert upon interaction with normal vs malignant cells. Regardless of whether the blocking antibody targets the receptor or the ligand, the same two objectives are desired: (a) to eliminate or decrease immune suppression orchestrated by the tumor and (b) to simultaneously unleash the anti-tumor power of immune cells, converting them to fully competent anti-tumor effectors. In achieving either objective, the presence and expression levels of PD-1 and/or PD-L1 on target cells will determine antibody binding and interaction with its target. The PD-1 Ab will target various types of PD-1+ immune cells, especially activated immune cells, as well as PD-1 expressing leukemic cells. All these PD-1+ cells will be sensitive to elimination by antibody-dependent cell-mediated cytotoxicity (ADCC) mediated by FcγR+ immune cells, e.g., NK cells, monocytes/macrophages, provided that the antibody is of an IgG1 or IgG3 isotype. This includes elimination of regulatory cells (Treg, MDSC) as well as conventional immune effector cells overexpressing PD-1 (activated T cells, NK cells, B cells, macrophages) at levels sufficient to be recognized by the antibody. Thus, the PD-1 Abs have a profound dual impact on the entire immunoregulatory system, on the one hand blocking negative PD-L1 signaling, and on the other hand targeting activated PD-1+ immune cells for immune destruction (Figure 2).

The end result of targeting PD-1 is likely to depend on the strength and persistence of environmental signals specifying the receptor expression and its functions. It is also possible that differences in levels of PD-1 expression between various immune cell subsets determine the cell sensitivity or resistance to checkpoint blockade. For example, Treg or MDSC, which overexpress PD-1 in the leukemic milieu could be especially sensitive to immune blockade as well as immune elimination, thus being very effectively removed or prevented from exerting immune suppression. Also, in the tumor microenvironment, overexpression of PDL-1 on tumor cells is used as a mechanism of tumor escape. Tumor infiltrating lymphocytes accumulating in the tumor microenvironment and overexpressing PD-1 lymphocytes are inhibited from mediating anti-tumor responses. PD-1 Ab blockade prevents tumor escape. In aggregate, the mechanisms through which a checkpoint antibody blockade exercises its immunorestorative effects appear to be complex and understanding of the molecular and cellular interactions involved in this process will require further examination.

The possibility that checkpoint blockade of tumor-induced immune suppression could make the delivery of other immunotherapies more effective has been also considered. In AML, combinations of PD-1 checkpoint inhibition with other immune-mediated therapies are under investigation. For example, the CD33/CD3-bispecific BITE antibody, AMG 330, is designed to redirect and activate T-cells to AML blasts, and it shows activity against AML cell lines and primary AML cells.[78] In primary AML samples cultured ex-vivo, PD-1 was upregulated on activated T-cells upon addition of AMG 330, and PD-L1 expression was increased in 16/19 of the primary AML cell cultures.[79] Antibody-dependent cytotoxicity (ADCC) of AML cells mediated by T cells in the presence of AMG 330 was significantly enhanced by PD-1/PD-L1 Ab blockade (75% vs 44% without PD-1 blockade).[79] This study emphasizes the therapeutic potential of combining anti-leukemia Ab therapy with checkpoint blockade. It can be safely predicted that up-regulation of the PD-1/PD-L1 pathway in leukemia would impair responses to vaccines and that PD-1/PD-L1 blockade may enhance effective immune response to vaccination. Specifically, several approaches to dendritic cell-based vaccination of patients with hematologic malignancies have been

recently evaluated in preclinical studies and early clinical trials [80] with promising results. It is likely that a combination of anti-leukemia vaccines with checkpoint inhibitors will improve and sustain immune responses generated by such vaccines. A clinical trial investigating a dendritic cell vaccine fused to autologous AML cells combined with PD-1 Ab blockade is currently underway in patients in complete remission. (NCT01096602)

