Book Chapter

Present and Future Role of Immune Targets in Acute Myeloid Leukemia

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Published **March 16, 2023**

This Book Chapter is a republication of an article published by Daniela Damiani and Mario Tiribelli at Cancers in December 2022. (Damiani, D.; Tiribelli, M. Present and Future Role of Immune Targets in Acute Myeloid Leukemia. Cancers 2023, 15, 253. https://doi.org/10.3390/cancers15010253)

How to cite this book chapter: Daniela Damiani, Mario Tiribelli. Present and Future Role of Immune Targets in Acute Myeloid Leukemia. In: Prime Archives in Cancer Research: 3rd Edition. Hyderabad, India: Vide Leaf. 2023.

Author Contributions: D.D. and M.T. equally contributed to conceptualization, writing and editing the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Conflicts of Interest: The authors declare no conflict of interest.

Simple Summary

Despite increasing knowledge of biological mechanisms leading to neoplastic transformation, identification of various molecular targets for innovative drugs, refinements in supportive care and a wider use of allogeneic transplant, prognosis of acute myeloid leukemia (AML) remains dismal. In the last years the role of bone marrow microenvironment and of the immune system in favoring leukemic cells persistence have emerged both as a cause of treatment failure but also as a potential setting for novel therapies. Aim of the present paper is to review biological evidence and clinical trials of immune-based treatments for AML.

Abstract

It is now well known that bone marrow (BM) cell niche contributes to leukemogenesis, but emerging data support the role of the complex crosstalk between AML cells and the BM microenvironment to induce a permissive immune setting that protect leukemic stem cells (LSCs) from therapy-induced death, thus favoring disease persistence and eventually relapse. Identification of potential immune targets on AML cells and modulation of the BM environment could led to enhanced anti-leukemic effect of drugs, immune system reactivation and restoration of AML surveillance. Potential target and effectors of this immune-based therapies could be monoclonal antibodies directed against LSCs antigens such as CD33, CD123 and CLL-1 (either as direct target or via several bispecific T cell engagers), immune checkpoint inhibitors acting on different co-inhibitory axes (alone or in combination with conventional AML drugs) and novel cellular therapies such as chimeric antigen receptor (CAR) T-cells designed against AML-specific antigens.

Though dozens of clinical trials, mostly in phases I and II, are ongoing worldwide, results have been still negatively affected by difficulties in identification of the optimal targets on LSCs.

Keywords

Acute Myeloid Leukemia; Drug Resistance; Immune Escape; Immune Therapy

Introduction

Acute myeloid leukemia (AML) is an heterogenous group of hematopoietic clonal diseases characterized by accumulation in the bone marrow (BM) and in the peripheral blood (PB) of immature myeloid cells with increased proliferation capacity and survival ability, resulting in bone marrow failure and increased hemorrhagic and infective risk [1]. AML is the most common type of acute leukemia in adults, its incidence is increasing by age, and despite a consistent proportion of patients initially respond to therapy, the long-term prognosis remains quite poor as over 60% of patients will eventually relapse and die from the disease [2]. Over the past twenty years, the role of clonal evolution in the leukemogenic process has emerged, identifying many recurrent molecular abnormalities, refining risk across the cytogenetic alterations and, at the same time, identifying potential therapeutic targets [3,4]. The progress in deciphering the molecular complexity of acute leukemia, the availability of target therapies and a wider use of allogeneic hematopoietic cell transplantation (HCT) promise better response in selected AML subsets [5], but the recent data of the American Cancer Society still estimate a 5-year overall-survival (OS) rate of 30%, and the prognosis is even worse in elderly patients, with a 1-year expected OS less than 10-15% [6]. The high relapse rate, even after HCT, is largely attributed to the persistence of leukemic stem cells, sharing, or hijacking the BM microenvironment of normal hematopoietic progenitors. It is well recognized that stem cell niche contributes to leukemogenesis, but accumulating evidence suggest that the complex crosstalk with leukemic cells contributes to create a permissive, protective immune environment able to affect response to therapy and facilitate relapse [7]. On this basis, efforts have been recently focusing on

the identification of potential immune targets on leukemic cells, and on tuning novel immune-based techniques to adjust the niche and to reactivate immune system, thus restoring anti-leukemia surveillance.

In this paper we summarize the current data on biological basis and clinical use of molecular or cellular therapies targeting leukemia cells to overcome drug resistance and to contrast immune evasion, potentially restoring the anti-leukemia immune surveillance.

Leukemic Cells in Their Bone Marrow Microenvironment: How They Induce Drug Resistance and Escape Immune Response

A hierarchical organization of bone marrow leukemic cell resembling the normal hematopoietic system, with a small subset of leukemic stem cells (LSCSs) on the top of the pyramid continually replenishing the more mature bulky population was first described 25 years ago by Bonnet and coll. [8]. As for the normal counterpart, leukemic stem cells are defined by their self-renewal capacity and their ability to initiate leukemia after serial transplantation in SCID mice [8,9]. Interestingly, LSCSs seems to originate from the acquisition of driver mutations not by normal immature hematopoietic stem cells (HSCs) but by committed progenitors. At least two distinct LSCs population have been identified in human AMLs: a more mature, emerging from granulocyte-macrophage progenitors (GMPs); a more immature, from lymphoid-primed multipotent progenitors (LMMPs) [10]. The recent identification of clonal hematopoiesis of indeterminate potential (CHIP) [11], and the recognized ability of more mature LSCs to revert to an immature state [12], has further complicated the scenario. However, the persistence of pre-leukemic hematopoietic clonal alteration in responding patients confirms the negative role of LSCs [13]. Although it is well known that LSCs are enriched within CD34+CD38- fraction, a clear definition of LSCs phenotype does not exist. Compared to normal HSCs, LSCs may have higher expression of CD25, CD32, CD44, CD96, CD123, CD200, GPR56, N-cadherin, Tie2, TIM-3, CLL-1, c-MPL, HDM2 [14-24]. Unfortunately, the high inter- and intra-individual heterogeneity

