EXPERT REVIEWS

The role of the immunosuppressive microenvironment in acute myeloid leukemia development and treatment

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Functional interplay between acute myeloid leukemia (AML) cells and the bone marrow microenvironment is a distinctive characteristic of this hematological cancer. Indeed, a large body of evidence suggests that proliferation, survival and drug resistance of AML are sustained and modulated by the bone marrow immunosuppressive microenvironment, where both innate and adaptive immune responses are profoundly deregulated. Furthermore, the presence of a number of different immunosuppressive mechanisms results in massive immune deregulation, which causes the eventual escape from natural immune control. Modulating the immune system, as documented by 40 years of stem cell transplantation, may improve survival of AML patients, as the immune system is clearly able to recognize and attack leukemic cells. The understanding of the factors responsible for the escape from immune destruction in AML, which becomes more prominent with disease progression, is necessary for the development of innovative immunotherapeutic treatment modalities in AML.

KEYWORDS: acute myeloid leukemia • immunotherapy • microenvironment • tolerogenic • tumor immunity

Acute myeloid leukemia (AML) is a clonal disease which is developmentally related to normal hematopoiesis and, similar to that, arises from a population of highly immature progenitors known as leukemic stem cells. Treatment for AML is intensive, with multiple cycles of cytosine arabinoside and anthracycline-containing combination chemotherapy regimens and the option of autologous or allogeneic stem cell transplantation for eligible patients. In response to chemotherapy, complete remission (CR) rates range from 60 to 85% in patients under 60 years of age. However, approximately 60% of patients will subsequently relapse and the 5-year overall survival (OS) is 40%. These results are worse in elderly patients, where OS falls to 10% due to the higher prevalence of unfavorable biological factors [1], such as poor risk cytogenetics.

In the attempt to improve the clinical outcome of AML patients, the identification of disease-specific alleles harbored by the malignant clone has triggered the development of molecular-based targeted therapies. Nonetheless, the efficacy of such approaches has proven to be limited in the long term, and far from being curative when employed as a single therapeutic agent.

Over the last few years, new biological insights have been provided supporting the notion that along with tumor cell autonomous defects, cell-extrinsic microenvironmental factors have a crucial role in leukemia development and maintenance [2,3]. In particular, inflammatory networks present in to the leukemia milieu appear to play a crucial role in tumor initiation and progression, as well as in the response to chemotherapy. Indeed, on the one hand, the hyperactivation of inflammatory networks has been indicated as a key contributor to tumor development. Also, the release of abundant inflammatory mediators within the tumor microenvironment, that is, during chemotherapy, has been shown to trigger pro-

inflammatory networks and enhance adaptive immune responses, promoting the presentation of tumor-associated antigens and attenuating tolerogenic pathways.

During the breakdown in cellular physiology that accompanies tumor development, tumor cells acquire some properties, which are defined through the interaction with the host environment (cell extrinsic). In particular, the immune system–tumor interaction plays a dual role in tumor development both by eliminating tumor cells and by facilitating tumor escape from immune control [4]. The genetic basis of this process, called cancer immunoediting, as well as its interplay with other aspects of malignant conversion, such as tumor cell proliferation and apoptosis, remain poorly understood. In this context, the immunological microenvironment seems to act as a fundamental background where cell-to-cell interactions and interplay may influence leukemia growth and response to chemotherapy.

The immunosuppressive microenvironment: novel pathways & targets

Similar to solid tumors [4], AML is capable of creating an immunosuppressive microenvironment, where both innate and adaptive immune responses are profoundly deregulated (FIGURE 1). Indeed, AML cells have been shown to reduce T and natural killer (NK) cell function and cytotoxicity [5–7] by altering the surface expression of relevant activating receptors and to induce/expand a population of immunosuppressive regulatory T cells (Treg) [8] through the expression of critical immunological checkpoint regulators [9]. Altogether, these results highlight the crucial role of AML cells in creating a defective anti-leukemic immune response, in the context of a tolerogenic microenvironment. Some recent reports have shed new light on the mechanisms underlying the induction of immunological tolerance by AML cells. In the next section, we will summarize these pathways, whose targeting may have therapeutic implications in AML management.

Small molecules catabolism: tryptophan & arginine

Tryptophan
Indoleamine 2,3-dioxygenase (IDO) is a key enzyme in the tryptophan metabolism that catalyzes the initial rate-limiting step of tryptophan degradation along the kynurenine pathway [10]. Tryptophan starvation by IDO consumption inhibits T-cell activation [10,11], while products of tryptophan catabolism, such as kynurenine derivatives and O_2 free radicals, regulate T-cell proliferation and survival [10–12]. So, IDO has immunosuppressive activity. A wide variety of human solid tumors have been demonstrated to express an active IDO protein: the transfection of IDO into tumor cells, in fact, prevents their rejection by pre-immunized hosts [13,14]. Several reports showed a clear correlation between IDO expression by cancer cells with reduced T-cell infiltration and poor prognosis [15,16]. As for hematological malignancies, AML cells may constitutively express IDO [17], which exerts its inhibitory effect on T-cell immunity by inducing *in vitro* the conversion of CD4+CD25−