5. Additional immune checkpoints in leukemia: LAG-3 and TIM-3

While PD-1 and CTLA-4 are the best understood checkpoint receptors with the most clinically advanced inhibitors, many additional immune checkpoints exist with functions that are non-redundant to PD-1 or CTLA-4. The notion that engagement of more than one checkpoint inhibitor or a series of checkpoint inhibitors might induce superior therapeutic responses has been introduced, based on the evidence for co-expression and synergy of inhibitory receptors on activated T cells[81]. This has already led to the introduction of combination therapies with, e.g., ipilimumab and nivolumab in patients with advanced melanoma[82]. In AML, co-expression of several inhibitory receptors on T cells has been evaluated in mouse models [41].

The lymphocyte activation gene-3 (LAG-3) is a homolog of CD4[83] that is expressed on subsets of T cells, NK cells, and B cells.[84] Dual blockade of LAG-3 with PD-1 was first demonstrated to enhance anti-tumor responses in the setting of ovarian cancer.[85] Since then, LAG-3 inhibition has been found to improve the effector function of adoptive immunotherapy in a murine model of leukemia.[86] A particular challenge of cancer immunotherapy is posed by a need to break tolerance to TAA that represent self or modified self. This challenge is recapitulated in the Abl:Gag mice that express the Gag protein on normal hepatocytes; the Gag protein is also a tumor-associated antigen expressed by Friend virus-induced erythroleukemia (FBL). In Abl:Gag mice with murine FBL leukemia, dual blockade of PD-1 and CTLA-4 extended the life span of Gag-specific CD8+ CTLs.[86] While additional blockade of LAG-3 did little to increase the persistence of the tumor-specific CD8+ T cells, it dramatically enhanced lytic activity of adoptively transferred CTLs and improved survival of mice compared to dual blockade of PD-1 and CTLA alone. Interestingly, triple-blockade of PD-1, CTLA, and LAG-3 in mice with FBL alone, even without adoptive transfer of Gag-specific CTLs, markedly increased survival compared to isotype controls.[86] In sum, these results reveal an important role for LAG-3 in modulating T cell effector function against leukemia and suggest that blockade of multiple checkpoint pathways may offer enhanced therapeutic potential.

Dysfunctional T-cells present at tumor sites in human cancers are frequently characterized by sustained overexpression of PD-1. T-cell immunoglobulin and mucin domain-contained protein 3 (TIM-3), another cell-surface molecule present on dysfunctional tumor-associated T cells, is commonly co-expressed with PD-1.[87, 88] In mice with solid tumors, TIM-3 and PD-1 co-expression was seen on the majority of tumor infiltrating lymphocytes, and these double-positive T cells were more dysfunctional than T cells expressing PD-1 alone.[89] Co-expression of TIM-3 and PD-1 on T cells also characterizes dysfunctional T-cell phenotype in mice with AML.[90] While treatment with a PD-L1 blocking Ab has led to a short period of improved leukemia control, TIM-3 inhibition did not reduce AML tumor burden. However,

dual blockade of TIM-3 and PD-L1 significantly reduced tumor burden and prolonged survival of mice with advanced AML.[90] Improved leukemia control seen with blockade of multiple immune checkpoints in preclinical models of leukemia implies cooperation and clearly offers greater potential for achieving remission. However, toxicities potentially associated with such dual or triple-checkpoint inhibition and its effectiveness in humans with leukemia remain to be determined.

