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Building upon the success of CART19: chimeric antigen receptor T cells for hematologic malignancies

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Abstract

Chimeric antigen receptor T cell (CART) therapy has dramatically changed the therapeutic prospects for B cell malignancies. Over the last decade CD19-redirected CART have demonstrated the ability to induce deep, long-lasting remissions and possibly cure patients with relapsing B cell neoplasms. Such impressive results with CART19 fostered efforts to expand this technology to other incurable malignancies that naturally do not express CD19, such as acute myeloid leukemia (AML), Hodgkin lymphoma (HL) and multiple myeloma (MM). However, to reach this goal, several hurdles have to be overcome, in particular: i the apparent lack of suitable targets as effective as CD19; ii. the immunosuppressive tumor microenvironment; iii. intra-tumoral heterogeneity and antigen-negative relapses. Therefore, new strategies that allow safer and more potent CART platforms are under development and may provide grounds for new exciting breakthroughs in the field.

The CAR immunotherapy revolution and the CD19 paradigm

Chimeric antigen receptor-based immunotherapy constitutes one of the most significant breakthroughs for the treatment of hematologic malignancies, in particular B cell neoplasms. [1] The revolutionary idea behind the success of this therapy is the development of a synthetic protein, the chimeric antigen receptor (CAR), that is able to redirect otherwise inoffensive T cells against cancer cells. [2,3] A CAR typically includes an antigen binding domain, most commonly a single-chain variable fragment (scFv) obtained from a monoclonal antibody (mAb), a co-stimulatory domain (commonly derived from 4-1BB or

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CD28) and the intracellular signaling domain of the T cell receptor (CD3 ζ chain) [4]. The introduction of the CAR transgene into patient's T cells enables them to engage a surface tumor-associated antigen (TAA) triggering T cell activation and cytotoxicity against the malignant cell.

CD19-specific CAR T (CART19) cells have led to unprecedented results in the treatment of B-cell acute lymphoblastic leukemia (B-ALL), with up to 90% complete remissions (CR) and durable molecular responses reported in relapsing/refractory (r/r) patients [5–9]. As a result, the United States of America Food and Drug Administration (FDA) recently approved the University of Pennsylvania/Novartis CART19 product (Kymriah(TM)/ tisagenlecleucel, formerly CTL019) for the treatment of children and young adults with r/r ALL. Sustained CR over 4 years after CART19 cell therapy have been described also in a subset of heavily pre-treated chronic lymphocytic leukemia (CLL) patients [10]. Recently, more than 70% responses, including more than 1 year CR, have been also reported in refractory diffuse large B cell lymphoma (DLBCL) [11–13]. Such an impressive success is partly explained by the unique nature of the tumor target CD19. CD19 is a surface antigen, highly and homogeneously expressed on malignant B cells and relatively tumor-restricted. The only non-malignant cells expressing CD19 are normal B lymphocytes, and their depletion (B cell aplasia) is clinically manageable in most patients. Therefore, identification of suitable TAA represents the first step in attaining clinical success in hematologic malignancies that do not express CD19, such as acute myeloid leukemia (AML), Hodgkin lymphoma (HL) and multiple myeloma (MM). However, synthetic biology and gene-editing technologies could increase the therapeutic index of CART for these malignancies using the currently available targets.

1. Extending the CART technology to hematologic malignancies that do not express CD19

Patients with relapsing or refractory hematologic malignancies have usually poor prognosis [14]. The current standard treatments for such patients often have limited clinical impact, thus highlighting an unmet need for more effective therapeutic strategies. Durable remissions and even cures in patients with AML, MM and HL attributed to the immune-mediated graft-versus-tumor effect following allogeneic HSCT (allo-HSCT) [15–25] underscore the notion that the immune system is capable of eradicating these malignancies. This, together with the promising results reported with CD19-specific CAR T cells in B cell neoplasms prompted the pre-clinical development and clinical investigation of CAR-based immunotherapy in other hematologic malignancies that do not express CD19.

2a. Pre-clinical and early clinical experience using conventional CAR-based approaches for AML, HL and MM

Acute myeloid leukemia—Despite significant advances in the understanding the cellular and molecular biology of AML over the past two decades, little progress has been made in the treatment strategies [26]. The development of effective CAR-based immunotherapy for AML is one of the biggest challenges in the field but it is hampered by the lack of suitable

targets and the diverse cellular architecture of AML [27,28]. Several targets are being evaluated for CART-based therapy of AML in both the preclinical and clinical setting.

CD33 is a transmembrane receptor of the sialic-acid-binding immunoglobulin-like lectin family involved in inflammatory and immune responses [29]. It is generally expressed on AML blasts [30–32], but also on normal hematopoietic cells, including the hemopoietic stem cell (HSC) and myeloid progenitors (Figure 1), and on Kupffer cells in the liver [33–35]. Gemtuzumab ozogamicin (GO), a calicheamicin-conjugated anti-CD33 antibody, has been associated with potent anti-leukemia activity, but also some clinical toxicity [36,37]. Similarly, CART33 cells featuring a GO-derived scFv exhibited potent activity against AML cell lines and primary AML cells *in vitro* and *in vivo*, leading to improved survival in AML xenograft models, although with evidence of hematopoietic toxicity [38]. Two clinical trials (NCT01864902 and NCT02799680) are currently recruiting relapsed/refractory CD33+ AML patients in China. The first patient treated with multiple autologous CART33 cell infusions achieved a transitory partial remission (PR) but rapidly progressed three weeks post CAR T cell transfer. Febrile reactions were reported in association with each cell infusion with high levels of IL-6, TNF-α and INF-γ. Profound pancytopenia and transient, mild hyperbilirubinemia were recorded after escalated CART cell dosing [39].

