Review

Renewing the immunological approach to AML treatment: from novel pathways to innovative therapies

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Citation: Alessandro Isidori, et al. Renewing the immunological approach to AML treatment: from novel pathways to innovative therapies. *Cancer Research Frontiers*. 2016 May; 2(2): 226-251. doi: 10.17980/2016.226

Competing Interests: the authors declare no competing interests.
Received Dec 29, 2015; Revised Mar 15, 2016; Accepted Mar 30, 2016. Published May 16, 2016

Abstract

Although in the last years major strides have been made in the understanding of the molecular basis of acute myeloid leukemia (AML), these relevant biological advances have had weak, if any, impact on the development of effective therapies for AML patients. Indeed, if the identification of molecular mutations within AML cell population has resulted in better risk-stratification, the vast majority of patients are treated with the same chemotherapy regimens and allogeneic stem cell transplant still represents the only curative option for intermediate and high-risk AML. In this context, increasing interest has gained the role that different cell components of the immune system may have for AML development and growth. In particular, a better knowledge of the mechanisms underlying the ability of AML cells of inducing immunological escape and systemic tolerance has been achieved. Based on these findings, the immunological way to the treatment of AML patients is becoming attractive and promising. The current review offers an overview of the tolerogenic mechanisms and pathways by focusing on those with potential clinical impact for the management of AML patients. Particularly, by moving from the biological significance of the underlying immunological pathways, we will discuss the clinical potential and application of a variety of different strategies, such as immunological checkpoint regulators, inhibitors of small molecules catabolism, i.e. indoleamine 2,3-dyoxigenase, anti-leukemia vaccines, adoptive immunotherapy with chimeric antigen receptor T cells and natural killer cells, monoclonal antibodies, including BiTEs engagers.

Keywords: acute myeloid leukemia, tumor immunity, immunotherapy, tolerance, monoclonal antibodies, vaccines, new drugs, cell therapy.

Introduction

Acute myeloid leukemia (AML) is a clonal disease characterized by the fast proliferation of immature myeloid cells in the bone marrow with an impaired differentiation program. Despite important progress in the therapy of AML and high rates of complete remission (CR) after induction chemotherapy, many patients will eventually relapse and die from the disease. At present, there is no established therapy for patients who relapse, except from allogeneic stem cell transplantation (allo-SCT). However, not every patient has a potential donor to proceed to allo-SCT, and this procedure has a substantial mortality, related to infections or graft-versus-host-disease. Moreover, a significant proportion of patients, ranging between 35 and 45%, could relapse even after SCT (1). Finally, patients who relapse have a worse performance status and could therefore be unfit for standard therapy (1). The prognosis is even worse in elderly patients, where overall survival at 1 year approximate 10%, due to the higher prevalence of unfavorable biological factors, such as poor risk cytogenetics [e.g: complex karyotype, abn(3q), t(11q23)] (2). Recently, the identification of disease-specific alleles harbored by the malignant clone has triggered the development of therapies targeting the molecular aberration, such as FLT3 inhibitors, DNMT 3A inhibitors, and few others, in order to improve the clinical outcome of AML patients. Nevertheless, the efficacy of these approaches has proven limited in the long-term, due to the clonal evolution of the disease leading to multiple molecular aberrations (3), and targeted molecular therapy is still not curative when employed as single therapeutic agent (4).

In the last years, a large body of evidence has been provided in support of the crucial role that the fine-tuned interplay between acute myeloid leukemia (AML) cells and the different cell components of the immune system may have for AML development and growth (5). In particular, a better knowledge of the mechanisms underlying the ability of AML cells of inducing immunological escape and systemic tolerance has been achieved (5). Such tolerogenic pathways, which create an immunosuppressive microenvironment, are being suggested both to critically hamper anti-leukemia immune response and to negatively impact on the anti-leukemia effects of conventional and experimental therapies. Some of these pathways are of particular relevance, since they have recently become the target for a new class of immunological drugs, i.e. checkpoint inhibitors, which specifically inhibit these mechanisms, thus resulting in increased anti-tumor immunity. Preliminarily, a brief overview of these tolerogenic mechanisms and pathways will be provided by focusing on those with potential clinical impact for the therapeutical management of AML patients.

1. Pathways and targets

The most relevant pathways are detailed in figure 1 and listed in table 1. Here, we will focus on the 5 pathways that are, in our opinion, the most important targets for an up-to date therapeutical intervention that can help to improve the clinical outcome for patients with AML.

1.1 Inhibitory surface molecules

a) PD-1/PD-1L axis: The T-cell receptor co-stimulatory pathways, such as B7-CD28, have important roles in regulating both T-cell activation and peripheral T-cell tolerance. Indeed, the interaction between programmed cell death protein 1 (PD-1) on T cells and its ligand, PD-L1 (B7-H1) on dendritic cells negatively regulates the proliferation and the cytokine production of T cells. The surface expression of PD-L1 on cancer cells inhibits cytotoxic lymphocytes due to elevated levels of PD1 on the surface of these T cells in several types of leukemia as well as in multiple myeloma. In AML, PD-L1 expression by AML blasts has been shown to protect AML cells from killing by cytotoxic T cells (6, 7). Moreover, PD-