into CD4+CD25+ Treg [18]. Such an inhibitory effect is not reversed by differentiating AML blasts into leukemic dendritic cells (DCs), which still express IDO and expand a fully functional population of Treg. Indeed, IDO expression can be regarded as a novel mechanism of leukemia escape from immune control. Interestingly, the expression of IDO by AML blasts has been correlated with poor clinical outcome in terms of a reduced response to chemotherapy, a higher frequency of relapse and a lower OS [19]. Therefore, IDO inhibition may represent a novel anti-leukemic therapeutic strategy. In preclinical studies, the IDO inhibitor 1-methyl-tryptophan reduced the tumor size of mice pre-immunized with a tumor antigen [13] and caused the regression of established murine breast cancers, when administered in combination with chemotherapeutic agents [20]. Recently, a novel IDO isoform, termed IDO2, was discovered. Like IDO1, IDO2 is expressed in tumors and tumor-draining lymph nodes [21]. Therefore, the pharmacological inhibition of IDO, in combination with chemotherapy or as a vaccine adjuvant, has become an appealing approach for the design of novel strategies of cancer immunotherapy [13,20–22].

The IDO inhibitor 1-methyl-tryptophan exists as two stereoisomers, 1-D-MT and 1-L-MT. Most preclinical studies have employed the racemic mixture 1-D/L-MT to inhibit IDO. Recent studies have shown that IDO1 is the preferential target of 1-L-MT, while 1-D-MT preferentially inhibits IDO2 [21–24]. An IDO inhibitor is also currently being investigated in patients with myelodysplastic syndromes, which often progress into overt AML [25]. However, despite the great interest generated from the possible inhibition of IDO, important challenges still remain regarding its clinical utility. So far, IDO inhibitors have been found to exert only minor effects on the enzymatic and cellular activities of IDO, and there are more questions than answers about the possible clinical impact of IDO inhibitors.

Arginine
Several human diseases and tumors, especially solid neoplasms, have been associated with the expansion of a myeloid cell population, known as myeloid-derived suppressor cells, which mediate immunological tolerance by acting as immunosuppressants. It is well established that arginase I expression in myeloid-derived suppressor cells is responsible for the suppressive activity of these cells [26,27]. Since AML blasts are proliferating myeloid-derived cells, it is conceivable to hypothesize that AML cells might act in a tolerogenic fashion through the expression of arginase. As a result, it has been recently demonstrated both *in vitro* and in mouse models that AML cells have an arginase-dependent ability to alter the leukemic immunological microenvironment [27]. In particular, AML blasts inhibit T-cell proliferation and modulate the polarization of surrounding bone marrow (BM) monocytes into a suppressive M2-like phenotype with tolerogenic properties [27]. Finally, this study showed that the immunosuppressive activity of AML blasts can be modulated through small-molecule inhibitors of arginase

and inducible nitric oxide synthase, suggesting a novel therapeutic target in AML [27].

Negative immunological checkpoint regulators: programmed death-1 & cytotoxic T-lymphocyte antigen 4

Recent reports have demonstrated that one of the mechanisms by which cancer cells evade immune-mediated control is by the critical regulation of the immunological checkpoints, such as programmed death-1 (PD-1) and cytotoxic T-lymphocyte antigen 4 (CTLA-4).

PD-1/programmed death-ligand 1 pathway

Physiologically, the engagement of PD-1 and CTLA-4 on activated T cells with its ligands, programmed death-ligand 1 (PD-L1; B7-H1) and CD80/86 (B7-1, B7-2), respectively, expressed on antigen-presenting cells and non-hematopoietic stromal cells, results in the maintenance of peripheral tolerance to self-antigens through the reduction of T-cell activation [28]. However, recent data indicate that tumor cells express PD-L1 and CTLA-4, which are capable of contrasting the efficient induction of antitumor T-cell responses. Indeed, the pharmacological inhibition of PD-1/PD-L1 ligation in several murine models results in an increased antitumor response and a reduced tumor growth [29–31]. Importantly, PD-L1 expression on tumor cells correlates with worse clinical outcome in various solid human malignancies. In the hematological field, PD-L1 expression was shown to be present also on AML cells [32]. Interestingly, PD-L1 expression was correlated with AML progression, independent of other biological prognostic factors [9]. Similar to the results reported for solid tumors, experiments conducted in a murine model of AML indicated that the PD-1/PD-L1 pathway promotes immune escape, thus resulting in AML progression. These data support a rationale for clinical trials examining anti-PD1 antibodies. Recently, target immunotherapy using PD-1 and PD-L1 monoclonal antibodies showed significant efficacy by inducing durable tumor regression and prolonged disease stabilization in patients with advanced solid tumors, including non-small cell lung cancer, a cancer considered to be non-responsive to immunotherapy [33–35]. Moreover, a Phase I study with CT-011, a humanized IgG1 monoclonal antibody that modulates the immune response through interaction with PD-1, demonstrated the safety of this compound in 17 patients with advanced hematological malignancies, comprising 8 AML patients. Even though a clinical benefit was observed in 33% of the patients with one CR, only 1/8 patients with AML experienced a minimal response, indicating that there is still a long way to go before finding the setting of AML patients who may benefit from this compound [36]. PD-1 blockade is currently being investigated in combination with a cancer vaccine in AML patients [37].