Recent reports of cumulative expression of as many as five different inhibitory receptors (CTLA-4, PD-1, TIM-3, LAG-3 and BTLA) on CD8+ T cells infiltrating human solid tumors (A. Zippelius, personal communication) suggest that plans for further improvements in therapy with checkpoint inhibitors might require the use of carefully selected combinations of inhibitors. Sustained expression levels and cumulative co-expression of inhibitory receptors on effector T cells might vary in different cancers, might be time dependent and may or may not correlate with progressive T-cell dysfunction and disease stage. Should coordinate overexpression of inhibitory receptors prove to be a correlate of T-cell dysfunction and disease progression, their role as biomarkers of response to therapy could be considered. Thus, overexpression of multiple inhibitory receptors on T cells could be taken as an indication that immunosuppressed patients with advanced malignancies will be unlikely to respond to monotherapy and will require therapy with combinations of checkpoint inhibitors. Further efforts are now in progress to evaluate therapeutic efficacy and prognostic importance of multiple inhibitory receptor blockade. There is a good reason to expect, and some preclinical evidence to support the expectation, that the same considerations apply to hematological malignancies, and that checkpoint blockade of multiple inhibitory receptors may augment the anti-leukemic responses.

6. Potential Efficacy of PD-1 blockade in other hematologic malignancies

In AML, a considerable volume of pre-clinical data, including studies of PD-1 blockade in leukemic mice, sensitivity of AML to immunotherapy despite of the existing immunosuppressive microenvironment and expression of PD-1 and/or PD-L1 on leukemic blasts, suggest that clinical PD-1 blockade is a promising therapeutic option. Given the acceptable tolerability, strong pre-clinical rationale, and immunological activity of PD-1/PD-L1 blockade, several clinical trials of anti-PD1 mAbs have been either completed or are underway in patients with a variety of hematological diseases (see Table 1 for selected studies). These studies explore PD-1 blockade as a single agent at various time points of disease progression and in combination with other immunomodulatory agents. As these studies mature, much anticipated data on the most effective antibody dose, a full toxicity profile, effects on the immune system and the potential as biomarkers for response to therapy in hematological malignancies should become available and facilitate a more rapid progress in translating this therapeutic strategy to AML.

7. Conclusion

In cancer immunotherapy, finding a reliable and effective means for unleashing the immune system from tumor-induced suppression has been a difficult and elusive objective. Many strategies to mobilize dysfunctional tumor-associated T cells to fight cancer have been

attempted, including activation of cellular pathways with various pharmacologic or biologic agents, TAA-specific vaccinations, adoptive immune cell transfers or elimination of regulatory cells responsible for immune suppression.[91] Therefore, the realization that antibody blockade of immune checkpoints leads to at least a partial restoration of immune competence accompanied by anti-tumor effects created great expectations. Rapidly progressing *in vivo* studies in animal tumor models of cancer as well as *in vitro* studies with human immune cells showed that checkpoint blockade was effective in restoring anti-tumor immunity and arresting tumor growth. More recently, results of monotherapy clinical trials with ipilimumab or nivolumab confirmed durable clinical benefits of therapy for some patients with advanced solid cancers. At the same time, research has uncovered a complex interplay of immune regulatory molecules (co-inhibitory and co-stimulatory) that govern T-cell activation via multiple pairs of receptors/ligands broadly co-expressed on immune and tissue cells. Overexpression of inhibitory checkpoint receptors, such as CTLA-4 or PD-1, and their synergistic signaling in immune cells responding to cancer provided a rationale for antibody therapy targeting these receptors. Simultaneously, overexpression of the ligands on tumor cells explained dysfunction of the receptor-positive T cells in the TME. Blockade of either PD-1 or PDL1 is expected to effectively remove inhibitory signals hampering T cells and restore their anti-tumor activity.