makes difficult their enumeration and their sequential monitoring. Despite this, the negative prognostic role of LSCs frequency at diagnosis or after therapy has been demonstrated by many studies, and different LSCs prognostic scores have been obtained by gene expression profile and whole exome plantation [25-27]. LSCs escape the effect of anti-leukemic drugs by nesting in the BM microenvironment in a quiescent state, and leukemic progeny gradually occupy the BM niche converting it into a "leukemic niche", able to support leukemic cells' survival and proliferation while decreasing its capacity to maintain normal hematopoiesis [28-30]. It is interesting to underline that BM failure is not due to a reduction of HSCs, but to its inability to produce an adequate number of progenitors, as a consequence of the release by mesenchymal cells (MSCs) of hypoxia-associated molecules that increase the stemness and prevent differentiation [31], mimicking the differentiation arrest, that is the hallmark of leukemia phenotype. The modifications of the BM niche by leukemic cells involve all the components of microenvironment. The endosteal niche is reshaped by the loss of balance between bone formation and resorption as the consequence of activation of RANK/RANKL pathway which promotes osteoclastogenesis and favor osteoclast survival [32]. Moreover, the secretion by LSCs of a bone morphogenic protein (BMP), of a negative regulator of osteogenesis (DKK), of a chemokine that decreases osteocalcin and switch MSCs differentiation from adipogenic to osteoblastic, with consequent accumulation of progenitor and immature osteoblast and defect of bone mineralization (CCL3, also known as MIP1□) [33,34], contribute to create a milieux facilitating leukemia cell growth and AML progression. Furthermore, in vitro data suggest that MSCs also support leukemia cell survival and promote resistance to chemotherapy inducing low cycling rate and anti-apoptotic signals [35,36].

AML vascular niche, increased IL-1β and TNF-α levels synergize with increased adhesive receptors E-selectin/CD44 and VCAM-1/VLA-4, thus ensuring the anchorage of LSCs to endothelial cells [37,38]. VEGF/VEGFR and Notch/Delta-like ligand 4 promote neo-angiogenesis, essential for AML progression and extra medullary homing [39,40]. Although less known compared to the other, the reticular niche is considered a

transitional niche, able to maintain HSCs in a proliferative state, such as the vascular, but in an undifferentiated state, like the endosteal one. CXCL12-abundant reticular (CAR) cells and Nestin-expressing cells are the most represented cells in the niche, localized in proximity of sinusoidal endothelial cells [41-43]. Most HSCs are in contact with CAR cells, but interaction with these cells is established also by B-lymphocytes, plasma cells, plasmacytoid dendritic cells, and NK cells, suggesting that reticular cells might act also as immune cells niche [44-46]. CAR cells secrete CXCL12 and SCF, crucial for HSCs trafficking and homing. In AML the CXCL12/CXCR4 axis regulates infiltration of leukemic cells in the protective bone marrow niche [47,48]. Nestin-expressing cells are associate to adrenergic nerves and regulate HSCs maintenance [43,49]. AML bone marrow is enriched of Nestin-expressing cells. Besides their role in developing AML, Nestin- expressing cells are implicated in induction resistance to chemotherapy by enhanced glutathione (GSH)-peroxidase (Gpx) activity [50].

Considered in the past only as passive bystanders, BM adipocytes actually play an important role in regulating normal HSCs and are implicated in hematopoietic recovery after irradiation or 5FU treatment by secreting SCF [51]. In AML, they promote leukemic cell survival during therapy and drug resistance by enhancing free fatty acid production [52,53] and by sequestering, inactivating and metabolizing chemotherapeutic drugs [54]. Among cell from myeloid resident in the bone marrow, also myeloid derived suppressor cells (MDSCs) and leukemia associated macrophages (LAMs) are implicated in generating an immunosuppressive microenvironment in AML. MDSCs are an heterogenous population, able to induce T cell tolerance by multiple mechanisms, still not completely elucidated. It is well known that MDSCs express high level of V-domain Ig suppressor of T cell activation (VISTA) and PD-L1, but also indoleamine 2,3-dyoxigenase (IDO), arginase, ROS, TGFβ and IL-10 [55-58]. In AML there is an increase of MDSCs by release of extracellular vesicles containing the oncoprotein MUC1, which induces MDSCs expansion via c-myc [59]; some clinical data suggest that MDSCs number correlate with disease progression and poor survival [60,61]. MDSCs are distributed through the three niches, in proximity of leukemic

cells. AS for MDSCs, resident BM macrophages are not localized in a specifical niche, but they are able to adapt to microenvironment, where they play a role in maintaining homeostasis and in processing and presenting antigens to adaptive T cell effectors. In AML microenvironment they may be involved in protection of leukemic cell from apoptosis induced by chemotherapy [62]. The increasing knowledge of healthy BM microenvironment and of its dynamic remodeling to become a leukemic "sanctuary" has completely changed the old view of leukemogenesis as a merely product of accumulating genetic defects, highlighting its dependence from an active crosstalk with BM niches. This perspective paves the way toward new challenging treatment strategies including targeting adhesion or leukemic cells/niche cells signaling pathways, reversing bone remodeling or signals, inducing vascular disruption or metabolic changes counteracting drug resistance.

Several clinical trials are currently ongoing to investigate the efficacy and the toxicity of many niche-targeting molecules. Their description is beyond the scope of this paper, but an exhaustive overview on this topic was recently publish by Kuek and coll. [63]. Besides the stromal component of the niche, an important role in creating a permissive environment is played by the immune cells resident in the BM. Although spatially distinct T and B compartments are not recognizable, it is well known that significant proportion (8-20%) of innate and adaptive immune cells transit and reside in the BM with a T cell/B cell ratio of 5:1 [64,65]. Marrow's lymphocytes are distributed throughout stroma and parenchyma and condensed in follicles; antibody-producing plasma cells account for about 1% of mononuclear cells. Despite most T cells have already encountered an antigen, making the BM an "immune memory reservoir", some circulating naive T cells can be primed in the BM [65]. In healthy individuals T cells benefit from BM environment and, at the same time, support hematopoiesis through cytokines secretion and expression of chemokines receptors [66]. BM T cells have an active role in allogeneic stem cells engraftment and memory CD8 T cells exert anti-leukemia effect by limiting the potentially harmful graft versus host disease (GVHD) [67-69]. Moreover, they are able of anti-viral activity providing protection from chronic infections [70]. As

well as BM effector T cells, also T regulatory cells (Tregs), that are present in the BM in higher numbers than in peripheral blood and lymphoid organs, contribute to hematopoiesis maintenance by regulating the endosteal niche [66]. Natural killer (NK) cells play a protective role against GVHD in transplant setting and a potent anti- tumor effect [71,72]. BM is also the primary B-lymphoid organ, where B precursors develop from HSCs and mature, in specific niche rich of CXCL12- an IL7-expressing cells [44]. Furthermore, the BM acts as reservoir for long-lived plasma cells, providing survival factors, and so contributing to maintain long term immunity [73]. Beyond remodeling stromal niche, marrow AML cells implement several unique strategies to escape immune surveillance by directly affecting the function of immune response players and preventing immune-mediated elimination. AML cells can develop immune-editing processes to reduce cell recognition, leading to epigenetic down regulation of HLA molecules, loss of HLA molecules, increased production of immunosuppressive/inhibitory molecules, increased expression of inhibitory ligands, expression of checkpoint inhibitors, altered expression of NK cell ligands, impaired capacity of T cells to form immune synapsis [58,74,75]. Moreover, they can induce expansion of Tregs, MDSCs and M2 polarization of macrophages [75]. A schematic representation of immune evasion mechanisms of AML cells is shown in figure 1.