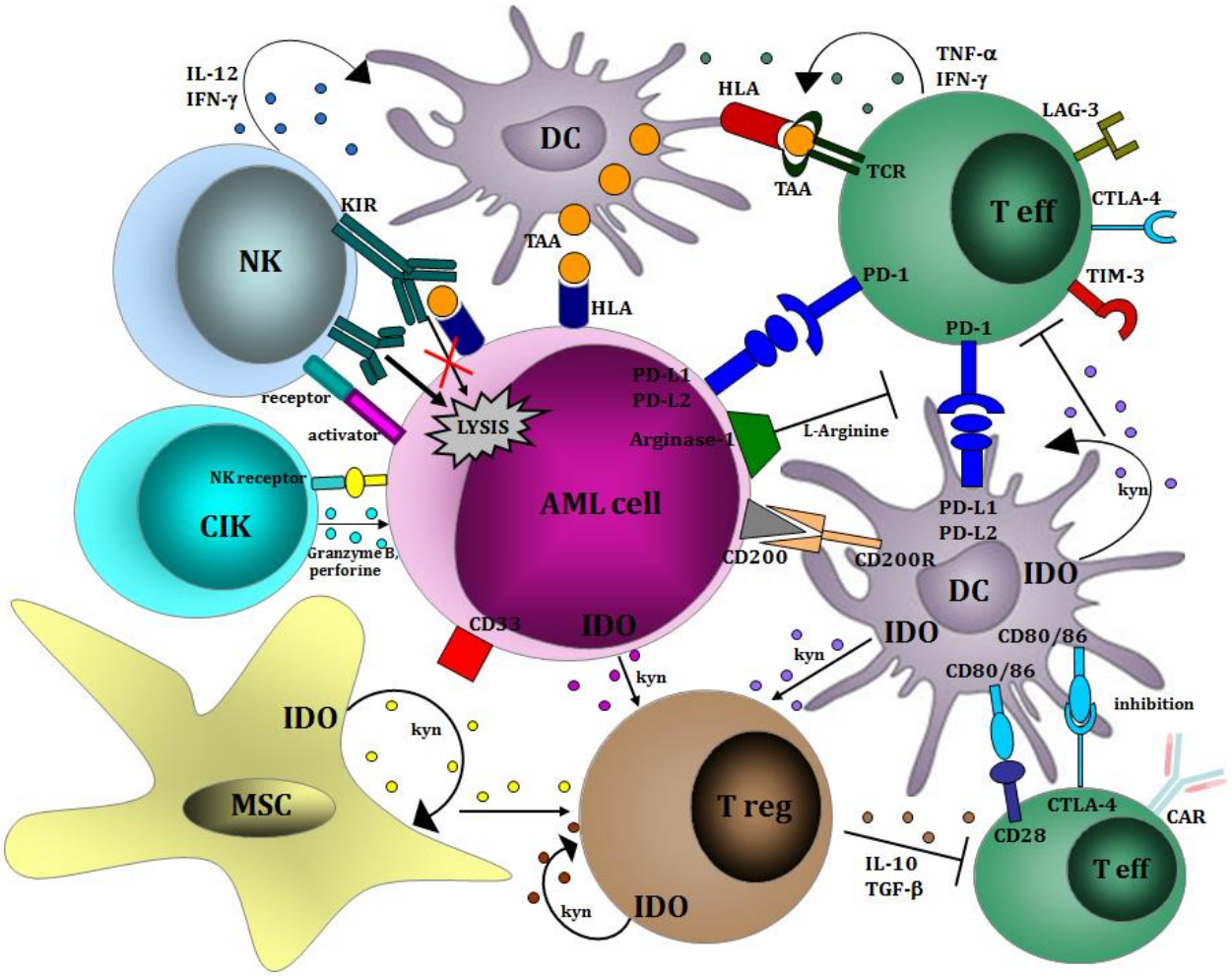

Figure 1. Relevant immunological pathways therapeutically targetable in AML.
Within leukemic microenvironment, AML cells interact with a variety of cells, such as T effectors, T regulatory cells, DCs, NK cells and mesenchymal stromal cells. AML is capable of creating a microenvironment, where both innate and adaptive immune responses are profoundly deregulated. The result of such complicated cellular network is the activation of the immune response or, alternatively, the suppression of anti-leukemia immunity. Major aim of the new therapies is to harness the immune system against AML both by implementing the cytotoxic effector pathways (CTLs, NK cells and CIKs) and/or by inhibiting the tolerogenic mechanisms, (T_{regs} and MSCs).

L1 expression was correlated with AML progression, independently from other biological prognostic factors (8). Similarly to solid tumors, the blockade of PD-1/PD-L1 axis results in increased anti-leukemia immune response and prevention of AML progression in experimental murine models (8).

b) Cytotoxic T-lymphocyte antigen-4 (CTLA-4): It is expressed on activated T cells and a subset of steady-state T_{regs}. Its ligation by CD80 and CD86 on antigen-presenting cells (APCs), such as DCs, results in decreased IL-2 production and consequent reduced T-cell proliferation (9). Inhibition of CTLA-4-driven pathway is a major task in the attempt to contrast one crucial tolerogenic mechanism, thus resulting in increased anti-tumor T-cell immune response. Indeed, monoclonal antibodies against CTLA-4

Table 1. Immunological pathways as target for immune therapies in acute myeloid leukemia.

Pathway		Therapeutical action	Effects	Refs
Type	*Mechanism*			
Surface molecules	**PD-1/PD-L1**	- mAb anti-PD-1 - mAb anti-PD-L1	- Increased T-cell cytotoxicity - Increased DC function as APCs	(6-8)
	CD33	- mAb anti-CD33	- AML cell lysis	(79-82)
	CTLA-4	- mAb anti-CTLA-4	- Increased T-cell cytotoxicity - Increased DC function as APCs	(9-14)
	CD200	- mAb anti-CD200	- Increased T/NK-cell cytotoxicity - Increased DC function as APCs	(15-19)
	KIR	- mAb anti-KIR	- AML cell lysis	(83-87)
Small molecules	**Arginine**	- human recombinant arginase	- Prevention of immune tolerance	(26, 27)
	IDO	- IDO1-inhibitor	- Prevention of immune tolerance	(20-25)
Cell subsets	**CIK cells**	- adoptive cell therapy	- AML cell lysis	(88-97)
	NK cells	- adoptive cell therapy	- AML cell lysis	(83-87)
	CAR-T cells	- adoptive cell therapy	- AML cell lysis	(100, 102-108, 124)
	TCR-edited T cells	- adoptive cell therapy	- AML cell lysis	(98, 99)
	T_{regs}	- lymphodepletion therapy	- Prevention of T-cell tolerance	(28-30)
Antigens	**TAAs (WT1, RHAMM)**	- immunotherapy-peptide vaccines	- Specific AML cell lysis	(48-56, 58-62, 64-67)

AML: acute myeloid leukemia; PD-1: programmed cell death 1; PD-L1: programmed cell death ligand 1; CTLA-4: Cytotoxic T-Lymphocyte Antigen 4; IDO: indoleamine 2,3-dioxygenase; NK: Natural killer; CIK: Cytokine-induced killer; DC: dendritic cell; CAR: chimeric antigen receptors; KIR: killer immunoglobulin receptor; TAAs: tumor associated antigens; WT-1: Wilm's tumor 1; RHAMM: hyaluronan-mediated motility receptor; mAb: monoclonal antibody; APC: antigen presenting cell.

can potentiate anti-tumor T-cell-based immune response in pre clinical models, resulting in prolonged tumor regression (10, 11) and, more importantly, the CTLA-4 antibody, ipilimumab, has proven efficacious also in the clinical setting, particularly in patients with metastatic melanoma and small-cell lung carcinoma (12, 13). In hematology, early-clinical trials targeting CTLA-4 in hematological malignancies, including AML, are underway (NCT010757639,

NCT00060372). Moreover, the rationale for exploiting anti-CTLA4 treatment in AML is provided by recent reports, which correlate a single-nucleotide polymorphism CT60, located in the 3'-untranslated region of the CTLA4 with a higher rate of leukemic relapse and lower overall survival at 3 years in AML patients (14).