Compared to CD33, the interleukin-3 receptor α chain, CD123, is more frequently expressed on the AML leukemia stem cell (LSC), but it is also present on normal hemopoietic HSC and progenitors (Figure 1) [40], monocytes and some subsets of endothelial cells. Antibody-based targeting of CD123 has shown anti-leukemic activity *in vitro* and in animal models [41,42] and some initial clinical activity in AML patients [43–48]. However, three fatal events associated with capillary leak syndrome (CLS) were reported following treatment with the SL-401, a fusion molecule composed of the catalytic and translocation domains of the diphtheria toxin fused to IL3 (NTC02113982). This side-effect could potentially be linked to CD123 expression in endothelial cells.Preclinical studies of CART123 cells showed, together with potent anti-AML activity, severe impairment of normal hematopoiesis [40], raising concerns about ‘on-target’, ‘off-tumor toxicity’. Several trials are currently evaluating anti-CD123 CAR immunotherapy. A case report from a Chinese group, showed reduction in BM blasts after infusion of a 4th generation CART123, associated with rigorous chills and fevers, low blood pressure and hypoxemia one day after infusion. [49] A phase I trial (NCT03190278) evaluating 3rd party, CART123 cells (UCART123, Cellectis) for r/r AML, was recently placed on hold by the FDA after the death of the first patient treated with due to CLS. In UCART123 cells, the endogenous TCRα gene is deleted using TALEN-based gene editing to prevent acute graft-versus-host disease [50]. While no GVHD was reported, lethal CLS occurred early post UCART123 infusion, confirming the preliminary clinical evidence with SL-401 suggesting a link between CLS and CD123-targeted immunotherapy. The toxicity of autologous CART123 cells is still under evaluation but safety measures were included to control CART123 toxicity, in particular: transient delivery of CAR constructs through RNA-electroporation (NCT02623582), co-expression of EGFR- (NCT02159495) and CD20-derived (NCT03190278) depletion genes (please also see section 3a) and inducible suicide systems (NCT03125577[49]). Based on the evidence that CAR cell toxicity against normal cells is proportional to potency against malignant cells [28], pursuing CD123 targeting may

perhaps require alternative CAR designs employing a different scFv clone associated with lower antileukemic activity *in vitro* to ensure an acceptable safety profile. [51].

CLEC12A (also known as CLL1) was proposed as a LSC marker [52] and has been recently shown to be expressed on chemorefractory AML blasts. Second generation CLEC12A-specific CAR T cells were highly reactive against CLEC12A+ AML cell lines and primary cells *in vitro*, leading to eradication of minimal residual disease and prolonged survival in AML-bearing mice that received induction chemotherapy followed by CART-CLEC12A immunotherapy. [53] Anti-CLL-1 CART were proven to be active against a subset of AML samples both in vitro and in vivo, without depleting the HSC but causing toxicity on mature myeloid cells. For this reason the authors suggest that depletion of CART-CLL-1 cells once leukemia is cleared could restore the myeloid compartment thanks to the intact HSC [54].

In keeping with the compelling need for more effective therapeutic targets in AML, the search for new candidates continues and a number of novel potential targets have been proposed [28,55]. The Lewis Y antigen [56–58], the NKG2D ligands [59], CD44v6 [60] and CD133 [61] are expressed by the AML cells and the hematopoietic system and, are also expressed on other types of cancer cells. However, expression of these antigens on healthy tissues calls for careful evaluation of potential toxicity of these CART approaches. Additional targets include the FMS-like tyrosine kinase 3 (FLT3) that is a well characterized antigen with high relevance to AML pathogenesis and strongly associated with poor clinical features. FLT3-targeted CAR T cells have shown promising anti-leukemic activity *in vitro* and *in vivo*[62]. As CD7 could be aberrantly expressed in AML blasts, an ongoing trial (NCT02742727) is also evaluating CD7-directed CAR-modified NK-92 cells in AML and T cell-malignancies. Further optimization may require additional manipulation, i.e. CD7-knockout of the desired T/NK effector cells prior to CAR engineering to prevent fratricidal killing and enhance antitumor activity [63].

Hodgkin Lymphoma—Classical Hodgkin lymphoma is a unique entity among mature B cell malignancies. Despite of their B cell origin, Hodgkin Reed-Sternberg (HRS) cells do not typically express B cell antigens like CD19. Therefore, direct tumor killing cannot be achieved with CART19. Instead, nearly all HRS cells express CD30 (Figure 1), a tumor necrosis factor receptor, that delivers pro-survival signals through activation of signaling pathways such as PI3-kinase/Akt/mTOR, ERK/MAPK and NF-κB [64]. CD30 has been the focus for therapeutic targeting by immunoconjugates like brentuximab vedotin with valuable clinical efficacy and acceptable toxicity [65]. CAR30 effectors, including EBV-specific cytotoxic T lymphocytes (CTL), were shown to effectively target CD30+ Hodgkin cells lines *in vitro* and *in vivo* [66–69]. Importantly, despite expression of CD30 by a fraction of activated CD30-specific CAR T cells and also by unmodified T cells, no apparent fratricidal killing, impaired cellular immune responses or reduced overall performance of CD30-specific CART cells were observed. On this basis, early clinical trials have been testing the safety and efficacy of CD30-specific CAR T cells in r/r HL patients, using different CAR designs, viral vectors and therapeutic schedules (see Table 1). In one study (NCT01316146), out of nine patients, one experienced CR and another 4 patients had stable disease (SD) resulting in an overall response of 67% [66,70]. In another study involving 18 HL patients with progressive disease, (NCT02259556 [71]) the overall response rate was 72% (13/18, of