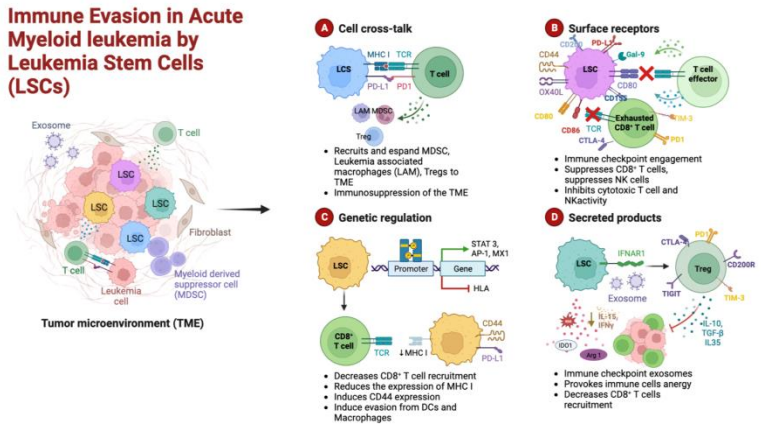

Figure 1: Cells and mechanisms of anti-tumor surveillance escape in AML. Figure create in Biorender.com.

Longitudinal investigation of immune landscape in leukemia patients had not only demonstrated a high inter-patient variability but revealed also that a selective immune signature predicts a negative outcome. Tang and coll. reported a negative event-free survival (EFS) and OS in presence of CD8+PD-1+ T cells [76]. Guo and coll. mapping a series of AML-derived BM immune cells confirmed the diverse immune assets among patients. They also identified many infrequent immune cell types, such as TH17/Treg intermediate population, CD8+ memory T cells, dysfunctional macrophages, and dendritic cell subsets, predicting a poor prognosis [77]. Rutella and coll. investigated AML-driven CD8+exhaustion/senescence, developing an immune effector dysfunction (IED) score associated with leukemia stemness and poor response to therapy and possibly predicting resistance to immunotherapy [78]. On this basis and following the success in lymphoid malignancies and in multiple myeloma, many immune therapies for AML are under development. The major challenge is the identification of the target, since the high heterogeneity of AML population, the difficulty in finding antigens restricted to LSCs, the lower antigen density compared to lymphoid cells, as well the best therapeutic strategy (in combination with chemotherapy and/or molecular therapy or sequentially) and the choice between an antibody-based or cellular-based therapy.

In the last few decades, different strategies have been developed to increase therapeutic potential: targeting LSCs restricted antigens, reactivating endogenous T-cell response through immune checkpoint inhibitors, harnessing T-cells and NK response independently of T-cell receptor (TCR) specificity through cell-engaging antibody constructs and genetic engineering of T cells [TCR-modified and chimeric antigen receptor (CAR) T cells].

Monoclonal Antibodies

Potential Targets on LSCs

The main mechanism of action of unconjugated antibodies is antibody-dependent cell- mediated cytotoxicity (ADCC). After formation of an immunological synapse with the target cell, NK cells trigger a cytolytic response through the exocytosis of

granules containing perforin and granzyme into target cell. Furthermore, they facilitate antibody-dependent phagocytosis (ADCP) by macrophages. Their major limit is the presence of inhibitory signal counteracting their action, and the limited potency of activation signals.

CD33

CD33 is a 67 kDa glycoprotein, member of the siglec family (siglec-3), expressed in normal hematopoiesis from early myelo-monocytic lineage committed progenitors to mature cells. It is also expressed on 99% of AML cells and in LSCs [79]. The use of unconjugated anti CD33 is challenging due to its low membrane density and to the low antibody-induced internalization, so that the first phase 3 trial (NCT0006045) with humanized IgG1 unconjugated antibody anti CD-33, lintuzumab (HuM195) was stopped for lack of efficacy [80]. Despite this, four phase I/II trials are ongoing to test a new anti-CD33 unconjugated IgG1 (BI 836858) alone or in combination with azacytidine (AZA), decitabine (DAC) or F16-IL2, in relapsed/refractory and newly diagnosed AMLs (NCT01690624, NCT03013998, NCT02032721, NCT03207191). The principal advantage of antibody drug-conjugates is the simultaneous selective attack of target cells and the on-site delivery of potent conjugated cytotoxic agent, not usable as free drugs in conventional chemotherapy schemes for their toxicity. After spontaneous withdrawal of the manufacturer due to the lack of survival improvement and the high induction mortality The anti-CD33-calicamycin conjugate gemtuzumab-ozogamicin (GO) has been revived for core binding factor (CBF) AMLs and for elderly patients with AML or high risk myelodysplastic syndrome (MDS), where it has demonstrated reduced relapse risk and survival advantage in patients with favorable and intermediate cytogenetic risk [81]. A phase I clinical trial testing safety and efficacy of GO in combination with venetoclax (VEN) in adult relapsed/refractory AMLs is currently ongoing (NCT04070768). A new anti-CD33 conjugate with DGN 462, a DNA- alkylating drug was active in preclinical model [82] and a phase I trial in CD33+ relapsed/refractory AMLs has terminated but results not yet available (NCT02674763).

CD123

CD123 is the interleukin (IL)-3 receptor alpha chain and is a type I transmembrane glycoprotein [83]. CD123 is present in 98% of CD34+CD38- LSCs, and on blast cells, not in normal hematopoietic progenitors making it a potential therapeutic target [17]. The unconjugated antibody (talacotuzumab) failed to demonstrate efficacy in AML and MDS (NCT0299860). The phase I/II with Anti CD123-DGN462 conjugate, alone or with AZA± VEN in MRD+ post induction therapy AMLs is still recruiting patients (NCT04086264).

CLL-1

C-type lectin-receptor1 (CLL-1) is an ITIM-containing inhibitory transmembrane protein code on chromosome 12p13.31. CLL-1 is not expressed on normal HSCs, but it is present on committed progenitors and in mature peripheral myeloid cell such as monocytes, granulocytes, and dendritic cells [84]. Its function, as well as its ligand, are not completely understood. It has been suggested its involvement in regulating some inflammatory situations, by down modulating granulocyte and monocyte function [85]. Being selectively expressed on LSCs, even more than CD123 which is present also in some CD34+CD38-normal progenitors, CLL-1 could be regarded as an ideal target for anti LSCs immune therapies [22,86]. Despite CLL-1 efficiently internalizes after ligand binding, in vitro experiments with an anti CLL-1 antibody suggested that an un-conjugated antibody cannot have anti leukemic activity because it does not activate ADCC [22]. In animal models two antibody-drug conjugates with a pyrrolobenzodiazepine (DCCL9718A) and isoquinolidinobenzodiazepine (CLT030) induced potent anti leukemic effect, with little off-target toxicity [87,88]. To enhance anti-CLL1 efficacy, different bispecific antibodies and cell-based therapies has been developed (see below).