Cytotoxic T-lymphocyte antigen 4

Activated T cells and a subset of steady-state Treg express CTLA-4, whose ligation by CD80 and CD86 results in reduction of T-cell effector function by decreasing IL-2 transcription, T-cell proliferation and the contact between T cells and antigen presenting cells [38]. Consequently, CTLA-4 represents an interesting target in the attempt to elicit T-cell response. In mice, monoclonal antibodies against CTLA-4 can potentiate antitumor T-cell–based immune response, which results in prolonged tumor regression. Importantly, the anti–CTLA-4 blocking antibody, ipilimumab (Yervoy; Bristol-Myers Squibb, USA), was recently approved by the US FDA for the treatment of patients with metastatic melanoma, given the encouraging results emerging from the Phase III studies. Furthermore, ipilimumab was also proven to be effective in patients with small cell lung cancer [39,40].

The impact of CTLA-4 as a negative regulator in AML is still under investigation. Interestingly, the recently described single-nucleotide polymorphism CT60, located in the 3′-untranslated region of the *CTLA-4* gene, was investigated in a cohort of AML patients and found to be associated with a higher rate of leukemic relapse and a lower OS at 3 years. These data may indicate a correlation between *CTLA-4* polymorphisms and AML relapse [41]. The impact of ipilimumab in stimulating the graft-versus-malignancy effect was investigated in 29 patients with recurrent or progressive hematological malignancies after allogeneic hematopoietic cell transplant [42]. Only two patients with AML were treated with ipilimumab, and none of them responded to treatment. Responses were only seen in a minority of patients with B cell hematological malignancies. At present, few trials are evaluating the safety and efficacy of ipilimumab in AML patients either at relapse (NCT01757639) or after allogeneic stem cell transplantation [43,44]. In this latter setting, Fevery *et al.*, in recent times, showed in a mouse model of minor histocompatibility-mismatched BM transplantation that the blockade of CTLA-4 induces a host-derived anti-leukemic effect without graft-versus-host disease [45].

Cell-based non-leukemic mechanisms: mesenchymal stromal cells

Mesenchymal stromal cells (MSCs) are recognized as essential elements of both normal and leukemic hematopoietic microenvironment. They are multipotent cells, with an extensive self-renewal capacity, which can differentiate into several mesenchymal lineages [46,47]. MSCs substantially contribute to the creation of the hematopoietic stem cells niche and play a crucial role in the development and differentiation of the hematopoietic system [48]. In recent years, there has been an increasing interest in the biological characterization of MSCs in hematological malignancies [49,50,33]. In these diseases, MSCs show an altered expression of some cell adhesion molecules and cytokines, and have a reduced capacity to support hemopoiesis *in vitro* [51]. It remains unclear, however, whether MSCs are directly implicated in leukemogenesis or they are affected as a consequence of the presence of leukemic cells. Controversial data have been obtained on this issue. Recently, Blau *et al.* [52] showed that patients with myelodysplastic syndromes and AML, who have genetic abnormalities in their *in vitro* expanded MSCs, had a worse overall and disease-free survival than patients with MSCs with a normal karyotype, suggesting that genetic alterations in MSCs may represent a specific mechanism of leukemogenesis. This interpretation is

reinforced by the observation that bone progenitor dysfunction can induce myelodysplasia or leukemia in murine models [53]. However, other papers reported that myelodysplastic syndromes-derived MSCs, in spite of harboring chromosomal alterations, maintain normal functional properties [54]. BM stromal culture derived from AML patients show both chromosomal alterations in approximately 50% of the cases [55] and reduced capacity to support hemopoiesis [56]. On the contrary, CML-derived MSCs do not express *BCR-ABL* or the Philadelphia chromosome and seem to retain their normal biological properties [57,58].

In addition, MSCs have a unique immune-modulating capacity. This feature of MSCs can generate an immune-tolerant microenvironment where lymphocyte proliferation, cytokine production and other functions are affected [34,59–61]. The exact mechanisms of MSC-mediated immunosuppression are still debated; many different factors are believed to be involved. Among these, the pivotal role of IDO1 has been particularly emphasized. MSCs up-regulate IDO1 expression *in vitro* after exposure to inflammatory cytokines, and thus can inhibit T-cell proliferation and modulate the function of major cell population involved in innate and adaptive immune systems [62–65]. As previously mentioned, IDO has a role in the induction of immune tolerance in different settings, including acute leukemia [66]. Emerging evidence suggests that during cancer progression, the activation of IDO-dependent pathway might act as a preferred nodal modifier for immune escape.

Finally, a critical aspect in hematological malignancies is represented by the interaction between transformed cells and MSCs within the leukemic microenvironment during chemotherapy. Indeed, MSCs favor the survival of leukemic cells by protecting them from apoptosis. Cell-to-cell contact, as well as diffusible molecules, appears to contribute to MSC-dependent supportive effect that may play a role in the resistance of leukemic blasts to chemotherapy [67–70]. In addition, chemotherapy itself may affect the frequency and/or some MSC biological properties, altering MSC/leukemic cell dynamic interactions. Recently, it has been published that MSCs derived from pediatric acute lymphoblastic leukemia show different proliferative and differentiative capacities at diagnosis, during the course of the disease and after achievement of CR [71]. Thus, the ability of MSCs to preserve cancer cells, combined with IDO-mediated immunosuppressive capacity, potentially constitutes a 'side effect' of the presence of MSCs in leukemic microenvironment. However, it has recently been reported that the overexpression of IDO in MSCs abolishes the anti-apoptotic effect on leukemia cells [72]. The investigation of signaling pathways involved in MSC-supportive properties could be promising for the development of novel therapies able to convert MSC-dependent microenvironment from protective to hostile to leukemia cells.