which 7 PR, 6 stable SD, no CR), including in patients who had undergone previous autoHSCT and treatment with brentuximab [71]. No cases of CRS were observed. Of note, the observed CART30 cell clinical activity was attained without prior chemotherapy preparatory conditioning and required repeated CAR T infusions. Overall, interim analyses from these 3 clinical studies suggest modest clinical efficacy with mild toxicity. Objective responses seemed to correlate with higher CAR T cell doses within all studies.

Multiple Myeloma—Multiple myeloma is an incurable plasma cell dyscrasia characterized by cellular and genetic heterogeneity. While molecules such as CD138, CD38 and CD56 are expressed on all or the majority of malignant plasma cells, their broader expression on healthy non-hematopoietic tissues makes them potentially problematic as CAR targets. By contrast, B-cell maturation antigen (BCMA), CD19 and CS-1 appear more promising thanks to their reduced off-tumor expression (Figure 1) [72].

BCMA (CD269) is a surface molecule that belongs to tumor necrosis factor receptor family and it is only expressed by late stage B lineage cells [73]. It delivers pro-survival signals to late-stage, mature B cells/plasma cells upon engagement by APRIL or BAFF ligands [74]. BCMA targeting by either therapeutic mAb [75] or CAR T cells [76–79] has generated significant anti-myeloma activity *in vitro* and *in vivo.* An ongoing phase I trial at National Cancer Institute (NCI) has demonstrated that BCMA-specific CAR T cells are capable of inducing CR in r/r myeloma patients with high disease burden. Results from 12 patients showed an objective response rate of 33% (1 CR, 2 VGPR and 1 PR), lasting up to at least 26 months, and SD in all remaining cases [80]. Similar to ALL and CLL patients treated with CART19 cells, the depth of remissions correlated with CAR cells dose and persistence as well as occurrence of cytokine-release syndrome (CRS). Importantly, in line with pre-clinical data [79], serum BCMA, which is abundant in myeloma, did not impact clinical outcome. However, tumor escape associated with appearance of BCMA-negative myeloma cells has been observed in 1 out of 12 cases [80]. In an effort to mitigate against immune escape, an APRIL-based CAR which can bind to and target both BCMA and TACI, another APRIL/BAFF receptor heterogeneously expressed on myeloma plasma cells [81,82], is in pre-clinical development [77]. Two recent reports from the American Society of Clinical Oncology (ASCO) annual meeting (see Table 1) showed high rate of responses after BCMA-CART (NCT03090659, NCT02658929). Fan F et al. (#LBA3001) treated 19 MM patients with their LCAR-B38M anti-BCMA CAR-T (Nanjing Legend Biotech Inc.) and obtained 100% ORR (1 PR, 4 VGPR, 14 CR). Berdeja JG et al. (#3010) had 100% ORR in 6 evaluable patients treated with bb2121 anti-BCMA CART (bluebird bio), including 2 sCRs and 2 MRD-negative responses.

The rationale of CAR targeting CD19 in MM is based on the notion that CD19 is expressed on the putative myeloma stem cell, with self-renewal capacity, myeloma-propagating potential and chemo-resistance features [83]. Despite the fact that only a small minority of malignant plasma cells expressed CD19 (as low as 0.05%), a pilot trial (NCT02135406) reported 80% ORR in 10 r/r myeloma patients receiving a single infusion of 5×10e7 CTL019 cells combined with standard auto-HSCT chemotherapy conditioning regimen, including 6 VGPR [84]. Toxicity was mild, with only one case of grade 1 CRS. Ultimately, all patients experienced disease progression, which occurred earlier than after the first HSCT

with the exception of 2 patients. Interestingly, the time to progression was found to correlate with CART19 cell peak in the bone marrow rather than their frequency and persistence in PB. On this basis, it has been speculated that CART19 would specifically kill the small CD19+, stem cell like clone while cytotoxic therapies target the bulk tumor. A phase II study is currently assessing the activity of 10-fold higher doses of CART19 cells in high risk patients following autoHSCT (NCT02794246).

CS-1 (SLAM7) is a SLAM receptor involved in the cross-talk between myeloma and surrounding stromal cells which is crucial for tumor initiation and progression [85]. CS1 is highly expressed on both malignant and normal plasma cells although also on activated NK and T lymphocytes [86]. The administration of the anti-CS-1 mAb elotuzumab has been proved to positively impact on clinical responses and outcome in combination with anti-myeloma agents, paving the way for further development of CS-1 targeted immunotherapy in MM [87,88]. Likewise, anti-CS1 CAR T cells have been successfully tested against primary myeloma cells *in vitro* and exhibited promising anti-tumor activity *in vivo* [88–91]. Further optimization, including TALEN-induced knock-out of CS-1 in T cells [92], aims to fully exploit the therapeutic potential of CS-1 targeted CAR strategies. This approach is expected to eliminate the risk of CS-1-mediated fratricidal effect of CS-1-specific CAR T cells that could negatively impact on CAR cell manufacturing, immunotherapy outcome as well as homeostasis and function of patients' immune system.