Other Current Clinical Trials of Toxin-Conjugate Antibodies

FLT3 (FMS-like tyrosine kinase 3) a member of the class III receptor tyrosine kinase family, which is highly expressed in the blasts of both AML and ALL patients. FLT3 plays an important but not absolute role in maintaining the survival of normal HSCs and its recurrent mutations (ITD-FLT3, TDK-FLT3) are expressed in many AML cases [89,90]. Working in conjunction with other growth factors, FLT3 promotes the proliferation and differentiation of myeloid and lymphoid cells. Transplant animal models with non-functioning and wild type FLT3 showed that hematopoiesis is almost normal in FLT3 knockout animals, while FLT3 mutations give a significant growth advantage. These facts suggest that selective FLT3 inhibition in leukemia cells can block the excessive FLT3 leukemia activation with acceptable hematopoietic side effects [90]. The phase I trial (NCT02864290) testing AGS62P1, anti FLT3 antibody-amberstatin 269 in relapsed/refractory adult AMLs has been closed for lack of efficacy. NCT03957915, phase I trial with INA03 drug-conjugate antibody targeting transferrin receptor (CD71) is active, not recruiting, and NCT01830777 testing brentuximab-vedotin in CD30+ relapsed AMLs, is completed (results not yet available). More recently, the use of monoclonal antibodies labeled with radionuclides has been proposed especially in the setting of HCT conditioning regimens, to obtain a more precise delivery of ionizing radiation to the disease site [91]. This method would maintain the advantages of high dose total body irradiation (TBI) and the low toxicity of reduced intensity regimens. At present many trials with radiolabeled antibodies are ongoing. In the phase III SIERRA trial (NCT02665065) patients are randomized to receive conventional myeloablative versus ^{131}-I-BC8, an iodine-conjugate anti CD45 antibody. The preliminary results on the first 25% patients, presented at the ASH Meeting in 2018, demonstrated the protocol feasibility, though non relapse mortality was relevant in the ^{131}-I BC8 arm, and a clear reduction of toxicity was not evident. The still recruiting NCT03867682 trial investigates the maximum tolerated dose and remission state in adult relapsed/refractory AML receiving a radiolabeled anti-CD33

conjugate (^{225}Ac-Lintuzumab) + VEN. The Phase I trial NCT 03441048 is recruiting patients with R/R AML to evaluate toxicities of the combination of CLAG-M regimen with ^{211}At-BC8, an anti-CD45 antibody. NCT 03670966, a phase I/II trial, investigates the toxicity, GVHD incidence, donor chimerism, engraftment rate and 100-day survival of the combination of 211-At-BC8 with a reduced intensity preparative regimen (fludarabine, cyclophosphamide, 2-Gy TBI) in haploidentical stem cell transplantation in patients with R/R AML. The same endpoints are investigated in the phase I/II NCT03128034 trial, in which 211At-BC8 is employed with fludarabine and 2-3 Gy TBI. The information from all these trials should allow a further reduction of toxicity through individualized treatment in line with the emerging "theranostics" concepts.

Immune Checkpoint Inhibitors

The immune system activity is closely modulated by interaction between co-inhibitory molecules and their ligands. These co-inhibitory molecules are involved in maintenance of immune tolerance, but in the neoplastic setting may represent one of the mechanisms employed by cancer cells to escape immune surveillance [92,93]. Many studies have reported an increased expression of co-inhibitory molecules in solid cancers and, more recently, similar results have been observed in AML patients [94,95]. Up-regulation of co-inhibitory ligands has been associated with poor clinical outcome in solid and hematological cancers [93,94] thus identifying a potential new class of therapy targets (Figure 2). Many molecules, such as ipilimumab, pembrolizumab, cemiplimab, avelumab, durvalumab, with different blockade targets, have been approved for the treatment of solid tumors, and are under investigation in AML [96]. Single agent treatments proved the ability of co-inhibitory blockade to improve immune response in many tumors, but at the same times highlighted the modest results of a monotherapy, promoting the design of various combination trials. About fifty trials, designed to provide definitive information on safety and long-term efficacy, are currently recruiting patients with AML, testing molecules blocking different co-inhibitory axes and different drug combinations.

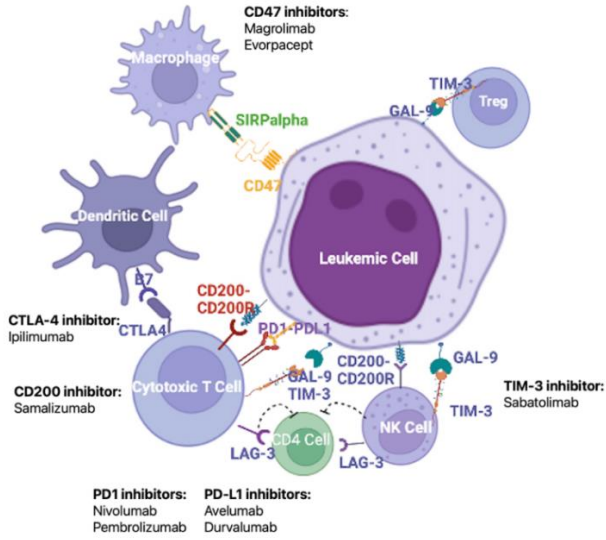

Figure 2: Major mechanisms of maintenance of immune tolerance and potential therapeutic targets. Figure create in Biorender.com.

The CTLA-4/B7 Axis

CTLA-4 (CD152) is a member of immunoglobulin-related receptors interacting with CD80 and CD86 ligands to deliver an inhibitory signal to terminate immune responses. Furthermore, it is involved in induction of Tregs lymphocytes, playing a pivotal role in regulating tolerance and autoimmunity [97]. Aberrant expression has been reported in AML, often in association with expression of other checkpoint inhibitors, and with negative impact on disease outcome [98]. In murine tumor models, blocking of CTLA-4 enhanced T cell activity, suppressing Tregs cells [99]. In clinical trials for melanoma, the anti CTLA-4 agent ipilimumab increased Teff/Tregs ratio, enhanced NK activity and restored T effector function, ultimately prolonging survival [100,101]. In vitro tests on AML cell lines and preliminary clinical studies confirmed the data on solid tumors [102]. The available "in vivo" data on ipilimumab efficacy is scarce. Davids and coll. reported 23% of complete remission (CR) and 9% or partial response (PR) in a series of hematological malignancies treated with single agent ipilimumab for relapse after HCT [103]. At present, a phase I trial exploring safety and toxicity of

ipilimumab combined with a definite dose of Treg-depleted donor lymphocyte infusion (DLI) for AML and MDS relapse after transplant is recruiting (NCT03912064).