However, the ever-present biological complexity may require additional mechanistic insights into the PD-1/PD-L1 pathway as it operates in AML before it can be therapeutically harnessed. In fact, checkpoint blockade in AML serves to illustrate multifactorial possibilities of this form of immunotherapy. In AML, sustained overexpression of PD-1, PD-L1, and PD-L2 on leukemic blasts and also on various activated immune cells in the bone marrow and in the periphery facilitates Ab checkpoint blockade. Such broadly-induced overexpression of the PD-1/PD-L1 pathway components together with co-expression of LAG-3, TIM-3 and potentially other inhibitory receptors presents an opportunity for more effective targeting and simultaneous engagement of several distinct mechanisms of disease control. These may include ADCC mediated by NK cells and/or monocytes, direct killing of blasts by re-activated LAA-specific CTL, simultaneous activation of Th1 effector T cells and dendritic cell for more effective antigen presentation and silencing of suppression mediated by Treg and/or MDSC. Synergy between all these mechanisms contributes a strong anti-leukemia environment. Also, chemotherapy-induced up-regulation of PD-1 on immune cells favors the implementation of checkpoint blockade following conventional leukemia therapies. While it is not yet clear that PD-1 is a dominant inhibitory receptor in AML, current research examining phenotypic and functional involvement of different inhibitory receptors is expected to provide the roadmap for a rational design of combinatorial check point blockade in patients with AML.

8. Expert opinion

Immunotherapy of AML emerges as a novel and potentially effective treatment option due to the rapid evolution of checkpoint blockade strategies. Based on pre-clinical data, it is expected but not yet proven that PD-1/PD-L1 blockade will eliminate immune suppression in AML. It is expected but not yet proven that PD-1/PD-L1 blockade in AML will show efficacy in inhibiting negative signaling in immune cells and, at the same time, induce death

of leukemic blasts, providing immunogenic TAA that could serve to generate long-term anti-leukemia immunity. In addition, the ability to monitor PD-1 and PD-L1 expression levels or their co-expression with other checkpoint molecules on immune cell subsets in the tumor microenvironment and the circulation during therapy will likely provide a series of biomarkers predictive of response to checkpoint inhibition. Yet another potential benefit might emerge from future combinations of checkpoint inhibition with other form of anti-leukemia immunotherapy, all based on the proof of principle that release from tumor suppression allows for effective responses to immune interventions. These objectives of checkpoint blockade in AML appear to be achievable in the near future. Clinical trials with nivolumab or pembrolizumab in patients with various hematological diseases are currently ongoing, providing preliminary evidence of tolerable toxicity and promising efficacy. This is a good basis for up-coming clinical trials with checkpoint inhibitors in AML patients. There is much optimism associated with this therapy, because of emerging conviction that a relief from immune suppression is necessary for restoration of anti-leukemia functions in immune cells, for up-regulating anti-leukemia responses and ultimately for achieving complete remission. The future of therapy with checkpoint inhibitors for AML and other hematological malignancies is likely to depend on a skillful combination of two or more antibodies to inhibitory receptors or ligands. In addition, combinations of checkpoint inhibition with conventional therapies and pharmacologic inhibitors designed to optimize or increase immune stimulatory as well as anti-leukemia effects will be evaluated for improved efficacy with reduced toxicity. The future selection of combinatorial therapies for AML will be based upon further investigations of the inhibitory receptors co-expression, cooperation and predictive significance in longitudinally monitored AML patients treated with checkpoint inhibitors. Given the anticipated increased response rates to therapy with checkpoint inhibitors in patients with AML, it will be possible to establish reliable correlations between immune and clinical responses, facilitating the development of biomarkers of response and outcome. Such biomarkers will allow for the selection of patients most likely to respond to checkpoint immunotherapy. Overall, the availability and further development of this new immune therapy has the potential of changing the future clinical practice and improving treatment options available for patients with AML.

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Article highlights

- PD-1, LAG-3, TIM-3 and possibly other inhibitory receptors are broadly co-expressed on various immune cells in the leukemic milieu
- PD-L1 and potentially other ligands of inhibitory receptors are overexpressed in leukemic blasts
- Mechanism responsible for recovery of anti-leukemia immune competence following checkpoint blockade involve ADCC, restored CTL and Th1 responses and reduced suppression by Treg or MDSC
- Sustained co-expression of multiple inhibitory receptors on T cells in progressive disease offers an opportunity for simultaneous targeting of these receptors with antibody combinations
- Chemotherapy up-regulates expression of checkpoint inhibitory receptors on T cells suggesting potential usefulness of checkpoint inhibitors as a follow-up therapy in leukemia
- Overexpression of inhibitory receptors on T cells in advanced leukemia stages might serve as a biomarker for poor response to checkpoint inhibition and for outcome