c) CD200: CD200 is a protein belonging to the immunoglobulin superfamily, which has been associated with a poor prognosis in lymphoproliferative disorders and in acute leukemia. In AML, recent data indicate CD200 as a bad prognostic factor with additive negative impact over conventional unfavorable features, such as adverse cytogenetics and secondary disease (15, 16). Immunologically, the immunosuppressive ligands PD-L1 and CD200 have been shown to be linked in AML T-cell immunosuppression, thus suggesting the presence of a new immunotherapeutic synapse (17). In particular, CD200 expression has been associated with expansion of T_{regs} and with direct suppression of memory T-cell function (17, 18). Moreover, CD200 expression by AML cells has been shown to exert immunosuppressive *in vitro* immune activity by reducing NK cell cytolytic function (19). These findings indicate CD200 as a possible pathway implicated in immunosuppressive AML microenvironment with potential clinical impact on prognosis (17-19).

1.2 Microenvironmental small molecules: the modulation by AML cells of small molecules, such as essential amminoacids like tryptophan and arginine, have been recently proposed as a novel mechanism of immunological escape.

a) Indoleamine 2,3-dioxygenase (IDO): AML have been shown to express IDO, which is a key enzyme in the tryptophan metabolism that catalyzes the initial rate-limiting step of tryptophan degradation along the kynurenine pathway (20-22). In AML cells, IDO expression results in tolerance induction through the induction of T_{regs} via the conversion $CD4^+CD25^-$ (23). Such tolerogenic effect is not reverted by differentiating AML blasts into leukemic dendritic cells, which still express IDO and expand a fully functional population of T_{regs} (24). Clinically, the expression of IDO by AML blasts has been correlated with reduced response to chemotherapy and higher frequency of relapse in patients who achieve CR (25).

b) Arginase: AML blasts have been demonstrated to modulate the metabolism of arginine through an arginase-dependent mechanism (26). As a consequence, AML cells inhibit T-cell proliferation and modulate the polarization of BM monocytes toward tolerogenic monocytes with a suppressive M2-like phenotype. These data are in agreement with the well-established role of myeloid-derived suppressor cells (MDSCs), whose immunosuppressive activity is to be related to arginase I expression, among other mechanisms (27). Since AML blasts are proliferating myeloid-derived cells, it is conceivable to hypothesize that arginase-dependent modulation of arginine within BM microenvironmnent may represent a general mechanism that underlies the tolerogenic activity of myeloid-derived, including leukemic cells.

1.3 Tolerogenic cell subsets

a) T Regulatory cells (T_{regs}): in the last years, the characterization and function of suppressor T-cell subsets have been profoundly revisited. In particular, the role of T regulatory cells in cancer, including leukemia, ?has been well-established. Physiologically, T_{regs} have a pivotal role in maintaining peripheral immunological tolerance by preventing autoimmunity and chronic inflammation. They are classified in 2 major sub-types according to their ontology: naturally occurring T_{regs} (nT_{regs}) and induced Treg (iT_{regs}). The first group constitutes a subset of $CD4^+$ T cells expressing high-levels of surface interleukin-2 receptor α-chain CD25, cytotoxic T-lymphocyte antigen-4 (CTLA-4 or CD152), glucocorticoid-induced tumor necrosis factor receptor (GITR) and which are negative for the IL-7 receptor alpha-chain(CD127) expression.

This subset expresses a master transcription factor essential for T_{regs} activity, FoxP3 and is generated in thymus. Inducible T_{regs} originate as $CD4^+$ cells and acquire CD25 and FoxP3 expression following adequate antigenic stimulation in a specific tolerogenic microenvironment. In AML, several reports indicate that newly diagnosed AML patients harbor a higher frequency of T_{regs} (5, 28). Interestingly, myelodysplastic syndromes (MDS), which may be considered a prodromic phase toward AML, have been clearly associated with an increase in T_{reg} number alongside the evolution from early, pauciblastic to late, "leukemic" phase (29). Moreover, the persistence of high numbers of T_{regs} in AML patients after induction/consolidation chemotherapy significantly correlates with poor clinical outcome due to early relapse even when CR is achieved. We previously demonstrated that AML patients with higher expression of IDO (23), which is known to critically induce a de novo population of Foxp3+ T_{regs}, show a concomitant increased frequency of circulating T_{regs}. Such finding was not correlated with clinical outcome, but, similarly to solid tumors (30), may intriguingly suggest a pathogenetic role of T_{regs} induction during the process of AML initiation and subsequent development. To corroborate this hypothesis, *in vivo* mouse experiments have shown that depletion of T_{regs} has a critical impact on the induction of anti-leukemia CTLs and on AML progression (5, 8). These data clearly suggest that T_{regs} are involved in AML development and may critically affect the efficacy of anti-leukemia treatments.

b) Mesenchymal stromal cells (MSCs): MSCs are multipotent cells, with both an extensive self-renewal capacity and the capacity to differentiate into several mesenchymal lineages (31, 32). MSCs are founding component of normal hematopoietic stem cells (HSC) *niche,* where they crucially contribute to the development and differentiation of the hematopoietic system (33). In recent years, several reports indicate a pathogenetic role of MSCs in the development of hematological malignancies (34). As for AML, several mechanisms have been proposed, including an altered expression of cell adhesion molecules and cytokines, such as IL-6 and vascular endothelial growth factor (35, 36) as well as a reduced capacity to support hematopoiesis, which seems to be correlated with disease status, worse overall and disease free-survival (37, 38). In particular, *in vitro* expanded MSCs from MDS and AML patients are characterized by an abnormal genetic expression pattern, including genes involved in downstream signaling from Toll like receptors, NFκB signaling and CCL/CXCL chemokine release (36). Importantly, MSCs from AML patients have been shown to harbor leukemia-associated chromosomal and molecular aberrations, which differ from those observed in same patients' AML cell population (37), thus suggesting that genetic alterations in MSCs may represent a specific mechanism of leukemogenesis (39).

Besides their role as crucial component of BM stromal microenvironment, MSCs have been recently shown to exert a potent immunosuppressive function (40, 41). Indeed, MSCs have been demonstrated to create a tolerogenic microenvironment, which may favor leukemia development. In such process, many different factors are believed to be involved. Among these, MSCs up-regulate IDO1 expression 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 (42-45). Moreover, MSCs may favor the survival of leukemic cells by protecting them from chemotherapy-mediated apoptosis. Cell to cell contact as well as diffusible molecules contribute to MSC-dependent supportive effect that may play a role in the blast resistance to therapy (46, 47). Targeting MSCs is an interesting area for both molecular and immunological-oriented therapeutical strategies.

