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Improving Therapy of Chronic Lymphocytic Leukemia (CLL) with Chimeric Antigen Receptor (CAR) T Cells

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Abstract

Adoptive cell immunotherapy for the treatment of chronic lymphocytic leukemia (CLL) has heralded a new era of synthetic biology. The infusion of genetically-engineered, autologous chimeric antigen receptor (CAR) T cells directed against CD19 expressed by normal and malignant B cells represents a novel approach to cancer therapy. The results of recent clinical trials of CAR T cells in relapsed and refractory CLL have demonstrated long-term disease-free remissions, underscoring the power of harnessing and re-directing the immune system against cancer. This review will briefly summarize T cell therapies in development for CLL disease. We discuss the role of T cell function and phenotype, T cell culture optimization, CAR design, and approaches to potentiate the survival and anti-tumor effects of infused lymphocytes. Future efforts will focus on improving the efficacy of CAR T cells for the treatment of CLL and incorporating adoptive cell immunotherapy into standard medical management of CLL.

Overview of CLL: The Current Rules of the Road for Cellular Therapies

CLL is a malignant disease of mature B cells, but the clinical course is variable; some patients never require treatment, and others have a rapidly progressive and fatal course. Accordingly, current guidelines suggest that therapy should be reserved for patients with symptomatic or progressive disease. The vast majority of CLL patients will at some point develop symptomatic disease requiring therapy. With the exception of cellular therapy with allogeneic stem cell transplantation (SCT), CLL remains incurable with standard treatment options. However, the advanced age of individuals and comorbidities at the time of CLL diagnosis (or need for treatment) can pose a significant barrier to transplant options. SCT carries significant risks of treatment-related mortality, due to toxicities of the conditioning regimen, graft versus host disease (GvHD), and immunosuppression. Many patients are unable to tolerate either the conditioning regimen or the medications used to prevent or treat

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GvHD. In addition, identifying suitably matched donors can be challenging, particularly in non-Caucasian populations. At best, sustained remission of high-risk CLL disease is observed in up to 50% of allogeneic transplant recipients ¹. Finally, the optimal timing of pursuing transplant options is a matter of discussion and research ² particularly because novel agents show significant therapeutic benefit.

Recently described targeted therapies that inhibit B cell signaling pathways such as ibrutinib (an inhibitor of Bruton agammaglobulinemia tyrosine kinase) ³ and idelalisib (PI3 kinase p110 δ inhibitor) ⁴ have demonstrated remarkable activity in CLL. Both ibrutinib and idelalisib (in combination with rituximab) were approved for the treatment of CLL patients who have failed at least one prior therapy, or in first-line when *TP53* is absent or mutated and fludarabine-based therapy is not effective ⁵. Even though these therapies effect robust responses in high-risk CLL, they are administered continuously and have not yet demonstrated the ability to induce cure ^{6,7}.

Patients with CLL who do go on to allogeneic hematopoietic stem cell transplant (HSCT) may achieve long-term durable remissions; these are almost always associated with some degree of chronic GvHD ⁸. Relapse can sometimes be treated with donor lymphocyte infusion, which can re-induce remission ⁹. These two findings suggest that T cells are the active agents in effecting long-term remission or even a potential cure of CLL. However, unmodified autologous T lymphocytes are unlikely to recognize or respond to CLL tumor cells due to immunological tolerance. Genetic manipulation and infusion of autologous T cell-based therapies is a way of breaking tolerance and has the tantalizing potential to induce long-term remission directly, without the risks of conditioning or GvHD that are associated with SCT.