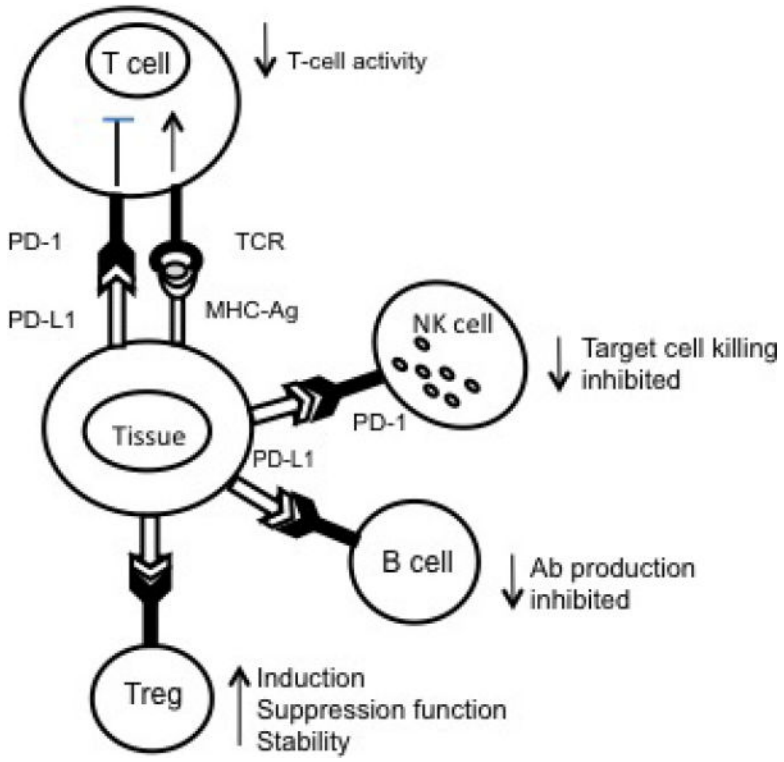

Figure 1.

A diagram demonstrating interactions between PD-1 receptor expressed on immune cells and PD-L1. A PDL-1 expressing antigen-presenting cell delivers a negative signal to the PD-1 receptor expressed on immune cells, which blocks their effector functions. In T-cells, Ag-specific responses are blocked. In NK cells, the ability to mediate cytotoxicity is decreased. In B cells, the Ab production is inhibited. In contrast, the same negative signal enhances the development and suppressor functions of Treg. Checkpoint receptor engagement directly blocks functions of immune cells and also has indirect suppressive effects mediated by Treg.

NK: Natural killer; PD-1: Programmed death-1; PDL-1: Programmed death ligand-1; TCR: T-cell receptor.

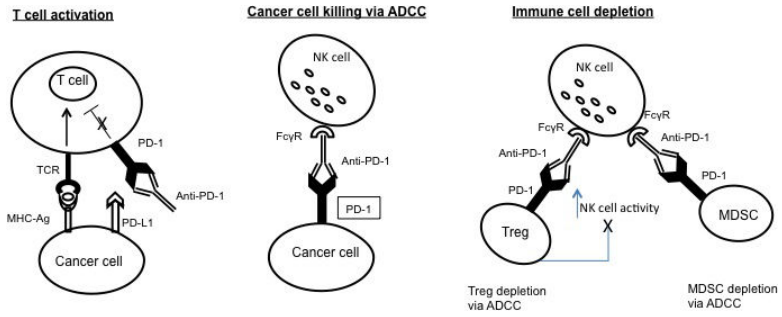

Figure 2.