2. Strategies to harness the immune system against AML

Both adaptive, i.e. T-cell mediated, and innate immune response have been clearly demonstrated to orchestrate anti-leukemia immunity upon different clinical settings, including allogeneic stem cell transplantation (SCT). Indeed, although the impact of conditioning regimen on the clinical outcome of transplanted patients still represents an important issue, in the recent years the focus has been pointed to the activity of immune cells, which are infused alongside reconstituting hematopoietic stem cells. Such approach has led to consider SCT mostly as a means of cell-based adoptive immunotherapy and, more importantly, as the field where to provide the proof-of-concept that immune cells, NK cells, T-cells and their different subsets, such as γδ T cells, play a critical role in the eradication of minimal residual disease. Based on the results from SCT, new immunological approaches are being actively investigated outside the SCT setting. In this context, the poor prognosis of AML patients, especially if elderly, stresses the importance of novel therapies with high clinical compliance, such as those based on the modulation of immune response.

2.1 Anti-AML vaccines: a significant number of relevant leukemia-rejection and associated antigens have been identified in the last years (48). This has prompted several groups to develop a variety of clinical strategies to activate *in vivo* anti-AML immunity though vaccination.

2.1.1 Peptide vaccines: different antigenic epitopes have been used as target. Among these, the greatest experience in the field is represented by the use of the Wilms' tumor 1 gene (WT1) as antigenic target. WT1 is strongly overexpressed in the majority of patients with AML (49) and is highly immunogenic, since patients with WT1-expressing AML produce WT1-specific antibodies and CTLs (50). Based on pre-clinical evidence of the capacity of WT1-derived peptides of inducing anti-leukemia response (51, 52), several groups have reported on the role of WT1 as a tumor-antigen in the clinical setting of cancer immunotherapy by using different WT1-derived peptides, both MHC-class I- and MHC-class II-restricted (53-56). The results of these studies indicate the feasibility of vaccinating AML patients against WT1. Although most of the studies documented an increase in WT1-specific T cells, including CTLs, only few significant clinical responses were observed. In particular, in most cases a reduction of BM blasts and/or the achievement of a stable disease were observed. Other target antigens have been addresses. PR1 is a peptide derived by proteinase 3, a serine protease, which is overexpressed in myeloid neoplasms, including AML. Several *in vitro* studies have demonstrated the presence of PR1-specific CTLs in AML patients (57) Based on these preclinical findings, clinical studies have addressed the feasibility and, in some cases, a preliminary efficacy of PR1 peptide vaccination in AML (58). Some immunological responses were observed, i.e. WT1-specific CTL induction as measured as intracellular cytokine staining and WT1-specific tetramer staining, but clinical results were poor and in most cases not sustained over time. The receptor for hyaluronic-acid–mediated motility (RHAMM) is another potential leukemia-associated antigen used in peptide vaccination that has demonstrated positive immune responses in AML patients (59) Taken together, these studies support the notion that antigen-specific immune response may be elicited in AML patients through vaccination. However, similarly to other settings, such as the use of vaccine in solid tumors, the clinical impact of these strategies is low.

2.1.2 Other vaccine formulations: AML patients have been vaccinated with a tumor-cell based vaccine after myeloablative chemotherapy (60), where vaccination has been previously demonstrated to skew the immune response toward tumor antigens (61). Some clinical trials using synthetic peptides derived from tumor-associated antigen have been conducted in AML patients and demonstrated some benefits in

vaccinated patients, which were associated with the induction of peptide-specific cytotoxic T lymphocytes (62). Other approaches include the use of autologous normal DCs, generated from leukemia patients in CR and loaded with tumor antigens (63) and/or the differentiation of leukemia blasts into leukemic DCs (64, 65).

The latter one provides a promising tool, which in vitro has been shown to increase the immunogenicity of leukemic cells and to induce CTLs against leukemia. However, the clinical experience is far too limited and future studies are highly warranted to assess the role and the efficacy of active immunotherapy in the clinical management of minimal residual disease in AML.

2.2 Monoclonal antibodies:

Monoclonal antibodies were the first targeted agents that have been approved in recent years in order to revolutionize the care of cancer patients. An increasing number of different types and classes of monoclonal antibodies has proven their efficacy and has been approved for indications in several hematological malignancies, comprising AML. In 2000, gentuzumab ozogamicin (GO), a humanized anti-CD33 monoclonal antibody conjugated with calicheamicin, was granted accelerated approval by the US food and drug Administration (66). However, the promising results of the phase II study conducted in relapsed, older adults with AML (67) were not confirmed in newly diagnosed patients by the randomized study of the SWOG, and therefore in 2010 the drug was voluntary withdrawn from the company when toxicity seemed to be relevant and efficacy not significantly improved. Still, targeting CD33 has proven challenging, because more recently 4 different randomized studies were done and, as a whole, effectively encourage the use of GO in newly diagnosed AML patients, with a safe toxicity profile (68-71). The example of GO demonstrates how long and winding is the road to develop and successfully commercialize a monoclonal antibody in AML. This is mainly due to two distinct problem: 1) the targeting of an "ideal" antigen, defined by few, well-established characteristics, that could result in the elimination of the chemoresistant leukemic cells and 2) the extra-hematological toxicity of monoclonal antibodies.

Given the vicissitudes of GO, several other antibodies targeting the CD33 antigen were developed in the last 10 years (lintuzumab, 213Bi-HuMI95, AVE9633). However, up to now, the clinical results with this antibodies, used alone or in combination with cytarabine in relapsed AML patients, were extremely disappointing, mainly due to a lack of efficacy, and the "GO brothers" dramatically failed to beat their founder (72, 73).

SGN-CD33A is a novel antibody-drug conjugate. It consists of a humanized anti-CD33 antibody with engineered cysteines conjugated to a highly potent, synthetic DNA cross-linking pyrrolo-benzodiazepine dimer via a protease-cleavable linker. An interim analysis of a phase 1 dose escalation study of SGN-CD33A in patients with relapsed, CD33 positive, AML or those patients who declined intensive therapy, the CRc rate was 29%. Seventy seven percent of patients who received doses of 40 mcg/kg or higher had at least a 50% reduction in bone marrow blasts (74). At present, SGN-CD33A is being tested in a randomized, double-blind phase 3 study versus placebo, in combination with azacitidine or decitabine in the treatment of older patients with newly diagnosed AML.