The PD1/PD-L1 Axis

PD1 (CD279) is a type I transmembrane protein preferentially expressed in almost all activated immune cells [92]. PD1 bind two different ligands: PD-L1 (CD274) and PD-L2 (CD273). PD-L1 is a member of B7 family of co-stimulatory/co-inhibitory molecules also present in hematopoietic cells and upregulated or aberrantly expressed in many tumors [92,104]. PD-L1 and PD-L2 over-expression in tumor cells have been associated with poor outcome [94]. PD1/PD-L1 engagement produces an inhibitory signal that causes T cell exhaustion and favors tumor cell immune escape [92]. Moreover, it induces apoptosis of tumor specific cells and favors Tregs differentiation and resistance to CD8+ mediated cytolysis [105]. In AML, PD1 up-regulation was described in T effector cells and Tregs, explaining the immune suppression observed during AML progression. PD-L1 and PD-L2 were found in blast cells and correlate with poor prognosis [106,107]. Despite the proven activity in solid tumors, PD1 and PD-L1 inhibitors are, to date, not approved for AML. The preliminary results of a phase II study of nivolumab maintenance in high risk AML patients in CR after induction and consolidation (NCT02532231), presented by Kadia and coll. in 2018 at the Annual Meeting of the American Society of Clinical Oncology (ASCO), report CR rates of 79% and 71% [108]. Many clinical trials combining epigenetic therapies with nivolumab or durvalumab in AML patients are recruiting. Results of combination therapy with AZA and nivolumab in relapsed AML (NCT02397720) presented in 2017 at the ASCO Annual Meeting reported 21% of CR/CRi, an hematologic improvement in 7% with a median OS of 9.2 months [109]. Finally, grade 2-4 immune adverse events were observed in 28% of patients. In another phase II clinical study associating nivolumab with cytarabine and idarubicin in newly diagnosed AML and MDS (NCT02397720), 72% achieved CR/CRi and 4-weeks and 8-weeks mortality were stable at 6% [110].

Tim-3/Galectin-9 Axis

T cell immunoglobulin and mucin domain 3 (TIM-3) is a cell surface molecule first identified on CD4+ TH1 cells and on CD8+ cytotoxic T cell (CTLs) and later on other innate immune cells, such dendritic cells, monocytes, macrophages, mast cells and in NK cells, and in many neoplastic cells [111-113]. TIM-3 gene is coded on chromosome 5q33.2, in the same region of IL4 and IL-5 genes. There are four known ligands for TIM-3: the first recognized and most widely studied is galectin-9 (gal-9), which induces apoptosis of TH1 cells [114], playing a crucial role in tumor cell immune evasion. Its high expression has been associated with worse prognosis in solid and hematologic neoplasms [92,94]. In AML, high levels of TIM-3 have been described in immune cells, associated with immune exhaustion, and on LSCs, where it represents a distinctive marker. Kikushige and coll. suggested that on LSCs TIM-3 and its ligand create an autocrine loop that regulate development and self-survival [21]. taken together, these data identify TIM-3 as an excellent candidate for therapy with monoclonal antibodies. Currently several monoclonal antibodies are used in clinical trials for solid tumors, such as MBG453 (sabatolimab), TSR-022, BMS-986258, LY3321367, SYM023, BGB-A425, SHR 1702 [115], but only MBG453 has shown preliminary efficacy and safety in AML and in MDS. Most of the trials are still ongoing. The interim data of trial NCT 03066648 (MBG453 in combination with HMAs) showed an ORR of 58% in MDS and 48% in newly diagnosed AML patients for the MBG453 + DAC arm and 70% in MDS and 27% in AML patients for the MBG453 + AZA arm, respectively. The most common grade 3/4 adverse events are thrombocytopenia, anemia, neutropenia and febrile neutropenia; in the MBG453 + DAC group four immune-related events were reported (ALT, arthritis, hepatitis, hypothyroidism), compared to none in the MBG453 + AZA cohort.

LAG-3/MHC Axis

LAG-3 (CD223), is a CD4-like molecule that binds MHC class II with higher affinity compared to CCD4, generating a signal that blocks T cell activation [100]. The molecule is expressed on activated T cells, Tregs, NK cells, B cells and dendritic cells

[94]. The engagement with its ligand reduces T cell activity and cytokine secretion, so blocking T cell activation and function[100]. Like other checkpoint molecules, LAG-3 has been identified on Tregs in cancer microenvironment. The frequent co-expression with PD1 suggest comparable function to PD1 [101,105]. Studies in AML are still limited [116]. It should be remembered that expression of MHC class II in AML cells can be involved in both immune suppression and antigen presentation processes. Antibody targeting LAG-3 are currently tested in solid tumors, lymphoma, and multiple myeloma [94,100]. No clinical trials have been activated in AML patients.

CD200/CD200R Axis

CD200 is a highly conserved 48kDa type-1 trans membrane glycoprotein, structurally related to B7 family, coded on chromosome 3q12-q13 near the region coding for CD80/CD86 proteins. Interaction with its ligand CD200R leads to the attenuation of many immune responsive effects, resulting in prolonged survival of transplanted allograph, but also in tolerance for tumor cells. CD200 overexpression has been described in several solid tumors, and in AML, where it marks LSCs but not the normal HSC counterpart [18,94]. CD200 overexpression in AML has been associated with poor outcome, especially in the favorable prognostic groups [117,118]. Furthermore, the expression of CD200 on leukemic cells suppresses the function of memory T cells, expands Tregs, and down modulates NK function [119-121]. “In vitro” and “in vivo” mouse models clearly demonstrated that inhibition of the CD200/CD200R axis with monoclonal antibodies restores anti-leukemia immune response [122]. At present, the monoclonal antibody anti-CD200 samalizumab is under investigation in solid tumors, but there are not active trials in AML.

CD47/SIRPs Axis

Leukocyte surface antigen CD47, encoded on chromosome 3q13.12, is a transmembrane glycoprotein that serves as the ligand for signal regulatory protein alpha (SIRPα), that is expressed on phagocytic cells, including macrophages and

dendritic cells. Upon activation CD47 initiates a signal transduction cascade resulting in inhibition of phagocytosis [123]. CD47 is broadly expressed in a variety of normal tissue, in normal circulating HSCs, as well on leukemic cells [124]. Moreover, its expression on leukemia cells has an adverse prognostic effect [125]. Preclinical studies demonstrated that block of CD47 exerts an anti-neoplastic effect on solid tumors and hematologic malignancies [126,127].