AML exosomes & RNA trafficking

Intercellular communication within the BM microenvironment has been investigated. It is conventionally thought that BM cell subsets deliver signals by direct cell-to-cell contact or secreted factors. Very recently, some reports identified a novel pathway for cellular cross-talk, which relies on the exchange of extracellular vesicles carrying protein and RNA [73,74]. Such a process implies the cytoplasmic delivery of mRNA, miRNA or protein independently from transcriptional and translational controls. Vesicles ranging in size from 30–100 nm (exosomes) to 100–1000 nm (microvesicles) have been detected in the urine and plasma of patients with diverse malignancies [75]. A recent cutting-edge report [76] indicates that AML cells may release exosomes, which result in gene expression change, protein secretion and functional effects on bystander cells. AML vesicles contain diverse RNA species, including mRNAs and miRNAs relevant to AML biology and with broad biomarker potential [76]. Further studies are warranted to confirm these preliminary data. Indeed, an exhaustive elucidation of the effects generated from the release of exosomes on microenvironment bystander cells, especially immune cells, could help to develop novel therapeutic targets with pleiotropic effects on the immunosuppressive milieu.

Inflammatory mediators: the purinergic signaling

Nucleotides and their specific receptors are emerging as novel and important modulators of inflammation and immunity and, as such, are potential players in host–tumor interaction. ATP can be released from the infiltrating inflammatory cells as well as from the tumor cells via different mechanisms, such as exocytosis, plasma membrane channels or lysis. Once in the extracellular environment, ATP serves as a ligand for purinergic receptor (P2Rs). P2Rs are plasma membrane receptors divided into two sub-families, P2XR and P2YR, and are widely expressed by hematopoietic cells. Regardless of the release mechanism, ATP may affect the development of tumors, including leukemias, by acting on tumor-associated immune response or directly on tumor cells [77].

ATP effects on immune cells. Recent studies show that ATP released by the dying tumor cells induces antitumor immunity. In the extracellular space, ATP activates P2X7 receptors on the DCs, thereby stimulating the activation of the NLRP3/ASC/Casp-1 inflammasome, driving the secretion of IL-1β. IL-1β is required for the priming of IFN-γ–producing tumor antigen-specific CD8+ T cells [78]. Extracellular ATP released after cellular/tissue damage can directly activate CD4$^+$ T cells. Under this condition, CD4$^+$ T cells receive a 'danger signal' and are ready to exert an inflammatory response. In order to avoid hyper-inflammation, the increasing ATP concentration induces a shutdown of the inflammatory response by inducing the recruitment of Treg on the site and the death of activated CD4$^+$T cells [79]. Balance regulation between ATP stimulatory (Treg) and inhibitory (activated CD4$^+$ cells) activities through ATP release could represent a tumor-escape mechanism from the immune system surveillance.

ATP effects on leukemia cells. AML cells express several functional P2XRs and P2YRs, and are responsive to ATP stimulation. Among the receptors engaged by extracellular ATP, P2X7R is the most intriguing. P2X7R acts as a survival/growth-promoting receptor and its tonic stimulation by

endogenously released ATP generates a trophic stimulus [80]. The overexpression of P2X7R in cancer cells has been well documented in several cancer cell types, including chronic B lymphocytic leukemia, prostate cancer, neuroblastoma, thyroid papillary cancer, myelodysplastic syndromes, and acute and chronic myeloid leukemias. Chong *et al.* demonstrated the correlation between high level of P2X7 and an aggressive phenotype in pediatric leukemia [81].

However, P2X7 receptor was first regarded as a cytotoxic receptor due to its ability to form cytolytic pore leading to necrosis or apoptosis. Very interestingly, and in contrast to hematopoietic stem cells from healthy donors, pharmacological ATP doses inhibit cell proliferation and colony formation in AML cells and reduce AML cell engraftment in immunodeficient mice. ATP exerts its anti-proliferative activity on leukemic blasts by repressing the overexpression of P2X7R, thus counteracting its proliferative function. Furthermore, higher doses of ATP exert pro-apoptotic effect on leukemia cells *in vitro*, and this effect is directly correlated with the expression of P2X7 receptor [82].

These observations indicate that extracellular nucleotides may be key players in the leukemic BM microenvironment and, therefore, represent potential novel target for therapy. The clinical manipulation of purinergic signaling is at an early stage. Preclinical and clinical studies with purinergic drugs are underway only for the treatment of rheumatoid arthritis and other inflammatory conditions [83].

Immune tolerance versus tumor immunity: clinical strategies to harness the immune system against leukemia

Based on these premises, it appears crucial to counteract some of the above-mentioned tolerogenic mechanisms induced by AML cells, in the attempt to skew the host immune response against leukemia. To this end, a wide variety of approaches, which will be briefly summarized, have been recently proposed (FIGURE 2).