As mentioned, additional targets with broader off-tumor expression like CD38, CD138 and others [72,93,94] have been developed for MM and are currently being evaluated pre-clinically or in early-phase clinical trials (NCT01886976; NCT02203825).

2. Novel CART cell approaches aimed to increase the therapeutic index of CART for hematologic malignancies

While CD19 has served as an optimal CAR target for B cell malignancies, the search for similar antigens for non-CD19+ hematologic malignancies is still on going. In AML most of the potential targets are also expressed on healthy hemopoietic progenitors and HSC (Figure 1), thus carrying a potential risk of myelotoxicity that could limit their clinical application. For MM, BCMA certainly represents a "CD19-like" antigen, however, BCMA-negative relapses were observed in early BCMA-CART clinical trials [80,95,96](Figure 1) [80]. For HL there is still little experience in the clinic with CART immunotherapy, but CART against CD30 have led to some clinical response and other targets like CD123 are being evaluated. Hence, while the quest for identifying the best antigen target continues, a conceptual shift is already taking place seeking to optimize CAR technology using the currently available targets (Figure 1). These approaches include: a. management of toxicity; b. increasing specificity; c. targeting intracellular targets; d. targeting the tumor microenvironment and e. targeting multiple antigen on tumor cells.

3a. Management of toxicity: controlling CART effector functions

As discussed, most of the currently available antigens to target AML, MM and HL are also expressed in normal tissues. Therefore, the ability to control T cell effector functions over

time would be highly beneficial to treat possible toxicities. To this end, three main strategies have been adopted in pre-clinical and clinical settings: i. suicide genes, ii. antibody-mediated depletion and iii. CAR RNA-electroporation.

The inducible caspase9 (iC9) includes a drug-dimerizer binding domain, cloned in frame with human caspase9 [97]. In the presence of the dimerizer drug, (e.g. AP-20187 or the clinical-grade equivalent AP-1903), the caspase9 pro-molecules dimerize and rapidly activate the mitochondrial apoptotic pathway, terminating allo-T cell-mediated GVHD in alloHSCT recipients as early as 30 minutes [98]. Another strategy for CART depletion, is the co-expression of depletion markers, such as truncated, biologically inert CD19, CD20 or EGFR, to allow selection/*in vivo* ablation by the corresponding mAb [91,92]. Lastly, CAR mRNA electroporation allows transient expression of the CAR as opposed to stable integration and expression following viral delivery. Such strategies have been explored either alone [38,99] or in combination [98] for CART123 and CART33 immunotherapy.

Safety switches may be key to implement clinical development of CAR immunotherapy in AML, MM and HL. However, there are some flaws inherent to each approach. In iC9-based systems, a population of caspase-resistant CAR T cells has been recently described, characterized by overexpression of anti-apoptotic molecules, such as Bcl2, compared to their caspase-sensitive counterparts [98]. By contrast, although not yet tested in the clinic, no 'escape' has been observed in association with mAb-based strategies in preclinical models [38,100,101]. Transient expression of the CAR construct after mRNA electroporation may result in either incomplete clearance of the malignant clone or loss of long-term immunosurveillance and consequent relapse. In facts, all depletion strategies can negatively impact CAR immunotherapy outcome by reducing the persistence of CART cells. However, compared to the progressive, stochastic loss of activity following CAR mRNA transfection, depletion genes offer the advantage to exert a temporal control of CART lifespan by triggering active termination exactly at the occurrence of severe adverse events, if any. In addition, it might be possible to identify a 'window of opportunity' that could be exploited for short-term interventions [100], allowing enough time to achieve disease eradication before the occurrence of relevant off-target toxicity [100], thus increasing the number of patients with deep and sustained remissions eligible for HSCT [38,101]. More recently, ON/OFF-switch CARs have been developed with the aim to remotely control and manipulate immune cell function. One version is based on constitutively inactive, heterodimeric CARs, consisting of one chain carrying the scFv-binding sequence and a separate chain with the signaling domains and a small molecule-dimerizer domains. Timely regulated administration of appropriate chemical compounds, would allow to control assembly and disassembly of the CARs thus modulating immune cell activation *in vivo* [102,103]. Similarly, tetracycline (Tet)-inducible systems have been investigated to modulate the expression of CAR constructs inserted into a pRetroX-TetOne third-generation vector. [104]. Another option is represented by downregulatory feedback circuits that dampen immune cell reactivity in response to signals of hyperactivation such as an excess of IL-6, thus limiting the risk of severe toxicity[102]. Although less practical, such technologies would have the advantage of preventing the permanent ablation of CAR T cells and the option of resuming their therapeutic effect and immunosurveillance in the long-term. Lastly, as shown in Figure 1, a novel interesting approach [105] includes genetic engineering of the normal HSC in order to

render them invisible to CART33. In a preclinical model, HSC were knocked out for CD33 using the CRISPR-Cas9 technology and engrafted in immunodeficient mice carrying CD33+ AML. Of note, upon CART33 infusion AML cells were cleared but HSC and hemopoiesis in general were spared.