Magrolimab is the first-in class monoclonal antibody against CD47 available for clinical use. In 2020 FDA granted breakthrough therapy designation to magrolimab based on positive results in MDS. In high risk MDS, the phase 1b trial of combination magrolimab + AZA demonstrated a favorable safety profile: the most common treatment-emergent adverse events (TEAEs) included constipation (68%), thrombocytopenia (55%), anemia (52%), neutropenia (47%), nausea (46%), diarrhea (44%). CR and ORR were 33% and 76% respectively. OS rates at 12 and 24 months were 75% and 52%, respectively. Favorable outcomes were observed both in patients with TP53 mutation (40%), and WT TP53 (31%). A phase 3 trial of magrolimab/placebo + AZA in MDS is recruiting (ENHANCE trial - NCT04313881). Similar tolerability and safety profile was observed in a phase 1b trial investigating the association of magrolimab +AZA in frontline patients with TP53-mutated AML. Common TEAEs were constipation (52.8%), diarrhea (47.2%), febrile neutropenia (45.8%), nausea (43.1%), fatigue (37.5%), decreased appetite (37.5%), thrombocytopenia (31.9%), peripheral edema (30.6%) and cough (30.6%). ORR was 48%, with CR33.3% and CRi 8.3%. 30- and 60-days mortality were 8.3% and 18.1%, respectively. Median OS was 10.8 months. In high-risk AML patients with mutated TP53 unsuitable for intensive therapy, magrolimab + AZA showed durable response and encouraging OS. A phase 3 trial of this combination vs standard of care in TP mutated AML is ongoing (ENHANCE2, NCT04778397). Another phase 2 clinical trial of magrolimab + AZA + VEN vs magrolimab + intensive chemotherapy (mitoxantrone, etoposide, and cytarabine – MEC) vs magrolimab + oral AZA (CC-486) is open and recruiting (NCT04778410).

Evorpacept (ALX148) is an engineered high affinity CD47-blockin protein, in which Fc domain has been modified to avoid red cell agglutination experienced with magrolimab. here are two ongoing trials assessing ALX148 in combination with AZA in high risk MDS (ASPEN02 trial - NCT04417517) and in combination with AZA + VEN in AML (ASPEN05 trial - NCT04755244); results of the phase 1 part of trial has been recently published, showing good tolerability of the combination evorpacept + AZA [128]. TEAEs observed in >1 subject included constipation and infusion related reaction (23%), and nausea or vomiting (15%); there were no evorpacept-related SAEs, and no patients discontinued treatment due to an AE. The combination will be further evaluated in the randomized phase 2 part of the study.

T Cells Engagers

Given the unsatisfactory results of "naked" and drug-conjugates antibodies in treating AML, over the past years combination strategies to overcome tumor cell and immune cell resistance have been developed. The purpose was to harness the immune response through targeting of specific tumor antigens and engaging the dormant effector cells (T cells, NK-cells, macrophages) against leukemic cells to obtain a fast and potent cytotoxic response and, ideally, generating also a durable immunologic memory [129]. On this basis, several bispecific T cell engagers (BiTEs) antibodies have been developed for hematologic malignancies and novel structures are constantly emerging [130]. Bispecific antibodies consist of a single heavy and light chain of the variable region of a tumor-associated antigen and CD3: by combining the two different specificities, they can activate exhausted T cell through sustained tumor antigen exposure, overcoming its possible low-density expression. At the same time, being the T cells activated only in the presence of target cells, they have limited off-target cytotoxicity. Moreover, BiTEs acts in a MHC-1 independent manner, generating a cytolytic synapsis between CD8+ T cells despite the tumor-induced MHC-1 down-modulation, and permits T cells activation even in the absence of co-stimulatory signals such as CD28 or IL-2 [131]. Starting from

blinatumomab, the first CD19/CD3 BiTE approved by FDA in 2014 for adult Ph+ acute lymphoblastic leukemia (ALL), many other BiTEs have been developed. Construct variants include dual affinity retargeting (DARTs) antibodies, bi- and tri-specific killer engagers (BiKEs and TriKEs). In their basic construct, BiTEs are connected by a linker molecule that defines the flexibility and the antigen-binding kinetics. In DARTs a c-terminal disulfide bridge is included to improve stabilization, resulting in stronger T cell activation and cell lysis [132]. BiKEs and TriKEs reactivate immune system by engaging NK cells via CD16; upon stimulation they release cytokines leading to tumor cell lysis and recruiting other immune cells to amplify immune response [131]. Furthermore, TriKes have an IL-15 crosslinker, which drives NK expansion and increases killing response [133].

Anti-CD33

The CD33 was integrated in AMG330 bispecific antibody. Pre-clinical studies demonstrated a potent T cell activation and cytokines release, high blast cell clearance and reduction of BM monocytic MDSCs [134,135]. Moreover, AMG330 lytic activity was not affected by CD33 polymorphisms nor by the over-expression of drug transporter proteins on AML cells, as it happens for gemtuzumab ozogamicin, and it does not modify CD33 membrane density, as conventional anti-CD33 antibody does [136]. On this basis AMG was administered in a phase 1, dose-finding trial (NCT02520427), for patients with relapsed/refractory AML. A total of 55 patients were enrolled in 16 cohorts receiving increasing doses. CR was obtained in 8/42 (19%) evaluable patients, despite half of responding patients was heavily pretreated. The cytokine release syndrome (CRS) and nausea were the most common adverse event (67% and 20%, respectively) [137].

AMG 673 is anti CD3-CD33 BiTE with a modified construct on Fc region to prolong its half-live, permitting weekly infusions. In NCT 03224819 phase I trial in R/R AML it has demonstrated efficacy in reducing BM leukemia burden in 44% of patients even if CR was achieved in only one patient. CRS was experienced by 50% of patients, transaminits by 17%,

leukopenia by 13% (febrile neutropenia by 7%) and thrombocytopenia by 7%.

AMV 564 CD3-CD33 BiTE was studied in animal models, demonstrating potent anti leukemic effect in BM and PB. In a phase 1 trial in R/R AML (NCT03144245) it confirmed anti-leukemic activity via T cell activation, irrespective to antigen expression level. Moreover, AMV 564 was able to deplete MDSCs in a dose dependent manner. Preliminary results on toxicity in 18 patients show acceptable profile, with CRS grade 2 in one patient, and febrile neutropenia in 7/18 patients (38%) [138].