A detailed discussion of the novel monoclonal antibodies in development in AML, targeting several, different antigen such as CD123, CD45, CD66 and others is not on the scope of this review. The list of the most relevant antibodies in pre-clinical or clinical development is listed in Table 2.

2.3 Bispecific T-cell engagers (BiTEs):

Bispecific T-cell engaging (BiTE) antibody constructs are a novel class of therapeutic antibodies, which have emerged as a means to

Table 2. Monoclonal antibodies in pre-clinical and clinical development in acute myeloid leukemia.

Target	Antibody	Pre-clinical activity / Clinical trials	References
CD33/CD33 BiTE	AMG 330	Against human AML cell lines and primary AML cells	(79-82)
CD44	H90	Against primary AML cell lines	(125, 126)
CD47	Various	No apoptosis detected of primary AML cell	(127)
CD96	Various	Against human AML cell lines	(128)
CLL-1	1075-7	Against human AML cell lines and primary AML cells	(129)
TIM-3	ATIK-2a	Against primary AML cell lines	(130)
CD300LF	IREM-1 MMRI 23	Against human AML cell lines and primary AML cells	(131)
CD45	^{131}I-BC8	Conditioning prior to ASCT in phase I-II trials	(132)
CD66	Various (not expressed or expressed at low level on AML blasts)	Radiolabelled-CD66 used as conditioning in phase I-II trials (cross-fire effect)	(133)
CD123	7G3	CR in 1/40 patients in a phase I trial	(134)
FLT3	IMC-EB10	Phase I trial terminated due to lack of efficacy	(135)
KIR	IPH2101	Safety shown in a phase I trial	(136)
VEGF	Bevacizumab	No efficacy when combined with CHT in untreated AML	(137)
CD52	Campath-1H	2/9 patiens achieved CR in I small trial	(138)

BiTE: Bispecific T-cell engagers; CLL-1: Human C-type lectin-like molecule-1; TIM-3: T-cell immunoglobulin and mucin-domain containing-3; FLT3: Fms-like tyrosine kinase 3; KIR: killer immunoglobulin receptor; VEGF: Vascular endothelial growth factor; ASCT: autologous stem cell transplantation; CR: complete remission.

harness polyclonal cytotoxic T-cells and cause highly efficient lysis of targeted tumor cells. BiTEs are made of two single-chain variable fragments, which target, at the same time, a tumor antigen on cancer cells and the invariant epsilon subunit of CD3 in the T-cell receptor complex (75). BiTE antibody constructs are able to effectively recruit polyclonal CD3+ T-cells in close proximity of target tumor cells irrespectively of their specificity (69). Nevertheless, these therapies might also elicit tumor cells to use immunosuppressive strategies to escape antibody-mediated tumor cell lysis (76), as the use of bispecific antibodies

basically leads to strong T-cell activation and, as a consequence, to the production of pro-inflammatory cytokines (76). The first compound, the CD19-directed BiTE antibody, blinatumomab, showed encouraging results in patients with extremely poor prognosis acute lymphoblastic leukemia (77, 78).

More recently, a bispecific CD33/CD3 BiTE molecule, AMG 330, showed high potency and efficacy in destroying CD33+ human AML cells in preclinical model. AMG 330 was shown to be very effective in recruiting and activating autologous T cells. Nevertheless, reduced T-cell activation and impaired tumor cell lysis was observed in some patient samples (79, 80). In vitro, CD33 was highest expressed in AML blasts of patients with NPM1 mutations and lowest in AML cells of patients with complex karyotype and t(8;21) translocations, indicating a particular benefit of patients with mutated NPM1 for CD33-targeted therapies. In this study, AMG 330 showed a strong cytotoxic activity against AML blasts, suggesting efficient therapeutic potential *in vivo* (76).

Phase 2 trials with blinatumomab have suggested that the tumor burden at the time of the therapy could affect the clinical activity of BiTE antibody in patients with poor-prognosis ALL (77, 78). However, the identification of specific biomarkers of response, in ALL as well as in AML, could help to overcome resistance to BiTE antibody constructs, through the development of combination therapies aimed to increase BiTE clinical activity. A recent study (81) found that the expression of T-cell ligands and signaling through T-cell co-stimulatory receptors, such as CD80, CD86 and CD28, or co-inhibitory receptors (PD-L1 and PD-L2), could modulate the cytolytic activity of AMG 330. By exploring the same field, Krupka and coworkers demonstrated a strong upregulation of PD-L1 on primary AML cells upon AMG 330 addition to *ex-vivo* cultures (83). Interestingly, the upregulation of PD-L1 was solely cytokine-driven, by interferon-gamma and tumor necrosis factor-alpha. Accordingly, the blockade of PD-1/PD-L1 axis allowed and increased lysis of AML blasts mediated by AMG 330, together with increased T-cell proliferation (82).

Taken together, these finding suggest that: 1) the expression profiles of one or more of T-cell ligands could serve as a biomarker of clinical response; 2) the strong T-cell activation led by BiTE triggers a pleiotropic secretion of pro-inflammatory cytokines that might be relevant for the activation of other mechanisms of immune-escape, thus favoring the development of mechanism of resistance to this therapy. As a consequence, it might be useful to develop therapeutic strategies using concomitantly AMG 330, or other BiTE antibody constructs, and checkpoint inhibitors or other drugs.

2.4 Cell-based therapies:

2.4.1 *NK cells:* the critical role of natural killer (NK) cells as key players in AML prevention and eradication has been clearly established, especially in the context of haploidentical SCT (83, 84). With that in mind, several groups have recently demonstrated the feasibility and, in some cases, a preliminary clinical efficacy of allogeneic NK cell infusion as a means of adoptive immunotherapy against AML outside the SCT setting. In a seminal study, Miller et al provided the proof-of-concept that an enriched NK cell population may be infused into AML patients with immunological and clinical response (85). More recently, highly-purified haploidentical NK cells have been infused into pediatric (86) and adult AML patients (87). These studies expanded the field and showed the potential impact of donor versus recipient KIR-L mismatch may have on the immunological and clinical activity, thus providing the rationale for exploiting NK-cell based alloreactivity as a novel platform for adoptive immunotherapy in AML.

2.4.2 Cytokine-induced killer (CIK) cells: CIK cells are a population of T lymphocytes enriched in $CD3^+CD56^+$ cells (NK-like T cells) (88), which

under optimal culture conditions can be expanded *in vitro* from human peripheral blood, bone marrow or cord blood mononuclear cells (89, 90) CIK cells have been shown to exert potent non-MHC-restricted anti-tumor activity both in preclinical and clinical models (91). In AML, various phase I studies have proven the safety and clinical applicability of CIK infusion (92-94). In most cases, the immunological responses are significant, but the clinical impact has been so far elusive. As a fact, the only clinical setting where CIK cells have shown some activity is their infusion after allogeneic SCT (92, 95), where they have shown very low GVH potential and some efficacy. In the attempt to potentiate the clinical efficacy of CIK infusion, several strategies are currently under active investigation, including the gene manipulation with chimeric antigen receptor against specific component of AML cell surface (96, 97).