Anti-leukemia vaccination after lymphodepletion (Treg depletion)

Although major advances have been made in the field of antitumor vaccination, in spite of great preliminary expectations and enthusiasm, to date, with some exceptions, the clinical results have been largely disappointing [84]. Several factors for such dismal results have been implicated. Among them, tumor-induced suppressors, such as Treg, have proven to play a predominant role in hampering the efficacy of vaccine-induced, tumor-specific T cells [85]. To disrupt such tolerogenic pathways, the induction of hosts' lymphopenia by the administration of cytotoxic drugs, which is known to eliminate Treg, may profoundly impact the capacity of vaccines to effectively induce and expand tumor-specific T cells [86,87]. In this context, in AML, anti-leukemia vaccination has been tested after myeloablative chemotherapy by using a tumor cell–based vaccine formulation [88]. This approach represents the translation into clinical practice preclinical results obtained in the murine

model, where vaccination, performed after chemotherapy during the aplastic phase, was associated with the skewing of immune response toward tumor antigen, resulting in increased efficacy of tumor vaccination [89]. From bench to bedside, a leukemia-specific immune response was induced *in vivo* by immunizing AML patients with a leukemia cell–based vaccine, during the immunological recovery after myeloablative autologous stem cell transplant [88]. These data represent the background for expanding this approach to non-myeloablative chemotherapy regimens in AML patients.

Altering the balance toward immunity over tolerance by adoptive immunotherapy

Another approach is based upon strengthening the effector mechanisms of the immune response in a way to efficiently counteract the tolerogenic pathways. In particular, the graft-versus-leukemia effect observed after allogeneic hematopoietic stem cell transplantation in AML patients is the proof of principle that leukemic cells are susceptible to the cytotoxicity mediated by activated T cells, especially in the context of minimal residual disease. However, it has clearly been demonstrated that graft-versus-leukemia is not all about T cells. In fact, the NK cells have proven, especially in the setting of haploidentical SCT, to act as anti-leukemia effectors [90]. NK cells are able to recognize and kill transformed cell lines in an MHC-unrestricted fashion, and thus play a critical role in the innate immune response. Several studies demonstrated that NK cell function, which is distinct from the MHC-restricted cytolytic activity of T cells, may be relevant for the immune control of tumor development and growth [91]. Although NK cell killing is MHC-unrestricted, NK cells display a number of activating and inhibitory receptors that bind MHC molecules to modulate the immune response [92]. NK cell receptors that recognize antigens at the HLA-A, -B or -C loci are members of the immunoglobulin superfamily and are termed killer immunoglobulin receptors (KIRs) [93]. Engagement of these NK cell receptors results in stimulation or inhibition of NK cell effector function, which ultimately depends on the net effect of activating and inhibitory receptors. Clinically, data from haploidentical T-cell depleted transplantation suggest that KIR mismatch with tumor MHC may significantly impact on tumor cell killing, particularly in AML [94]. In particular, high-risk AML patients with a KIR-ligand mismatch in the graft-versus-host direction had a relapse rate of 0% compared to KIR-ligand matched patients who had a relapse rate of 75% [94,95]. Preclinical and clinical investigations demonstrated that haploidentical KIR-mismatched NK cells play the main role as anti-leukemia effector cells in this setting [95]. Also, haploidentical KIR-mismatched NK cells administered to AML patients as cell-based immunotherapy, outside the transplantation setting, have the potential to kill leukemia cells, resulting in the elimination of residual disease. Such an approach has been recently translated into clinics in a cohort of adult and pediatric AML patients with promising results [96,97]. Recently, our group from Bologna and Pesaro reported the results of a Phase I study

using haploidentical NK cells after immunosuppressive chemotherapy in elderly AML patients [98]. The results of this study showed that NK cell based immunotherapy is safe and effective in a setting of AML patients with high-risk disease, who are not eligible for standard therapy or BM transplantation [98].

Pharmacological approaches targeting the microenvironment: lenalidomide

Lenalidomide is an immunomodulatory drug active in several hematological disorders. Its mechanism of action remains in part unknown, although its activities in different diseases include activation of cellular innate immunity, enhancement of humoral antitumor immune response, inhibition of protein phosphatase 2A, induction of expression of the tumor suppressor SPARC, anti-angiogenesis and cytokine inhibition [99,100]. The mechanism of action of lenalidomide can be subdivided into a cancer cell-intrinsic, a stromal and an immunological component. Indeed, lenalidomide not only exerts direct cell cycle–arresting and pro-apoptotic effects on malignant cells, but also inhibits their interaction with the tumor microenvironment and mediates a robust immunostimulatory activity [99,100]. In particular, lenalidomide has been shown to stimulate the cytotoxic functions of T lymphocytes and NK cells, limit the immunosuppressive impact of Treg, and modulate the secretion of a wide range of cytokines, including tumor necrosis factor α, interferon γ, as well as IL-6, IL-10 and IL-12 [101,102]. Currently, lenalidomide is approved by the FDA for use in patients affected by multiple myeloma, or low or intermediate-1 risk myelodysplastic syndrome associated with the cytogenetic 5q abnormality and transfusion-dependent anemia.

In AML, the efficacy of lenalidomide was tested in a few studies both as a single agent, at high dose (50 mg/day), and in combination with other drugs, at lower doses (10–25 mg/day) [103–107].

Among the other studies, our group in Pesaro demonstrated the efficacy of the combo of low-dose oral lenalidomide 10 mg/day for 21 days and low-dose cytarabine in 33 very elderly (>70 years) AML patients [107]. The overall CR rate was 38%, with a median OS of responding patients significantly longer than that of non-responders (491 vs 64 days, p < 0.0001). Interestingly, by studying the global miRNA and gene expression profile, we identified a molecular signature, including 114 genes and 18 miRNAs associated with the clinical response (CR vs no CR). By linear discriminant analysis, we identified a minimal set of genes (five genes) still capable of discriminating the two groups. Of note, the involved genes belonged to relevant functional categories such as angiogenesis, cell cycle regulation and immune response. Based on the expression of five genes, we developed an algorithm to predict treatment response, successfully validated by showing an 87% overall accuracy [107].