G333 is a novel CD3-CD33 BiTE, modified with a linker to increase T cell binding to AML cells. In preclinical studies it has demonstrated low effect on normal HSCs [139], while a phase I trial in R/R AMLs is ongoing (NCT03516760), with results not yet available.

JNJ-67571244, CD3-CD33 BiTE, was tested in a phase I, dose escalating trial in R/R AML and high-risk MDS (NCT03915379), that enrolled 68 patients; the trial was completed on March 28th, 2022, but results hed still not been reported.

GTB-3550 is a CD16-CD33 BiKE that, in preclinical studies, demonstrated the ability to overcome MHC-1 inhibitory signal, to exert anti-leukemic activity and to reverse MDSCs-induced suppression of NK cells. A phase I trial in R/R AML, MDS and advanced systemic mastocytosis is currently ongoing (NCT03214666). No data are available currently.

Anti-CD123

Vibecotamba (XmAb14045), is a potent CD3-CD123 bispecific antibody, showing in preclinical models high anti leukemic activity on PB and BM blasts, and a long half-life [140]. Initial results of a phase I dose-escalation study in R/R AML (NCT 02730312) enrolling 104 patients showed an ORR of 14%, a CR rate of 4% and stable disease in 71% of patients. CRS was

observed in 59% of patients. CD123 expression intensity on AML blasts seemed not to affect response [141].

APVO436, another CD3-CD123 bispecific antibody is currently in clinical trial of AML and MDS (NCT03647800). One of its peculiar features seems to be a low induction of cytokines release while maintaining the ability to reactivate T cells [142]. However, preliminary results from ongoing trial suggest a limited efficacy [143].

Flotetuzumab (MDG006), a CD3-CD123 DART antibody, has been tested in a phase I/II clinical trial for R/R AML (NCT02152956), demonstrating anti-leukemic activity in 30 heavily pretreated patients, with an ORR and a median OS of 10.2 months among responders [144]. Interestingly, in vitro studies demonstrated an up-regulation of PD1/PD-L1 following flotetuzumab, prompting its combination with an anti PD antibody, currently under investigation [145]. More, MGD006 have demonstrated preferential binding of leukemic cells and blast cell lysis at low doses, along with the maintenance of BM cellularity and HSCs compartment [146].

Recently, the dual-targeting triple-body SPM-2, targeting CD33, CD123 and engaging NKs as effector cells via CD16, has been tested on blast cells from 29 patients with AML. The new construct has demonstrated lytic activity across different AML subtypes at nanomolecular concentration, raising the expectation that SPM-2 may also be capable to eradicate LSCs [147].

Other targets

MCLA-117 is a CD3-CLEC12A (CLL-1) bispecific antibody, currently under investigation in a phase I trial of R/R AML and newly diagnosed elderly AML patients (NCT03038230). In vitro studies demonstrated its ability to lyse cells at a low E:T ratio, even blast cells with low CLL-1 expression [148]. Its extended half-life permits weekly or bi-weekly infusions [149]. CLEC12a TriKEs has been developed to target LSCs lacking CD33 expression, but expressing CLL-1, demonstrating lower off-

target toxicity and similar lytic capacity compared to CD33 target, preserving normal HSCs [150].

AMG427 is an anti-CD3-FLT3 (CD135) bispecific antibody constructed to target the same receptor expressed from leukemic cells. It has demonstrated in vitro T cell dependent cytotoxicity but also an increased production of PD1 expression which can act as an immune escape mechanism without concurrent administration of anti-PD1 therapy [151].

Published results of clinical trials of bispecific antibodies are summarized in Table 1.

All the bispecific antibodies are still in early clinical trials and many questions remain to be answered. Available clinical data indicate an acceptable safety profile, suggesting that bispecific antibodies will soon enter in the current clinical practice. Despite the limited use, some weaknesses have been already identified. First, the ideal "format" of bispecific antibodies is still not defined; small molecules have a short half-life and require continuous or close infusion and frequent dose adjustments. Construct modifications increasing molecular weight may prolong half-life by preventing a rapid renal clearance, and thus permitting to delay infusions, but may increase off-target toxicity. Second, antibodies themselves can favor the maintenance of as permissive microenvironment by inducing the upregulation of inhibitory costimulatory molecules, such as PD1/PD-L1, and by vanishing T cell activation. In this instance, concomitant administration of anti-PD1 antibodies could help, specifically in those leukemia subtypes known to have PD1 or PD-L1 upregulation. Finally, the best target on leukemic cells is far from been identified. Few antigens restricted to LSCs are known, their expression (yes or no) and their intensity can be variable and may change under chemotherapeutic pressure or at disease relapse. It must be underlined that the currently available information has been obtained in relapsed and often heavily pretreated subjects.

Table 1: Published results of clinical trial of bispecific antibodies in AML.

	Target	**Agent**	**Phase**	**Disease**	**Outcome**	**NCT ID**
BiTE	CD33xCD3	AMG330	I	R/R AML	CR+CRi:19%	NCT02520427
					CRS:67%	
	CD33xCD3	AMV564	I	R/R AML	CR+CRi:66.7%	NCT03144245
					CRS:5.7%	
	CD33xCD3HLE	AMG673	I	R/R AML	Blast reduction:44%	NCT03224819
					CRS:50%	
	CD123xCD3	APVO436	I	R/R AML	Blast reduction: 7%	NCT03647800
					CRS:18%	
	CD123XCD3	XmAb14045	I	R/R AML	CR+CRi:14%	NCT02730312
		(Vibecotamab)			CRS:59%	
DART	CD123xCD3	Flotetuzumab	I/II	R/R AML	CR/CRi:27%	NCT02152956
					CRS13%	
TRiKE	CD33xCD16XIL15	1-GBT-3550	I	PIF R/R AML	SD:50%; POD:25%	NCT03214666
					No toxicity	

BiTE: bispecific T cell engagers; DART: Dual affinity Re-Targeting Molecules (BiTE); HLE: half-life extended; TRiKE: tri-specific killer engager; R/R: relapsed/refractory; CR: complete remission; CRi: complete remission with incomplete hematologic recovery; SD: Stable disease: POD: progression of disease; PIF: primary induction failure; CRS: cytokines release syndrome.