CAR-based Therapy for CLL

T cells can be re-directed against tumor targets by endowing them with new, specific, antigen receptors, based on either natural T cell receptors (TCRs) or chimeric antigen receptors (CARs). The first clinical trials of CAR T cells for CLL have been directed to the pan-B cell antigen, CD19. Clinical outcomes of CLL patients treated with anti-CD19 CAR T cells have recently been reported from various academic centers ¹⁰⁻¹⁵. The National Cancer Institute infused four patients who had relapsed following fludarabine and cyclophosphamide treatment with CAR T cells directed to CD19. In this trial, clinical responses were variable, including on-target toxicities (i.e., CAR T cell activation, B cell aplasia) and one case of a complete response (CR) lasting more than fifteen months ¹³. Similar clinical outcomes for CD19-specific CAR T therapy were reported by Memorial Sloan-Kettering Cancer Center and Baylor College of Medicine. These were characterized by objective responses in some cases, but more often resulted in prolonged periods of disease stabilization, reduction in lymphadenopathy, and/or B cell aplasia ^{12,15}. The first clinical trial, coming from our group at the University of Pennsylvania, involved the treatment of three CLL patients with anti-CD19 CAR T cells (CTL019) and demonstrated impressive complete and durable remissions in two out of three patients; notably, the CAR T cells expanded *in vivo* and mediated their effects, both responses and toxicities, in a relatively delayed fashion (several weeks following infusion) ^{10,11}. More recently, our

colleagues at the University of Pennsylvania reported the results of two separate CTL019 trials in relapsed/refractory CLL: in a dose-escalation trial, eighteen patients were treated and an overall response rate (ORR) of 39% (three CR and four partial response (PR)) was observed; dose did not seem to have an impact on efficacy or toxicity. In the second trial, an ORR of 57% was reported, and all of the responding patients experienced a cytokine-release syndrome (CRS) and B cell aplasia^{16,17}. Interestingly, many of the PRs were defined by clearance of disease from the blood and the bone marrow, with residual lymphadenopathy.

There are many factors that could account for the differences in clinical outcomes among the aforementioned trials, including variances in the potency of the CAR T cell products and clinical differences in the patients, such as disease status and conditioning regimen¹⁸. In the context of establishing correlates CAR T cell treatment efficacy, interrogation of the *in vivo* phenotype and functionality of infused cells has been difficult to accomplish and this may be attributed to the relatively poor persistence of these lymphocytes observed in many adoptive cell therapy trials. Thus, broad and systematic characterizations of long-term persisting modified lymphocytes are needed for the establishment of measures of CAR T cell bioactivity.

Although CAR T cell therapy can have a significant impact on the disease, it is largely dependent on the function of the adoptively-transferred cells. Indeed, CAR T cells are “living drugs”: they can proliferate, produce cytokines, and kill tumor cells, both in the lab and upon re-infusion into the patient. CAR T cells are also likely to be susceptible to modulation in a similar fashion to unmanipulated T cells: that is, their functions may be modulated by inhibitory signals, cytokines and chemokines, a hostile or favorable microenvironment, and lymphotoxic drugs. Their overall numbers may be controlled by normal homeostatic mechanisms. However, it is possible that the signaling domains in the CAR T cells alter the sensitivity or functional responses to other receptors. It is logical to assume, however, that the final CAR T cell product potency depends, at least in part, on the phenotype and functionality of input cells used for clinical manufacturing. The best correlate of objective clinical responses to date is the degree of expansion of CAR T cells in patients, which is a measure of T cell function. Accordingly, we have observed that all responding patients have a significant expansion of CTL019 cells after infusion^{19,20}. Therefore, there is a critical need to understand the nature and function of T cells in CLL, and what factors could drive the expansion of genetically-redirected cells to be effective in CLL disease.

Components of a CAR T cell product

The major components and determinants of adoptive T cell therapies include (1) the inserted antigen receptor, (2) the gene transfer approach, (3) the input T cell population (phenotype and functional characteristics), and (4) the *ex vivo* culture system used during CAR T cell manufacturing.

CARs are synthetic molecules that combine the effector functions of T lymphocytes with highly specific antibodies^{21,22}. One advantage of antibody-based antigen receptors is that they recognize pre-defined surface antigens in a non-major histocompatibility complex (MHC)-restricted manner^{23,24}; this makes the design of the antigen receptor applicable to

all patients regardless of human leukocyte antigen (HLA) type. Furthermore, CARs recognize intact membrane proteins, independent of antigen processing, which can be defective in tumor cells. The antigen-recognition region of antibody-based CARs is typically reduced to a single chain variable fragment (scFv), containing the variable light and heavy chains of an antibody joined by a peptide linker of approximately fifteen residues in length²⁵. Chimeric receptors bearing CD3 ζ signaling modules are not sufficient to drive proliferation nor cytokine production in peripheral blood T cells; however, addition of co-stimulatory domains engineered into the CAR can recapitulate natural co-stimulation²⁶ and enhance T cell functions such as proliferation and cytokine production. The “first generation” of CAR T cells contained only the signaling domain of CD3 ζ , while CAR constructs with a single co-stimulatory molecule are known as “second generation,” and those with more than one additional co-stimulatory portion are known as “third generation” CARs (**Figure 1**).