2.4.3 Leukemia-specific TCR-transferred T cells: To specifically target a population of effector T cells against a leukemia-associated antigen, recent studies reported about the feasibility of transferring high-avidity T cell receptor (TCR) genes isolated from rare tumor-specific lymphocytes into polyclonal T cells (98). After cell culture expansion, TCR-edited cells have been shown to better recognize leukemia antigen as compared to donor-matched, unedited TCR-transferred cells. Moreover, TCR-gene transferred T cells did not mediate off-target reactivity, thus demonstrating improved biosafety profiles. A very recent paper supported in a xenografted leukemia mouse model the therapeutical utility of infusing both CD4+ and CD8+ TCR-gene transferred T cells. In particular, leukemia growth was remarkably inhibited by the combination of $CD4^+$ and $CD8^+$ TCR-edited T cells, thus correlating with improved survival and enhanced induction of memory T cells. These still preclinical data strongly suggest that this strategy may be clinically advantageous for the treatment of human leukemia (99).

2.4.4 CAR-T cells: to overcome tolerance to tumors that results from deficiencies in the T cell receptor repertoire, T cells are genetically modified to express chimeric antigen receptors (CARs) for a specific cell-surface antigen. The development of CARs is changing the applicability and the clinical success of cellular therapy. CARs are synthetic molecules resulting from the fusion, through a spacer region, of an extracellular antigen-binding domain (most often derived from variable heavy and light regions of a monoclonal antibody) and intracellular signaling domains, capable of activating T cells. Three generations of CARs were developed until now for clinical trials. The first-generation CARs, mainly used in patients with HIV, contained a single-signaling domain derived from the TCRζ chain or the FcR chain. Second- and third-generation CARs is the number of intracellular signaling domains (1 versus 2 or 3). Moreover, second- and third-generation CARs were developed with the aim of providing costimulation by incorporating inside the cytoplasmic domains one or two costimulatory motifs, such as CD28, 4-1BB, or OX40, therefore resulting in superior cytokine and proliferative responses against the tumor (100). Several groups have developed CAR T cells specific for the B cell antigen CD19, and have observed encouraging antitumor responses in phase I-II clinical trials, with acceptable side effect in a very high risk patient population (101). However, the choice of specific cell-surface antigen in AML is more demanding, as classic leukemia-associated antigens (LAAs) are also expressed in the normal myeloid cell compartment (102). A possible antigen to target with novel therapies in AML is CD123, the transmembrane α chain of the interleukin-3 receptor, which is over-expressed in AML compared with normal hematopoietic cells (103, 104). CD123 expression is mainly restricted to cells of the myeloid lineage, is absent in T cells and shows limited expression on hematopoietic stem cells. Monoclonal antibodies specific for CD123 have displayed favorable safety profiles in 2 phase I trials

(ClinicalTrials.gov ID#NCT00401739 and #NCT00397579), making CD123 an attractive target for CAR-mediated T-cell therapy. Recently, a myeloablative CAR-based therapy targeting CD123 (CART123) have shown, in a mouse model, a potent effector activity against cell-line and primary AML, evincing antigenic-specific proliferation, degranulation, cytoxicity and elaboration of multiple effector cytokines (105). Moreover, CART123 led to long-term survival of mice engrafted with AML cell line, or with primary AML (106). Furthermore, infusion of CART123 resulted in the establishment of a T-cell memory pool able to reject disease (105). Mardiros *et al* (104) developed and evaluated 2 CARs containing a CD123-specific single-chain variable fragment, in combination with a CD28 costimulatory domain and CD3ζ-signaling domain, targeting different epitopes on CD123. These second generation CD123 CAR T cells activated T-cell effector functions against poor-risk primary AML patient samples. Furthermore, CD123 CAR T cells were able to significantly decrease the growth of clonogenic myeloid leukemia precursors *in vitro*, without any toxicity on the formation of myeloid or erythroid colonies. Additionally, T cells obtained from patients with active AML and genetically modified to express CAR 123 were able to lyse autologous AML blasts *in vitro*. Finally, a single injection of CD123 CAR T cells exhibited a significant antileukemic activity *in vivo* against a xenogeneic model of disseminated AML (104). From bench to bedside, a trial with anti-CD123 CARs for relapsed or refractory AML patients was recently started and is actively recruiting patients (NCT02159495). Other AML antigens may also be potential target for CAR-expressing T cells. Folate receptor β is an interesting target on AML cells due to its upregulable expression by all-trans retinoic acid (ATRA) and histone deacetylase inhibitors. Importantly, ATRA did not impact FRβ expression in healthy HSCs or monocytes, suggesting that the combination with ATRA represent an opportunity for increasing the FRβ-specific CAR T cells efficacy with a low toxicity for healthy myeloid tissues. However, due to the high baseline expression of FRβ in myeloid/monocytic AML (M4 and M5), these patients may benefit the most from FRβ-directed CAR therapy (106). The CD33 differentiation antigen is also predominantly expressed on myeloid cells and immunotherapies targeting CD33 such as gentuzumab ozogamicin, AMG 330 and a CD33 CAR are currently used in clinical and preclinical setting. CD33 is expressed on a subset of T cells. As a consequence, the antileukemic activity of anti-CD33 targeting therapies results in slow recovery of hematopoiesis and citopenias and does not make it an ideal target for a CAR based therapy. A Chinese phase I clinical trial have studied the feasibility of anti-CD33 CAR in the treatment of relapsed or refractory AML. Only one patient was treated, and he showed a decrease in blast count for a short time with severe side effects as fever, cytokine release syndrome and pancytopenia (107). Recently, a small study tested the feasibility and the safety of CAR anti-LeY (dicofusylated carbohydrate antigen) therapy in patients with relapsed AML, in whom the blasts were shown to express LeY. The trasducted and expanded autologous CAR T cells, were successfully and safely infused in 4 patients with high-risk AML, showing tissue specific localization, long-term persistence and antileukemic efficacy (108).