Cancer immunogenic cell death

In the last few years, a large body of evidence has clearly demonstrated that cancer cell death has critical effects on the immune system. The classic notion that necrosis is immunogenic whereas apoptosis is not has been recently questioned. Indeed, it is now clear that during apoptosis, dying cells undergo a wide variety of biochemical and molecular events, resulting in modifications of local immune cell infiltration and in systemic effects on the immune response [108]. Recent data in solid tumors suggest that cancer cell death, induced by ionizing radiations and some chemotherapeutic agents (i.e., anthracyclines), is immunogenic. As a consequence, cancer cell death results in the efficient loading of tumor-associated antigens into antigen-presenting cells, especially DCs, which, in turn, actively initiate an antitumor T-cell–based immune response. The biology of chemotherapy-induced immunogenic cell death has been actively investigated, especially in solid tumors. At early stages, calreticulin translocates from the endoplasmic reticulum to the cell surface, initiates the apoptotic process via caspases and heat shock proteins (HSP90 and HSP70), binds tumor antigens and influences the maturation of DCs. At late stages, the pro-inflammatory factor high mobility group box 1 which targets toll-like receptor 4 on DCs, is released from the nucleus into the extracellular milieu. From bench to bedside, a specific polymorphism in human toll-like receptor 4, which results in decreased binding of high mobility group box 1, was associated, in melanoma and colorectal cancer patients, with shorter time to progression overt metastatic disease following a standard protocol including anthracyclines [109–111]. Only very few studies have been conducted in AML and, at present, the results are controversial [109,112]. In particular, the *in vitro* induction of immunogenic cell death pathways after chemotherapy treatment through calreticulin expression by leukemic blasts has not been clearly established [109]. Future studies are highly warranted to better elucidate the possible effect of chemotherapy in promoting a leukemia immunogenic cell death.

Expert commentary

Over the last years, great expectations have been raised by the intriguing hypothesis of switching-off leukemic cells by targeting relevant and crucial cell-intrinsic genetic alterations. Although technological development has made genomic investigation much easier, albeit not always reliable, a molecular-driven approach to leukemia therapy has proven limited, mainly due to the extraordinary genetic complexity of AML cell biology. If the deeper knowledge of the molecular mechanisms underlying leukemic transformation still represents a fundamental task for leukemia research, in particular for patients' risk stratification and prognosis definition, it is time to re-appraise a 'holistic' approach to leukemic development, which takes into account cell-intrinsic as well as cell-autonomous defects. Under this viewpoint, increasing interest is shown on the contribution made by microenvironmental factors in leukemia development and maintenance, as well as in disease progression and drug resistance, conditions in which both innate and adaptive immune responses are profoundly deregulated. The consequent evasion of AML blasts from immune destruction is indeed based on several mechanisms, including the degradation of metabolically important amino acids by key regulatory enzymes such as IDO, with the consequent expansion

of Treg; the production of immunosuppressive cytokines; and the expression on cancer cells of negative immunological checkpoint regulators, such as PD-1 and CTLA-4. Altogether, these mechanisms underline the crucial role of AML cells in creating a defective anti-leukemic immune response, in the context of a tolerogenic microenvironment. In other words, it is not all about clonal evolution in AML, but there is also a lot of room for the interplay between AML blasts, a tolerogenic tumor microenvironment and the immune system.

With this in mind, it is crucial to counteract the tolerogenic mechanisms induced by AML cells in the attempt to skew the host immune response against leukemia. In this review, we discussed quite a lot of different possible therapeutic modalities with which to target the tolerogenic mechanisms in AML. From blocking inhibitory pathways such as PD-1/PD-L1 or CTLA-4 to blocking functional enzymes such as IDO, from cancer vaccines to adoptive NK cell immunotherapy, there is a lot of room for immunotherapy in AML.

However, as predominant molecular mutations may change during disease progression, favoring one of the AML subclones insensitive to standard therapy, the same may happen during immunotherapy, in which the loss of expression of antigens such as IDO, PD-L1 or FoxP3 during vaccination therapy is a mechanism to favor again tolerogenic pathways. In this

Figure 1. Immunosuppressive mechanisms in acute myeloid leukemia. Leukemic cells can suppress immunity by contact-dependent and contact-independent mechanisms. AML is capable of creating an immunosuppressive microenvironment, where both innate and adaptive immune responses are profoundly deregulated. This immunosuppression is not only generated by LSC themselves but also by the expansion and attraction of regulatory immune cells, namely Treg, tolerogenic MSC and tolerogenic DCs. Expression of negative co-stimulatory ligands, increased levels of immunosuppressive cytokines and increased IDO expression are the other important mechanisms by which leukemia cells evade from immune surveillance.
AML: Acute myeloid leukemia; BM: Bone marrow; CTLA: Cytotoxic T-lymphocyte antigen; DC: Dendritic cells; IDO: Indoleamine 2,3-dioxygenase; LSC: Leukemic stem cells; MSC: Mesenchymal stem cells; NO: Nitric oxide; OB: Osteoblasts; PD: Programmed death; PD1: Programmed death 1; PD1-L: Programmed death 1-ligand.

view, even if the use of chemotherapy in combination with immunotherapy has been controversial in the past, there is emerging and convincing preclinical evidence that immune surveillance is reinstated by successful antitumor therapy. To this end, chemotherapy could have various ancillary effects on the immune system [113], and the combination of chemo- and immunotherapy, concomitant or sequential, may result in a domino effect which could eventually and finally target the leukemic stem cells also, which are commonly considered poorly sensitive to the effect of cytotoxic drugs, and open novel frontiers in the cure of AML.