A diagram demonstrating blocking by anti-PD-1 Ab of PD-1 receptor-PD-L1 interactions. In the presence of a PD-L1+ tumor, checkpoint blockade with anti-PD-1 Ab not only unleashes immune cell effector functions from inhibition but provides a mechanism for tumor cell destruction by ADCC. In the presence of anti-PD-1 Ab, which targets all PD-1+ cells (i.e., immune cells, including Treg and MDSC, tumor cells), activated FcgR+ NK cells and monocytes effectively mediate ADCC. Tumorassociated antigens released by dying tumor cells are presented to activated, unleashed T effector cells and, in the absence of Treg, which are partially or completely depleted by ADCC, and which are no longer expanded or stabilized by PD-L1 signaling, swift anti-tumor immune responses are generated. The illustrated ADCC mechanisms will be effective only if mediated by the Ab with IgG1 or IgG3 isotypes.

ADCC: Antibody-dependent cytotoxicity; MDSC: Myeloid-derived suppressor cell; NK: Natural killer; PD-1: Programmed death-1; PDL-1: Programmed death ligand-1; TCR: T-cell receptor.

Table 1

Ongoing Clinical Trials of PD-1 Checkpoint Blockade in Hematologic Malignancies.

Hematologic Disease	Phase	Disease Stage	PD-1/PD-L1 Inhibitor	Combination Therapy	Reference
Blood Cancer	Phase 1B	Relapsed/Refractory	Pembrolizumab	Single-agent	NCT01953692
Multiple Myeloma	Phase 1	Relapsed/Refractory	Pembrolizumab	Lenalidomide and dexamethasone	NCT02036502
CLL and Indolent NHL	Phase 2	Relapsed/Refractory	Pembrolizumab	Single-agent	NCT02332980
Multiple Myeloma	Phase 2	After HDT/ASCT	Pembrolizumab	Revlimid	NCT02331368
Mycosis Fungoides or Sezary Syndrome	Phase 2	Relapsed/Refractory	Pembrolizumab	Single-agent	NCT02243579
Multiple Myeloma	Phase 1/2	Relapsed/Refractory	Pembrolizumab	Pomalidomide and dexamethasone	NCT02289222
HD, NHL, or Multiple Myeloma	Phase 1	Relapsed/Refractory	Nivolumab	Ipilimumab or Lirilumab	NCT01592370
NHL	Phase 1/2	Relapsed/Refractory	Nivolumab	Urelumab	NCT02253992
AML	Randomized Phase 2	CR after chemotherapy	Nivolumab	Single-agent	NCT02275533
CML	Phase 1B	Progressing after ≥2 TKIs	Nivolumab	Dasatinib	NCT02011945
CLL, FL, DLBCL	Phase 1/2A	Relapsed/Refractory	Nivolumab	Ibrutinib	NCT02329847
HD	Phase 2	Relapsed/Refractory	Nivolumab	Single-agent	NCT02181738
FL	Phase 2	Relapsed/Refractory	Nivolumab	Single-agent	NCT02038946
DLBCL	Phase 2	Relapsed/Refractory	Nivolumab	Single-agent	NCT02038933
Multiple Myeloma	Phase 1/2	Relapsed/Refractory	Pidilizumab	Lenalidomide	NCT02077959
DLBCL, FL	Phase 1B	Relapsed/Refractory	MPDL-3280A	Obinuzutumab	NCT02220842
DLBCL, MCL	Phase 1B/2	Relapsed/Refractory	MEDI-0680	MEDI-551 (Anti-CD19 Antibody)	NCT02271945

Abbreviations: CLL, Chronic lymphomic leukemia; NHL, Non-Hodgkin's Lymphoma, AML, Acute myeloid Leukemia; CR, Complete remission; CML, Chronic myelogenous leukemia; HD, Hodgkin's lymphoma; TKI, Tyrosine kinase inhibitor; FL, Follicular lymphoma; DLBCL, Diffuse large B-cell lymphoma; MCL, Mantle cell lymphoma