We and others have observed that CD137 (4-1BB) signals are critical for the long-term proliferation of CD8⁺ T cells^{27,28}. Our own studies based on a CAR directed against CD19 and incorporating 4-1BB co-stimulation (CTL019), have resulted in dramatic responses in both CLL and acute lymphoblastic leukemia (ALL)^{10,11,20,29}. It should be noted that in other CLL trials at different academic centers, T lymphocytes bearing CD3 ζ alone or including a CD28 co-stimulatory domain were used. The significance of this sole variable should be interpreted with caution, as there were also differences in patient populations, host conditioning, and gene transfer methods. Nevertheless, the inclusion and type of signaling domains are hypothesized to play a role in the persistence of the CAR T cells. Other second generation CARs could include CD2, CD134 (OX40) or inducible T cell co-stimulator (ICOS). ICOS-based stimulation has been shown to enhance CAR T cell persistence and potentiate T-helper 17 (Th17) differentiation, possibly resulting in superior anti-tumor activity compared to Th17 cells co-stimulated via CD28^{30,31}.

T cells can be engineered to either constitutively or transiently express a transgene of interest using a number of different approaches. These strategies may employ viral- and non-viral-based gene transfer methods. Gamma-retroviral and lentiviral transduction based on the use of replication-incompetent vectors results in permanent integration of the transgene into the chromosomal DNA of host cells, without the production of immunogenic viral proteins^{32,33}. Because of these features, chromosome-integrating vectors are widely used in gene therapy research and for clinical trials of adoptive T cell therapies. Non-viral approaches such as electroporation with *in vitro* transcribed messenger RNA (mRNA) can be used to express proteins, such as CARs or transgenic TCRs for approximately one week³⁴; however, longer persistence of CAR T cells has been associated with increased efficacy³⁵. In addition to antigen receptors, genetic engineering can be used to endow lymphocytes with a number of other features, including enhanced proliferative capacity, prolonged survival/persistence *in vivo*, and the increased ability to migrate to tumor sites¹⁸. Some groups are also exploring the potential of allogeneic T cells genetically modified to reduce the risk of GvHD.

Several efforts have been directed toward identifying the optimal starting population of T cells for genetic engineering and subsequent adoptive transfer. It is now believed that a less

differentiated “seed” population such as T stem cell memory (T_{SCM}) or central memory T cells (T_{CM}) may exhibit enhanced anti-tumor efficacy compared to terminally differentiated effector memory T cells (T_{EM}) that have largely lost plasticity and proliferative capacity. Indeed, the goal of immunotherapy based on the redirection of T lymphocytes is to achieve cellular self-renewal and effector differentiation *in vivo* upon tumor exposure. In certain disease states such as CLL, however, the peripheral T cell repertoire may be skewed to more differentiated and antigen-experienced populations³⁶. Furthermore, the manufacturing process itself requires multiple rounds of replication to obtain therapeutically sufficient numbers of lymphocytes³⁷, which may in itself alter the phenotype and function of the final T cell product. This issue is particularly evident in CLL, where the starting population of CD8+ T cells is already in a state of pseudoexhaustion³⁶. This phenotypic and functional profile of terminally differentiated CD8+ T cells in CLL patients may be the result of chronic stimulation by low-affinity self-peptides. For this reason, long periods of *ex vivo* expansion for CAR T cell manufacturing may result in an inferior lymphocyte product that has acquired a phenotype associated with senescence, anergy or exhaustion³⁸.