Five-year view
Harnessing the immune system against cancer, including leukemia, has been exploited for a very long time. The great scientist, Paul Ehrlich, stated in 1897, 'If it is possible to protect small laboratory animals in an easy and safe way against infectious and highly aggressive neoplasms, then it will be possible to do the same for human patients'. However, although major strides have been made in the last few decades as for the
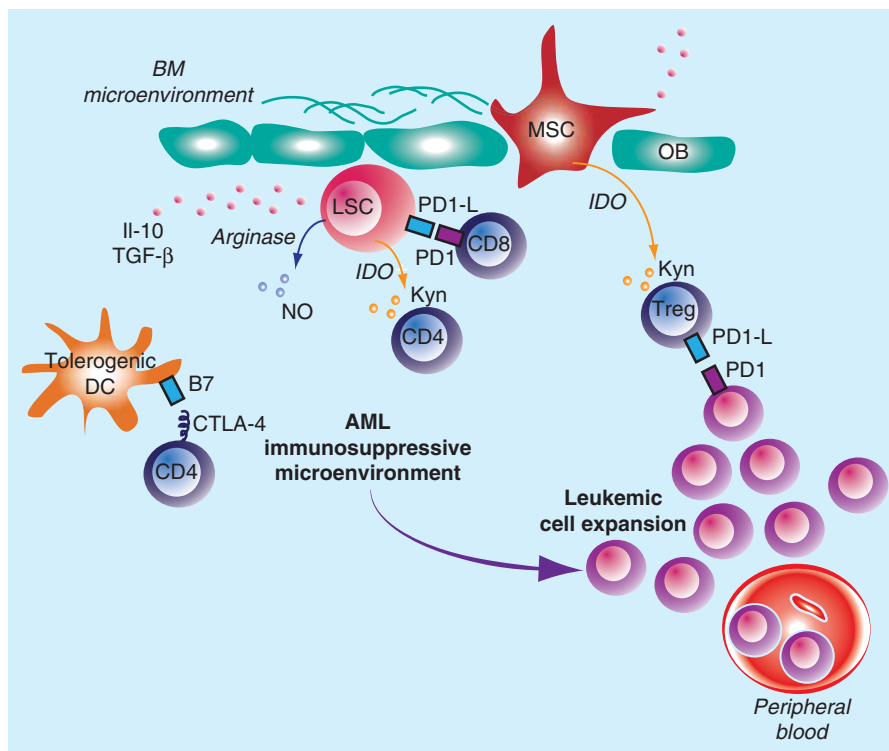
comprehension of the mechanisms involved in antitumor immunity, most of the immunological-based translational approaches resulted in dismal clinical results. As an example, although the identification of the role of DCs and their different subsets in the regulation of immune response represents an historical achievement of cellular immunology, which was celebrated by awarding the Nobel Prize to Prof. R. Steinman in 2011, the clinical results deriving from such DC-based antitumor vaccines have been largely disappointing [84]. The main reason for these results relies on the fact that in cancer patients, the efficacy of vaccine-induced, tumor-specific T cells is significantly hampered by the presence of tumor-induced suppressor pathways [85], whose disruption may profoundly impact the capacity of vaccines to effectively induce and expand tumor-specific T cells [86,87].

Over the last few years, great advances have been made in the deeper elucidation of the intercellular relationships and cross-talks within BM microenvironment upon leukemic breakdown. Furthermore, novel and important pathways of immunological escape by tumors have been recently established. In
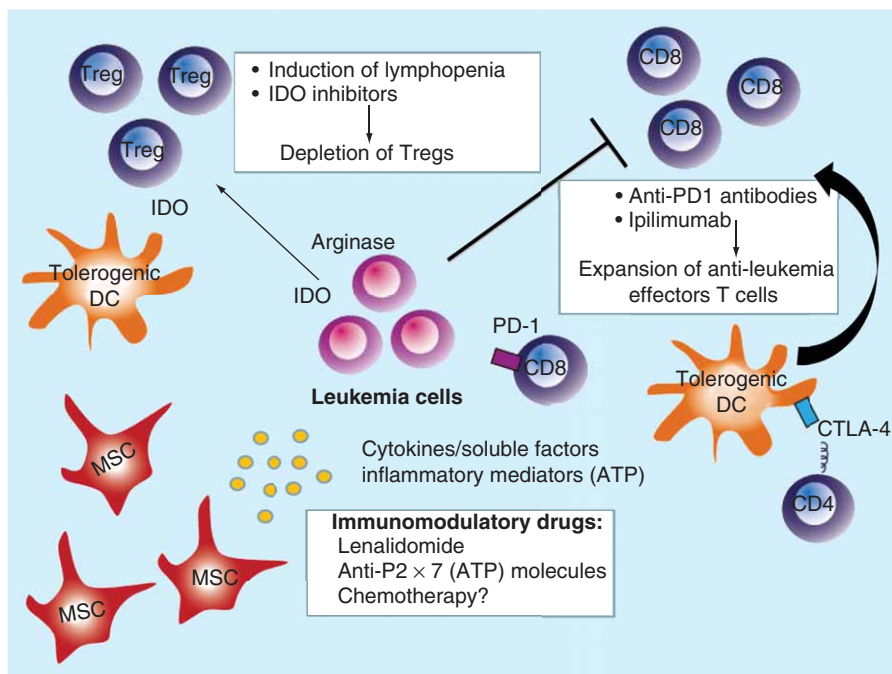
Figure 2. Strategies to overcome immunosuppressive mechanisms in acute leukemia (see details in the text).
CTLA: Cytotoxic T-lymphocyte antigen; DC: Dendritic cells; IDO: Indoleamine 2,3-dioxygenase; MSC: Mesenchymal stem cells.