2.4.4 Checkpoint inhibitors:

CTLA-4 and PD-1, provide crucial inhibitory signals that down-regulate T-cell function in the context of antigen recognition (109-111). Indeed, the pharmacological inhibition of PD-1/ PD-L1 axis in mice models of solid tumors resulted in an increased anti-tumor response and a reduced tumor growth (112, 113). Similarly 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 (8). From bench to bedside, Yang et al recently demonstrated that PD-1 and its two ligands, PD-L1 and PD-L2, as

well as CTLA4, are aberrantly upregulated in 8 to 34% of bone marrow CD34+ cells from patients with myeloid leukemias, with a trend towards increased expression also in myelodysplastic syndromes (114). Patients with lower expression of PD-L1 showed a trend towards better survival, that however was not statistically significant (31.5 months versus 16.2, p=0.24) (114). In addition, two relevant observation were reported: 1) PD-L1 expression was correlated with progression to AML in MDS patients, independently from other biological prognostic factors; 2) PD-L1, PD-L2, PD-1 and CTLA4 were induced by treatment with hypomethylating agents (HMA) in a concentration dependent manner, but not by treatment with cytosine-arabinoside (AraC) (114). Interestingly, another group recently reported that demethylation of the PD-1 promoter occurring during HMAs treatment correlated with a significantly worse overall response rate (8% vs. 60%, p = 0.014), and a trend towards a shorter overall survival (p = 0.11) in 27 MDS patients (115). This is probably due to the fact that the expression of PD-1 on activated T cells is regulated by DNA methylation (116), and PD-1 promoter demethylation correlates with an increase in PD-1 expression (116). Therefore, the activation of the PD-1 checkpoint during HMAs treatment can be a possible resistance mechanism to these drugs, and this information provide a strong rationale for combining therapy with checkpoint inhibitors with HMAs in both AML and MDS patients. Recently, clinical trials with PD-1 and PD-L1 inhibitors showed significant efficacy by inducing durable tumor regression and prolonged disease stabilization in patients with advanced solid tumors, and more recently also with hematological malignancies (117, 118). The first phase I study with CT-011, a humanized IgG1 PD-1 inhibitor, demonstrated the safety of this compound in 17 patients with advanced hematological malignancies, comprising 8 AML patients. Even if it was observed a global clinical benefit in 33% of the patients, only 1/8 patients with AML experienced a minimal response (118). Up to now, the groundbreaking results obtained in patients with Hodgkin lymphoma, with 87% of heavily patients receiving nivolumab, another PD-1 inhibitor, achieving a clinical response, are only a result of a wishful thinking for AML. However, several trials are now testing checkpoint blockade therapy in AML. Nivolumab is currently being investigated, in a randomized phase II study, as intravenous maintenance therapy every 2 weeks up to 46 courses in patients in CR after standard therapy aged 18 or older (except young, < 60 years AML patients in European LeukemiaNet favorable group) (NCT02275533). CT-011 is being tested in combination with a dendritic cell cancer vaccine in AML patients aged 18 or older in first or second CR (NCT01096602) to evaluate toxicity and disease free survival. Ipilimumab, involved in CTLA-4 blockade, is currently under investigation in 2 phase I/Ib studies (NCT01757639, NCT01822691) testing its intravenous efficacy for up to 8 courses in patients with resistant/relapsed AML, MDS or CMML. Finally, two compounds involved in PD-L1 blockade, namely MK-3475 and MEDI4736 are now being studied in high-risk MDS patients, both untreated or previously treated with HMAs (NCT01953692 and NCT02117219, respectively). In conclusion, there are a daunting number of possible avenues for future research on checkpoint blocking agents in AML. However, we probably have to think about expanding our knowledge on the expression of PD-1 and PD-L1 and PD-L2 in different AML subsets in order to select patients most likely to respond to this type of therapy.

2.4.5 Hypomethylating agents

Hypomethylating agents (HMAs), namely 5-azacytidine and decitabine, have both been investigated either alone or in combination in elderly patients with AML (2). The rationale for using these agents in AML relies on the demonstration of alterations of DNA methylation in AML, frequently resulting in the hypermethylation of different genes (2). Hypermethylation is involved in silencing the

promoter regions of tumor suppressor genes in AML. HMAs inhibit DNA methyltransferase inducing the hypomethylation of DNA, which results in a direct cytotoxic effect on leukemic cells and/or affects cellular differentiation and apoptosis (2).

Treatment with HMAs has proven survival benefit in selected groups of AML patients, but the mechanism of action is only partly understood. Recently, few papers investigated the possible indirect immunological effects of treatment with HMAs.

Goodyear et al (119) demonstrated the upregulated expression of melanoma-associated antigens (MAGE) in AML cell lines treated with azacitidine and sodium valproate. Subsequently, they measured CTL responses to MAGE antigens in 21 patients with AML before and after treatment with azacitidine and sodium valproate. Interestingly, they documented CTL responses to MAGE antigens in the peripheral blood of only 1 patient before treatment, but in 10/21 patients after treatment with azacitidine and sodium valproate (119). Moreover, they were able to find an association between major clinical response and CD8+ T cell response to MAGE antigens in 8/11 patients (72%). This is the first demonstration that up-regulation of epigenetically silenced tumor antigens may induce an immunologically mediated antitumor response, thus contributing to the clinical activity of HMAs (119).

The same research group headed by Charles Craddock (120) was able to demonstrate a CD8+ cytotoxic T cell response to several tumor antigens, such as MAGE A1, MAGE A3, BAGE-1, RAGE-1 and WT1 in AML patients receiving azacitidine after allogeneic stem cell transplant. Interestingly, only 2/15 patients who showed a CD8+ T cell to the tumor antigens studied did relapse at the time of publication (120). This could be due to an augmented GVL effect after transplantation generated by the up-regulation of target antigens expression. Moreover, the Authors demonstrated also an increase in T_{regs} number within few months from allogeneic transplantation in patients receiving post-transplant azacitidine (120). As a whole, azacitidine could thus increase in the one hand the GVL effect, by inducing up-regulation of target antigens, and in the other hand reduce the risk of acute and chronic GVHD by expanding circulating T_{regs} after transplant.

Discussion

The hypothesis of harnessing the immune system against cancer, including leukemia, has been postulated for very long time and several clinical attempts have been made in this field. In the last years and decades, a large body of evidence from the preclinical and biological ground has demonstrated that leukemia cells, including AML, are critically influenced by the immunological microenvironment, which clearly plays a role in leukemia growth and progression (4). Although these findings have certainly increased our knowledge about the mechanisms underlying the interplay between AML and immune cells, (4) the clinical hematology community has mostly failed in the attempt to translate basic immunology from bench to bedside. As a representative example, the results from DC-based anti-tumor vaccines have not confirmed the great expectations that experimental models had arisen, although the preclinical data were extremely promising (121).