T- and NK Cell Therapies

CAR-T cells are engineered autologous peripheral T cells equipped with a synthetic target-antigen receptor (CAR), able to expand after transfusion in a target-antigen dependent matter and designed to persist after infusion, eventually generating a long-term anti-leukemic memory. Binding CAR to the target antigen initiates an intracellular signaling leading to TCR-independent T cell activation. CAR structure consists of four main components, each of which can affect CAR-T functionality: (1) an extracellular target-antigen binding domain, (2) a hinge region, (3) a transmembrane domain, (4) one or more intracellular signaling domains. Extracellular domain structure is crucial not only for antigen recognition, but also for affinity and specificity of binding. Too low or too high affinity may result in activation induced death of CAR-T cells and trigger toxicities. The hinge region confers flexibility, allowing the antigen binding domain to access the target epitope and to form immunological synapses. The most used hinge regions are derived from aminoacidic sequences from CD8, CD28, IgG1 and IgG4. Transmembrane domain influence CAR expression level and stability. Most transmembrane domains are derived from natural proteins including CD3ζ, CD4, CD8α, CD28. A CD3ζ transmembrane domain probably facilitates T cell activation, mediate CAR dimerization and incorporation to endogenous TCRs [152]. However, a CD3ζ domain is less stable than the CD28 one. Hinge region and transmembrane domain influence CAR-T cell cytokine production and activation induced cell death (AICD), while intracellular domains influence activation magnitude and persistence of CAR-T cells. The first generation of CAR-T cells has a single signal domain (CD3□), the newer generations have additional costimulatory structures, such asCD28 or CD137 and 4-1BB for 2nd generation CAR-T and CD28plus, CD137 or CD134 (OX40) for the 3rd generation CAR-T [153]. The highly suppressive microenvironment and the fact that target antigens are frequently expressed also in normal hematopoietic counterpart represent a big challenge for CAR-T cell therapy. Nonetheless, preliminary result of CAR-T cell therapy in AML demonstrated positive results, and more than 20 trials are currently ongoing. A summary of principal active trials is summarized in Table 2.

Table 2: Summary of preliminary results of CAR T /CAR NK cell therapy for AML.

Product	Target antigen	Phase	Disease State	Clinical Outcome	NCT ID
CAR T (2nd gen)	Lewis-Y Ag	I	R/R	Transient Blast decrease 1/4pts	NCT01716364
	CD33	I	R/R	Marked blast decrease 1pt	NCT02799680
CAR T	NKG2D	I	R/R	CRh+CRi 3/7pts	NCT02203825
Compound CAR T	CLL-1 and CD33	I	R/R	MRD neg 1 pt	NCT03795779
CAR-NK	CD33	I	R/R	Decrease of MRD 1/3pts	NCT02944162

R/R: relapsed/refractory; MRD: minimal residual disease; CRh: hematologic complete remission; CRi complete remission without complete hematologic recovery

Target antigens under investigation in CAR-T cell therapy trials are CD33, CD123, CLL-1, CD38, NKG2D, and CD7.

CD33 is expressed in almost all AML subtypes. A Chinese clinical trial investigating the feasibility of anti-CD33 CAR-T therapy suggests a potential benefit but reporting severe side effects (fever, pancytopenia CRS) [154]. Anti-CD123 CAR-T cells showed potent anti leukemic activity in vitro, but difficulties in differentiating CD123 expression on leukemic cells and normal hematopoietic cells limit their utilizations and new strategies are needed to protect normal cells. CD44v6 is an adhesion protein supposed to favor leukemogenesis and to contribute to LSCs phenotype. Anti-CD44v CAR-T cells have showed potent in vitro anti leukemic effect, sparing normal HSCs. Feasibility and safety of CD44v6 CAR-T cells are under investigation in a phase I ongoing trial (NCT040097301). Even CLL-1 has been proposed as a target antigen for CAR-T cell therapy: in vitro studies and mice models showed encouraging results, with good distinction between normal and leukemic cells [155]. With these premises a phase I trial using CD33-CLL1 dual CAR-T cells has been activated (NCT03795779).

Preliminary results presented at the 2020 European Hematology Association Annual Meeting reported MRD negativity in 7/9 treated patients [156]. Clinical therapeutic and safety of CD38-targeted CAR-T cells were investigated by Cui and coll. in AML patients relapsing after allogeneic HCT (NCT04351022). Four weeks after infusion 4/6 patients achieved CR/CRi with manageable side effects and LFS was 7.9 months [157]. Ultra-CAR-T, simultaneously expressing CD33 and IL-15 were used in R/R AML and high-risk MDS patients in a phase I, dose finding, trial (NCT03927261): ORR was 50%, with good safety and tolerability [158]. Preclinical studies evaluating double targeting of FLT3 with CAR-T cells and the FLT3 inhibitor gilteritinib seems to suggest potent leukemia clearance and normal HSCs protection [159]. Appelbaum and coll. explored the use of controlled anti-CD33 CAR-T cells (SC-DARIC33), genetically modified to express a Dimerizing Agent Regulated Immunoreceptor Complex (DARIC) controlled by post-infusion intermittent doses of rapamycin, in a phase I trial in pediatric and young adult patients with R/R CD33+ AML (NCT05105152). Finally, NK CAR cells targeting different AML cell antigens (CD70, CD33 and FLT3, CLL-1) exhibit potent anti-leukemic effect "in vitro" for their powerful cytotoxic effect with limited off-target side effects and unique recognition mechanism [160-162], and are at present in preclinical evaluation. CAR-T cell therapies are expected to revolution the treatment of hematologic malignancies; however, beyond the enthusiasm for being the first truly innovative cellular therapy since allogeneic transplantation, many obstacles remain to be overcome. In AML the ideal target antigen should be specific for LSCs, constantly expressed through all disease phases and easy to be monitored. At the same time, CAR-T cell production requires refinements to improve target antigen recognition, to assure a binding strength sufficient to activate T cells without causing activation-induced cell death, so limiting off target toxicity and prolong CAR-T cell persistence.

Conclusions

The enormous advances made in the past years regarding molecular and cellular mechanisms leading to immune dysfunction and leukemia tolerance, the encouraging results

obtained in lymphoproliferative diseases and in solid tumors, and the advances in molecular analysis and sequencing technologies have generated great expectations on the possibility to manipulate immune system and BM niches in AML. Despite interesting preliminary data, we are far from having a "magic bullet" to eradicate leukemia cells. Effective targets on AML cells must be identified, but this effort is complicated by the fact that LSCs and HSCs share various antigens, making immune therapy toxicities often unacceptable, and by a mutation rate that in AMLs is 40-fold less compared to solid tumors, significantly reducing the chance of appearance of neo-antigens. This could be counterbalanced by a much easier accessibility to AML cells and by their susceptibility to killing.

The possibility to target the BM niche, thus reversing the immunosuppressive microenvironment, is a promising approach. Combined therapies need to be studied to harness immune function, taking advantage of peculiar activities of conventional drugs, such as cytarabine, which is known to increase expression of MHC class II and CD80 costimulatory molecules and to downregulate PD-L1, or hypomethylating agents that increase expression of many immune-related genes, or multi-tyrosine kinase inhibitors that can induce release of cytokines potentiating T and NK response.

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