3b. Increasing specificity: modulating CAR affinity and recognition

CAR switches represent the first requirement to improve safety of CAR T immunotherapy. Yet additional strategies need to be considered to improve precision and achieve higher specificity. Modulation of scFv affinity and CAR expression can be used to discriminate between high- (malignant) and low- (normal) antigen-expressing cells in a temporally controlled manner [106–109]. A low affinity CD123-specific CAR was designed to trigger robust lytic response only against targets expressing >1600 CD123 molecules, i.e., typically leukemic cells [110]. Another anti-CD123 CAR characterized by reduced lysis of normal HSC, but full anti-AML activity *in vivo* was generated by combining different VH and VL chains derived from four CD123-specific mAbs [111]. A similar ‘light chain exchange’ approach has been successfully applied in other contexts, e.g. CD38-specific CAR T cells tested against multiple myeloma cells [112]. Although more functional studies would be required before testing such approach into clinical settings, these preclinical findings suggest that tuning of CAR affinity and antigen expression on malignant cells may represent a valid option to increase the therapeutic index and the range of potential applications of CAR immunotherapy.

Finally, synthetic biology has been recently applied to generate smart CAR platforms and micro-circuits that would provide T cells with the ability of discriminating between healthy and cancer cells *in vivo* by integrating the information from a defined pattern of antigens. CAR AND-gate circuits consist of two distinct CAR constructs, one endowed with the CD3z activating domain and the other with a costimulatory motif, to allow full activation only against tumor cells expressing the ‘right’ combination of antigens engaging both CARs at the same time [113–115]. Since the presence of a single antigen might still be sufficient to trigger a cytotoxic response [102], in an alternative approach one construct was created to serve as a ‘sensor and primer-CAR’ provided with a Notch-derived regulatory transmembrane region and an intracellular transcriptional activator motif (SynNOTCH) [116,117]. Upon engagement of the first antigen, such CAR undergoes intramembrane cleavage, followed by translocation of the intracellular domain to the nucleus and induction of an ‘effector’ CAR. Further T cell proliferation and killing occur when the opposing cell also presents the ligand for the second receptor. Another strategy to potentially achieve more specific tumor recognition is with CAR NOT-gate circuits combining one conventional activating with one inhibitory CAR (iCAR), i.e., a receptor equipped with an intracellular CTLA-4- or PD-1-derived domain overriding the signal delivered from the first construct, thus preventing reaction against bystander (normal) cells. [118]

3c. Expanding the available antigens: targeting intracellular targets

To circumvent the relative dearth of suitable cell surface targets, T cells could also be redirected against intracellular antigens. Indeed, there is indirect evidence that AML, HL and MM are suitable for such approaches. For instance, adoptive transfer of *ex vivo*

expanded autologous EBV-specific CTLs, recognizing the EBV-derived latent membrane proteins 1 and 2 (LMP1 and LMP2), induce objective responses in patients with r/r EBV+ HL [119,120]. More recently, T cells specific for intracellular tumor-associated antigens, i.e. PRAME, SSX2, MAGEA4, NY-ESO-1 and survivin, were shown to induce complete responses in patients with HL in the absence of conditioning chemotherapy [121,122]. Spontaneous as well as post vaccination humoral and cellular immunity against the NY-ESO157-165/HLAA*02:01 complex have been also observed in myeloma patients [123]. Similarly, antibody and CTL responses have been detected in AML patients against a pool of immunogenic peptides, which are relevant to the tumor biology and correlate with poor prognosis [124,125]. Building upon these encouraging results, considerable efforts have been initially devoted to the clinical development of TCR-like CARs provided with a single scFv recognizing the MHC:peptide complex. Anti-WT1/HLA-A*02:01 CARs have been already successfully tested in AML patients [126–128]. Additional candidates under pre-clinical investigation include cancer testis antigens NY-ESO-1, in MM [129] and AML; LAGE-1, MAGE-A3, PRAME, proteinase 3, RHAMM and Flt3 in AML [28,62,130,131].

3d. Targeting the tumor microenvironment

Targeting the ‘right antigen’ might be not be sufficient to eradicate cancer cells if CAR T cells have to reach and function in highly immunosuppressive contexts. Therefore, it may be beneficial to redirect CAR T cells against components of the TME. In MM, the myeloid-derived suppressive cells (MDSC) may induce with immune paresis, ultimately promoting myelomagenesis and relapse [60,132,133]. Dependence from pro-survival signals within the TME has been equally demonstrated in HL, where the HRS cells typically represent a small minority of the tumor mass, nurtured and sheltered by the surrounding infiltrate. In addition, tumor-associated macrophages (TAM), as well as HRS cells, generally express increased levels of PD-1 ligands in association with a recurrent genetic amplification at chromosome 9p24.1, thus suggesting that the PD-1/PD-L pathway might represent a key determinant of immunosuppressive TME in HL. Accordingly, PD-1 blockade significantly improved the outcome of r/r HL patients, who reported >65% response rate upon treatment with the PD-1 inhibitors nivolumab and pembrolizumab [134–137].

In order to attack tumor cells, T cells must overcome the immunosuppression of the TME to physically reach them. In a preclinical *in vivo* model of HL co-expression of CD30-specific CAR and exogenous chemokine receptor CCR4 have proved to enhance homing of CAR T cells to the tumor site [138], leading to improved anti-tumor activity with no systemic toxicity [139–141]. Alternatively, CAR-mediated cytotoxicity could be directed against the neighboring immunosuppressive cells. Indeed, CAR T cells recognizing CD123 on TAM within the HL microenvironment as well as in HRS cells effectively killed both TAM and HL cell lines *in vitro* and displayed potent therapeutic activity against disseminated disease in an *in vivo* model of HL (Figure 1) [142,143]. Another proposed approach aimed to overcome the TME immunosuppression in HL includes EBV-specific CTL expressing exogenous IL-12 [144]. Given the promising responses to nivolumab and pembrolizumab, strategies combining CAR technology with checkpoint inhibitors might have a higher therapeutic impact.