Again, in AML as well in other diseases, the great recent advances in basic immunology are offering a new opportunity to translate the findings provided by preclinical scientists into eventually effective therapeutical strategies. In particular, a better knowledge of the mechanisms leading to immunological tolerance, as well as the identification of critical regulators, such as immunological checkpoints, are pacing the way for a fast-track development of a huge amount of novel drugs and therapeutic strategies. It is of crucial importance that clinicians, who are responsible for the clinical application of these innovative therapies, would approach the new generation of immunological strategies, known as immune-

oncology, by learning from past mistakes and taking into account the specificity of these drugs. Here, through a survey of the most relevant advances in the field, we attempt to start a discussion about the future of immune-oncohematology by focusing on AML as a paradigmatic disease model, where to investigate a novel immunological approach to therapy.

It is well known that very few strides have been made, in the last years, in terms of novel effective therapies against AML, and drug approval for this disease has mainly been a boulevard of broken dreams (122). At the same time, diagnostics, such as "omics" technology and basic science have provided a deep and comprehensive picture of the disease, in an era of inexpensive high-throughput sequencing. In particular, the demonstration of a clonal heterogeneity and evolution within the AML cell population, the role of cell-extrinsic factors deriving from bone marrow microenvironment, and a more-in-depth understanding of the molecular pathogenesis of the disease, represent great advances with the potential to subvert the conventional approach to AML therapy. As an example, given the clonal and sub-clonal architecture of AML cell population, the relevance of minimal residual disease detection after induction chemotherapy, as a basic prognostic factor for assessing eligibility to allogeneic stem cell transplantation, is likely to be of weak clinical impact if one single marker, not representative of the whole cell population, is assessed.

With this in mind, it is time to reappraise thinking of a total, and at the same time individualized, approach to AML management, which moves from the biology of the disease and attempts to personalize the treatment to the single patient. Under this viewpoint, an immunological approach, albeit integrated, fulfills most of these characteristics and may open new avenues in the management of patients with AML.

However, such an innovative approach is likely to rely on different platforms than conventional treatments. Recent reports in solid tumors clearly indicate that the mutational landscape is crucial for determining the sensitivity to PD-1 blockade (123). Very interestingly, such genomic pattern is completely different to that is used to define the response to conventional chemotherapy. Similarly, to determine the response to immunotherapy with CAR-T cells in acute lymphoblastic leukemia the commonly used biologic factors, such as cytogenetics, have been of poor significance and impact (114).

These examples from different settings tell us that an immunological approach will require a novel list of prognostic and biologic factors, specifically related to the novel immunological pathways and mechanisms. At the same time, the methods, including timing and criteria of evaluation, for assessing clinical response to immune-oncology, is likely to be different from those commonly used to test response to conventional chemotherapy or cytotoxic drugs. As an example, experimental models have demonstrated that upon treatment with checkpoint inhibitors, the regression of tumor mass follows an initial expansion due to the infiltration of immune cells and the inflammatory reaction. Such pattern is not observed after treatment with cytotoxic drugs, and should be considered in planning the timing for disease assessment after treatment with novel immunological drugs.

Moreover, it is necessary to bear in mind that the immune system probably represents the most complicated and discordant system in human pathophysiology. At the same time, the immune reaction is an extremely powerful tool, which may result in rapid eradication of tumor disease. Moving from past and disappointing experience of clinical application of immunology into the clinical setting, it is worth discussing a novel method for translating into the clinics the novel immunological therapies. In this context, it is possible that, given the complexity of immune-oncology, the principles of evidence-

based medicine, which aims to reduce the complexity of single patients to few comparable cohorts, will not be fitting to the aims of clinical translational research, which, at the end, aims to provide useful tools to take care of single patients.

In conclusion, the challenge represented by immunological therapies in hematology, and beyond, is supposed to prompt the clinicians to wonder whether the community is offering to patients the most useful method to address the point of "taking care" and, more importantly, of "cure". Given the great advances in the understanding of the biology of the disease, AML is probably one of the best setting where to start working on such a new approach, in order to stop to "walk these empty street on the boulevard of broken dreams".

Acknowledgements
Financial support: the research projects of the Authors are partly funded from: Progetto Università-Regione, Call 2102, Associazione Italiana contro le Leucemie, Section of Pesaro; Associazione Italiana contro le Leucemie, Section of Bologna; Fondazione Fatro.

Abbreviations
Allo-SCT, allogeneic stem cell transplantation
AML, acute myeloid leukemia
APC, antigen-presenting cell
AraC, cytosine-arabinoside
ASCT, autologous stem cell transplantation
ATRA, all-trans retinoic acid
BM, bone marrow
BiTE, bispecific T-cell engager
CAR, chimeric antigen receptor
CART123, CAR-based therapy targeting CD123
CD, cluster of differentiation
CD200 R, CD 200 receptor
CIK, cytokine-induced killer
CLL-1, Human C-type lectin-like molecule-1
CMML, chronic myelomonocytic leukemia
CR, complete remission
CTL, cytotoxic T lymphocytes
CTLA-4, cytotoxic T-lymphocyte antigen 4
DC, dendritic cell
FcR, Fc receptor
FLT3, fms-like tyrosine kinase 3
FRβ, folate receptor β
GITR, glucocorticoid-induced tumor necrosis factor receptor
GO, gentuzumab ozogamicin
GVHD, graft versus host disease
GVL, graft versus leukemia
HLA, human leukocyte antigen
HMA, hypomethylating agent
IDO, indoleamine 2,3-dioxygenase
IFNγ, interferon gamma
IL, interleukine
iT_{reg} , induced regulatory T cells
KIR, killer-cell immunoglobulin-like receptor
KIR-L, killer-cell immunoglobulin-like receptor ligand
KYN, kynurenine
LAA, leukemia-associated antigen
LAG-3, Lymphocyte-activation gene 3
mAb, monoclonal antibody
MAGE, melanoma-associated antigen
MDSC, myeloid-derived suppressor cell
MDS, myelodysplastic syndrome
MHC, major histocompatibility complex
MSC, mesenchymal stromal cell
NPM, nucleophosmin
NK, natural killer
PD-1, programmed cell death protein 1
PD-1L, programmed cell death protein 1 ligand
RHAMM, receptor for hyaluronic-acid-mediated motility
SCT, stem cell transplantation
SWOG, Southwest Oncology Group
TAA: tumor associated antigen
TCR, T cell receptor
TGF-β, transforming growth factor beta
TNFα, tumor necrosis factor alpha
T_{reg}, regulatory T cell
Teff, effectory T cell
TIM-3, T-cell immunoglobulin and mucin-domain containing-3
VEGF, vascular endothelial growth factor
WT-1, Wilms' tumor 1

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