particular, a better knowledge of the role of immunological checkpoint regulators, such as PD-1 and CTLA-4, in the induction of immunological tolerance against tumors, represents an important step forward in the definition of critical pathways for the manipulation of antitumor immune response. In that context, it is conceivable that in the next 5 years, further experimental approaches will increase our knowledge of those mechanisms, among which the contribution of the immune system is of great relevance. At the clinical level, the relevance of such findings is corroborated by the active investigation of novel and more efficacious compounds capable of disrupting these tolerogenic pathways and whose results are likely to provide important advances in the near future. However, at present, clinical trials reveal that these drugs targeting the immune response, such as ipilimumab or anti-PD1/anti-PD-L1 and with the only exception of lenalidomide (which has a pleiotropic effect), are at least not encouraging in AML, given the extenuating circumstance that they were used as a single agent in a patient population with an extremely poor prognosis. Our personal opinion is that a different scenario may come out in the next 5 years, in which these drugs will probably find their way in combination with standard chemotherapy or novel targeted drugs during induction/consolidation therapy, or as maintenance therapy in intermediate-risk patients, possibly after autologous stem cell transplantation. Finally, it should be mentioned that other co-inhibitory receptors, such as TIM-3 and LAG-3, are emerging as potential cancer targets. The combined targeting of negative regulatory receptors and their ligands will

be a possible, interesting strategy to further improve antitumor immunity.

Last but not least, alongside the translation into clinical practice of the more recent biological advances regarding the mechanisms of tumor escape, it is of great relevance for the expansion of the field toward novel and dramatically powerful therapeutic cellular products to be used as adoptive immunotherapy. In particular, the development of two distinct technologies has brought again to the limelight the killing abilities of T-lymphocytes. First, the availability of chimeric antigen receptor T cells re-directed to tumor (including leukemia) surface antigens provided, for the first time, a new population of T cells with potent activity against tumor cells and, more importantly, less influenced by tumor-induced suppressor mechanisms. The combination of adoptive immunotherapy with chimeric antigen receptors T cells and novel compounds inhibiting tolerogenic pathways is likely to significantly impact the management of AML patients. Second, the development of bispecific T-cell engager antibodies has emerged as a means to harness polyclonal cytotoxic T cells and cause highly efficient lysis of targeted tumor cells. The first compound, the CD19-directed bispecific T-cell engager antibody, blinatumomab, showed encouraging results when administered as a single agent in a population of patients with extremely poor prognosis acute lymphoblastic leukemia (refractory and relapsed). A first candidate for AML is the CD33/CD3 molecule, AMG 330, for which a number of up-to-date preclinical studies demonstrated high potency and efficacy in destroying CD33+ human AML cells.

Many questions remain to be addressed, but in the next 5 years it is expected that both chimeric antigen receptors and bispecific T-cell engager antibodies may be exciting new compounds that will be actively investigated in a disease for which the outcomes still remain unsatisfactory for too many patients.

Key issues

- Acute myeloid leukemia (AML) is a clonal disease, which is developmentally related to normal hematopoiesis and, similar to that, arises from a population of highly immature progenitors known as leukemic stem cells.
- Treatment for AML is capable of inducing a high rate of complete remission, but long-term survivors constitute less than 30% overall.
- In the attempt to improve the clinical outcome of AML patients, the identification of disease-specific alleles harbored by the malignant clone has triggered the development of molecular-based targeted therapies.
- Nonetheless, the efficacy of such approaches has proven limited in the long term and they are far from being curative when employed as a single therapeutic agent.
- Similar to solid tumors, AML is capable of creating an immunosuppressive microenvironment, where both innate and adaptive immune responses are profoundly deregulated.
- The presence of a number of different immunosuppressive mechanisms results in massive immune dysregulation, which causes the eventual escape from natural immune control.
- Harnessing the immune system against cancer, including leukemia, has been exploited for a very long time, as the immune system is clearly able to recognize and attack leukemic cells.
- The understanding of the factors responsible for the escape from immune destruction in AML, which becomes more prominent with disease progression, is necessary for the development of innovative immunotherapeutic treatment modalities in AML.
- Novel and important pathways of immunological escape by tumors have been recently established. In particular, a better knowledge of the role of immunological checkpoint regulators, such as programmed death-1 and cytotoxic T-lymphocyte antigen 4, in the induction of immunological tolerance against tumors, represents an important step forward in the definition of critical pathways for the manipulation of antitumor immune response.
- The availability of chimeric antigen receptor T cells re-directed to tumor, as well as of bispecific T-cell engager harnessing polyclonal cytotoxic T cells to kill targeted tumor cells adds to the immunological treatment armamentarium to beat AML.

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