3e. Avoid antigen-negative escape: targeting multiple antigen on tumor cells

Following the paradigm of multi-agent chemotherapy, in order to mitigate antigen escape and improve discrimination against healthy cells, ongoing efforts focus on developing CAR approaches that target simultaneously more than one TAA [145]. Although yet-to be tested in clinical settings, several co-targeting approaches have been proved superior to mono-specific strategies in solid and hematological malignancies and could be grouped in two categories: i. “pooled CART” - a mixtures of CAR T cells endowed with different specificities [146], or ii. “multi-specific CART” - a single CAR T cell that can engage multiple antigens thanks to the presence of either 2 different CAR molecules (bi- or dual-CART) [147–149] or a single CAR molecule with two recognition regions (tandem CARs) [150–152]. Early preclinical experience suggests that, provided optimal spatial configuration, dual targeting by tandem CAR may prevent immune escape more effectively than the combination of distinct CARs [153]-[154]. Alternatively, switchable CAR like UniCAR, consist of an array of soluble modular, tagged scFv domains, equally fitting a constant CAR stalk and supplied as required, to add extra flexibility and multi-specificity in a timely controlled manner [155]. Such an approach was specifically developed to simultaneously target CD33 and CD123 on AML blasts [156], and potentially allowing the control of myeloablation.

3. Conclusions and future perspectives

Overall, the majority of MM patients, more than half AML patients and 10-15% of patients with HL will develop r/r disease [14,157]. For these patients there is little chance of attaining long-term remission with conventional treatments. The development of CART immunotherapies for these diseases is currently rapidly advancing but it has become clear that significant optimization of the current CART dogma is required in order to reproduce the clinical success of CART19 in the setting of AML, HL and MM. Synthetic biology and gene-editing technologies are now tools available to researchers to generate CART products specifically crafted for a defined disease. Ultimately, as for CART19, early clinical trials will guide the required improvements for the successful development of CART for AML, HL and MM.

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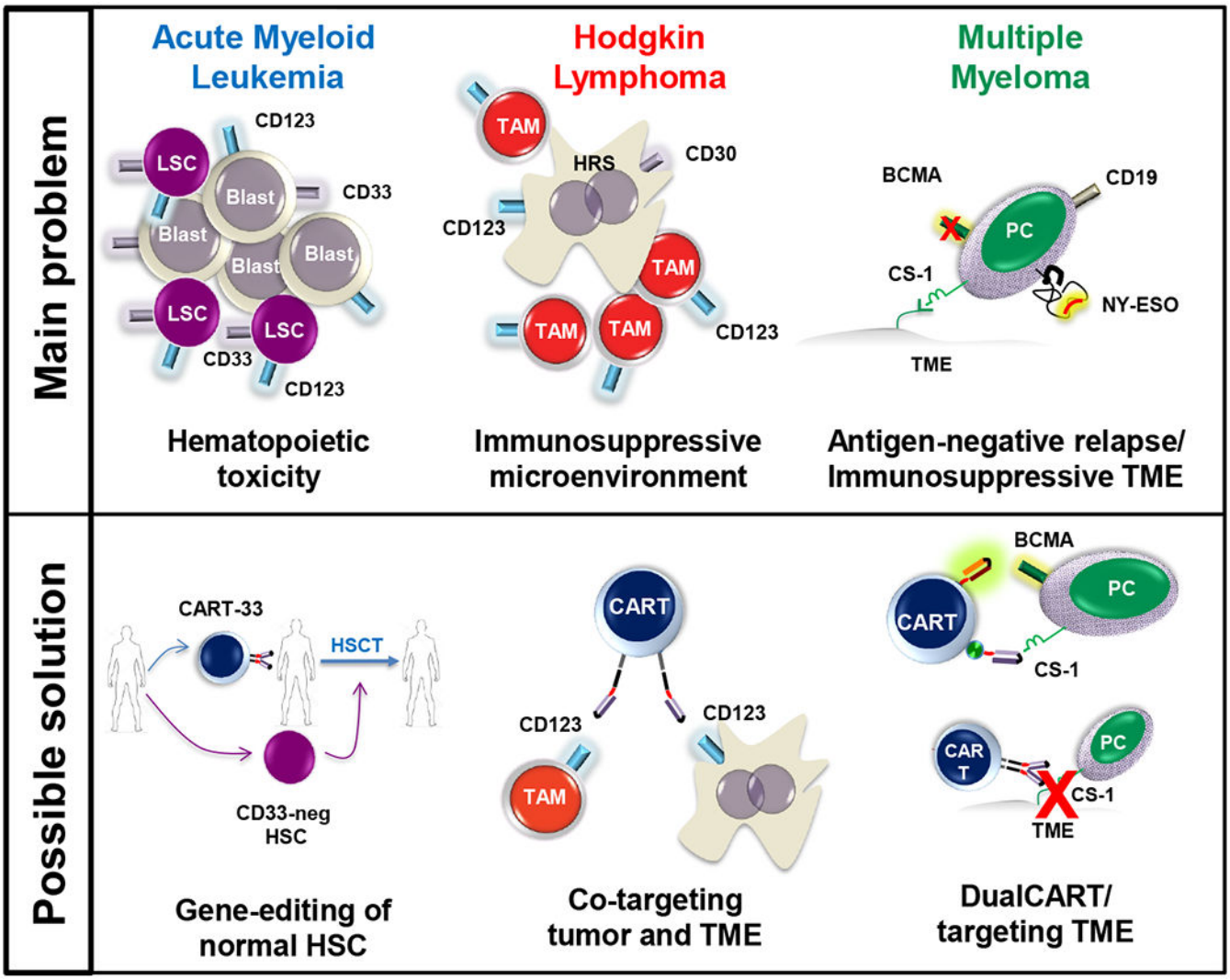

Figure 1.
CAR-cell immunotherapy in hematologic malignancies: challenges and novel approaches.

Table 1.

CAR cell clinical products currently tested in AML, HL and MM patients (as of September 30, 2017)

	Product	Target	Tumor	Start date	Academia	Industry	Vector	Construct (scFv-Hinge-TM-CD-SD)	Additional features	Active clinical trials	Reports
1	CART-LeY	LeY	Myeloid malignancies	Oct-16	Southwest Hospital, China	NA	NA	NA	NA	NCT02958384	NA
2	CM-CS1 T	NKG2DL	AML, MDS, MM	Mar-15	DFCI, USA	Ceylad	Retro	NKG2D-CD3ζ	Single infusion of autologous CM-CS1 cells	NCT02203825 *	Nikiforow S, 2016[158]
3	CART33	CD33	AML	Apr-13	Chinese PLA Hospital, China	NA	Retro	AM402974.1-41BB-CD3ζ	Autologous	NCT01864902	Wang Q-S, 2015[39]
4	CART33	CD33	AML	Oct-15	Chinese PLA Hospital, China	NA	NA	NA	Allogeneic	NCT02799680	NA
5	CD33.CAR T	CD33	Myeloid malignancies	Oct-16	Southwest Hospital, China	NA	NA	NA	NA	NCT02958397	NA
6	Allo-CARpNK-33	CD33	AML	Oct-16	*FPHH*, China	PersonGen BioTherapeutics	Viral	NA-CD28-41BB-CD3ζ	NK-92 clinical grade cell line	NCT02944162	NA
7	CD33.CAR T	CD33	AML	Aug-17	MDACC, USA	NA	Lenti		Autologous, EGFR-derived kill switch	NCT03126864	Song D, 2016 [99]
8	CART-133	CD133	Solid tumors[1], AML, ALL	Sep-15	Chinese PLA Hospital, China	NA	Lenti	HW350341.1-CD137-CD3ζ	NA	NCT02541370	Feng KC, 2017[159]
9	RNA CART123	CD123	AML	Dec-15	UPenn, USA	Novartis	mRNA	NVS2-41BB-CD3ζ	mRNA	NCT02623582 *	NA
10	CART123	CD123	AML	Dec-15	NCI, USA	NA	Lenti	NA-CD28-CD3ζ-EGFRt	Autologous or donor-derived, EGFRt	NCT02159495	NA
11	CD123.CAR T	CD123	Myeloid malignancies	Oct-16	Southwest Hospital, China	NA	NA	NA	NA	NCT02937103	NA
12	CAR123 T	CD123	AML	Mar-17	AHAMMS, China	Shenzhen Geno-Immune Medical Institute	NA	CD123CAR-41BB-CD3zeta-EGFRt	Allogeneic	NCT03114670	NA
13	UCART123	CD123	AML	Jun-17	NA	Cellectis	Lenti	NA-41BB-CD3ζ	TALEN-mediated CD52-TRAC ablation	NCT03190278 **	NA
14	Anti-CD7 CAR-pNK	CD7	AML T cell malignancies	Mar-16	NA	PersonGen BioTherapeutics (Suzhou) Co., Ltd.		NA-CD28-41BB-CD3ζ	Allogeneic, clinical-grade NK-92 cell line	NCT02742727	NA
15	4SCAR123	CD123	AML	Jul-17	FAHZU, China	NA	Lenti	NA/CD28/CD137/CD27/CD3ζ-iCasp9	4th generation iCaspase9	NCT03125577	Luo Y, 2015[49]
16	CART123 CART33	CD123 CD33	AML,MDS ALL	Sep-17	Zhujiang Hospital, China	NA	NA	NA	Combined with Eps8 peptide specific DC	NCT03291444	NA
17	CAR.CD30	CD30	HL, NHL	May-11	BCM, USA	NA	Retro	NA-CD28-CD3ζ	Autologous CAR.CD30 EBV specific-CTLs	NCT01192464 *	NA
18	CAR.CD30	CD30	HL	Oct-11	BCM, USA	NA	Retro	NA-CD28-CD3ζ	NA	NCT01316146 *	Ramos, 2015[70]
19	4SCAR30273	CD30	HL	Mar-14	Peking University, China	NA	Lenti	NA-CD28-CD137-CD27-CD3ζ	4th generation iCaspase9	NCT02274584	Ying Z-T, 2015[160]
20	CART30	CD30	HL	Oct-14	Chinese PLA, Hospital, China	CBMG	Lenti	AJ878606.1-CD137ζ -GFP	Repeated infusions	NCT02259556	Wang C-M, 2017[71]
21	ATLCAR.CD30	CD30	HL, NHL	Jun-16	BCM, USA	NA	Retro	HRS3-CD28-CD3ζ	3rd line therapy	NCT02690545,	NA
22	ATLCAR.CD30	CD30	HL, NHL	May-16	BCM, USA	NA	Retro	HRS3-CD28-CD3ζ	2nd line therapy	NCT02663297	NA
23	CD30.CAR T	CD30	HL, NHL	Oct-16	Southwest Hospital, China	NA	NA	NA	NA	NCT02958410	NA
24	Anti-CD30 CAR T	CD30	Hl, NHL	Mar-17	NCI, USA	NA	Retro	NA	Autologous T cells, fully human anti-CD30 CAR	NCT03049449	Kochenderfer J, 2017 [161]
25	CD30.CAR T	CD30	HL, NHL	May-17	BCM, USA	NA	Retro	NA-CD28-CD3ζ	No previous autologous tHSCT	NCT02917083	NA

	Product	Target	Tumor	Start date	Academia	Industry	Vector	Construct (scFv-Hinge-TM-CD-SD)	Additional features	Active clinical trials	Reports
26	CART	CD123, CD30, BCMA	Myeloid/lymphoid malignancies	Dec-16	NA	Hebei Senlang Biotechnology	NA	NA	Autologous	NCT03121625	NA
27	Anti-BCMA CAR-T	BCMA	MM	Aug-14	NCI, USA	NA	Retro	Anti-BCMA-CD28-CD3z	Autologous	NCT02215967	Ali SA, 2016[80]
28	CART-BCMA	BCMA	MM	Sep-15	UPenn, USA	Novartis	NA	Anti-BCMA-41BB-CD3z	Autologous	NCT02546167	NA
29	LCAR-B38M	BCMA	MM	Oct-15	NA	Nanjing Legend Biotech	NA	NA	NA	NCT03090659	Frank XF, 2017 [162]
30	bb2121	BCMA	MM	Jan-16	NA	Bluebird Bio	Lenti	Anti-BCMA-41BB-CD3z	Autologous	NCT02658929	Berdeja J, 2017[163]
31	CART-138/BCMA	CD138/BCMA	MM	Sep-16	FAHSU, China	NA	NA	NA	Autologous or allogeneic	NCT03196414	NA
32	Anti-BCMA CAR-T	BCMA	MM, Leukemia, Lymphoma	Oct-16	Southwest Hospital, China	NA	NA	NA	NA	NCT02954445	NA
33	Anti-BCMA CAR-T	BCMA	MM	Feb-17	SAHHUTCM, China	NA	Retro	Anti-BCMA-41BB-CD3z	Autologous	NCT03093168	NA
34	Anti-BCMA CAR-T	BMCA	MM, PCL	Feb-17	MSKCC, USA	Juno Therapeutics	NA	EGFRt/Anti-BCMA-41BB-CD3z	NA	NCT03070327	NA
35	bb21217	BCMA	MM	Aug-17	NA	Bluebird Bio	Lenti	Anti-BCMA-41BB-CD3z	CART cells cultured with a PI3k inhibitor	NCT03274219	NA
36	P-BCMA-101	BCMA	MM	Sep-17	NA	Poseida Therapeutics	piggyBAC transposon	Anti-BCMA.FN3-CD8a-41BB-CD3z	Selection and safety switch genes	NCT03288493	Hermanson DL, 2016[164] Barnett BE, 2016[165]
37	CART-138	CD138	MM	Jun-13	Chinese PLA Hospital, China	NA	Retro	Anti-CD138-41BB-CD3z	Autologous or allogeneic	NCT01886976	Guo B, 2016[166]
38	CART-19	CD19	MM	May-14	UPenn, USA	Novartis	Lenti	FMD63scFv-41BB-CD3z	Autologous, r/r MM	NCT02135406	Garfall A, 2015[167] Garfall A, 2016[168]
39	CART-19	CD19	MM	Jun-16	UPenn, USA	Novartis	Lenti	FMD63scFv-41BB-CD3z	Autologous, day+60 post first-line autoHSCT	NCT02794246	NA
40	CAR-K+ T	κ light chain	MM, CLL, NHL	Jul-09	BCM	NA	Retro	NA-CD28-CD3z	Autologous	NCT00881920	Ramos, 2016[169]

*not recruiting;

**suspended participant recruitment

[1]Liver, pancreatic, brain, breast, ovarian, colorectal

DFCI, Dana-Farber Cancer Institute; *FPHH*, The First People's Hospital of Hefei; *MDACC* MD Anderson Cancer Center; *UPenn* University of Pennsylvania; *NCI* National Cancer Institute; *AHAMMS* Affiliated Hospital to Academy of Military Medical Sciences; *FAHZU*, The First Affiliated Hospital of Zhejiang University; *BCM* Baylor College of Medicine; *FAHSU*, The First Affiliated Hospital of Soochow University; *SAHHUTCM* The Second Affiliated Hospital of Henan University of Traditional Chinese Medicine, *MSKCC* Memorial Sloan Kettering Cancer Center, *TM* trans-membrane domain, *CD* costimulatory domain, *SD* signaling domain, *autoHSCT* autologous hematopoietic transplantation. *NA* not available, *PCL* plasma cell leukemia. Retro: retroviral vector; Lenti: lentiviral vector. r/r: relapsing or refractory; TRAC: TCR-α constant region; DC: dendritic cells; FN3: human tenascin fibronectin type III consensus sequence