T lymphocyte subsets may be functionally defined by their expression of surface molecules, cytokines or master regulator transcription factors. They can be phenotypically defined as naïve, T_{SCM} , T_{CM} , T_{EM} or effector cells. One method of obtaining a starting population of T lymphocytes with enhanced plasticity for cellular therapy is to isolate, genetically redirect and expand specific subsets *in vitro* such as T_{CM} cells. This strategy relies upon cell sorting or other selection procedures prior to engineering specificity against the desired antigen³⁹; it is being examined by investigators at the University of Washington³⁹. In contrast, we have proposed the use of bulk T cells with culture conditions that augment CD137 (4-1BB) and CD28 co-stimulatory signaling in order to enrich for T_{CM} cells and promote their maintenance^{34,39,40}. This latter strategy obviates the need for sorting or other means of physical purification that could contribute to excessive manipulation of the cellular product prior to re-infusion, but it may require higher total doses of the CAR T cell product than a more selected population.

The use of T_{SCM} cells with a prolonged replicative capacity and multi-potency to produce diverse lineages of T lymphocytes with potent effector functions⁴¹⁻⁴⁴ may represent a superior seed population for ACT approaches. This is supported by the observation that adoptively-transferred T_{SCM} cells mediate an enhanced anti-tumor response compared to other CD8+ T cell subsets⁴². Although the use of T_{SCM} cells for tumor immunotherapy appears to hold great promise, it is not clear that the absolute numbers of these lymphocytes are consistent in the peripheral blood across large numbers of diverse cancer patients¹⁸. Furthermore, validated and clinically- approved culture systems will be required to isolate and maintain T_{SCM} cells during the genetic modification and *ex vivo* culture process. Ultimately, improvements in cellular reprogramming methods may potentiate the induction of plasticity in T cells to expand the pool of T_{CM} or T_{SCM} cells⁴⁵, and could perhaps produce a continuous source of effector progeny³⁸.

Optimal *ex vivo* cell culture methods for ACT procedures recapitulate the natural processes by which antigen-presenting cells (APCs) trigger TCR signaling and concomitant co-stimulation. The type of culture system used and its different components depends upon (or

determines) the particular T cell subset that is being expanded for the associated therapeutic application. Recent studies suggest that dendritic cells (DCs) can mediate anti-tumor responses when appropriately activated and induced to present tumor-associated antigenic peptides⁴⁶⁻⁴⁹. However, although potentially suitable in the context of therapeutic vaccination, the use of DCs for adoptive T cell therapy trials is limited due to high manufacturing costs and logistical restrictions associated with maintaining independent culture systems. In addition to these scale-up issues, the efficiency of generating autologous DCs that can mediate a sufficient level of T cell activation often varies from patient-to-patient. Therefore, despite their obvious potential, *ex vivo* approaches based on natural DCs to expand T cells for adoptive immunotherapy can be hampered by difficulties in obtaining large numbers of these terminally differentiated, short-lived cells in manner that is compliant with good manufacturing practices (GMP)⁵⁰⁻⁵². For these reasons, culture systems for ACT platforms have been focused on the use of artificial APCs which are either bead- or cell-based.

A highly effective approach for T cell expansion involves co-culture using magnetic beads coated with clinical grade anti-human CD3 and CD28 antibodies to induce TCR cross-linking, while simultaneously providing unopposed CD28-mediated co-stimulation (**Figure 2.**). This expansion method based on bead-immobilized antibodies also allows for T cell activity to be enhanced due to removal from a tumor-associated immunosuppressive milieu⁵³⁻⁵⁶, and has the added benefit of not requiring exogenous growth factors or feeder cells to generate a scalable, clinical-grade product. Similar beads may be coated with peptide-MHC molecules⁵⁷. Alternatively, cells lacking endogenous MHCs can be engineered to express MHCs and/or other co-stimulatory factors^{57,58}.

Successful adoptive transfer, engraftment, and persistence of genetically-modified lymphocytes within the host are complex immunological processes that depend not only on the cellular replicative capacity, but also upon homeostatic signals *in vivo*. The historic paradigm was based on the administration of large doses of effector cells, mainly because infused T cells exhibited sub-optimal proliferation and poor long-term survival. Thus, these first attempts effected weak and temporary therapeutic responses⁵⁹⁻⁶¹, resembling short-acting drugs rather than persistent memory T lymphocytes. In contrast, the results of recent clinical trials based on genetically-redirected T cells have demonstrated that in many cases, small numbers of engineered lymphocytes that successfully replicate and engraft in the host may be sufficient. In this way, the majority of T cell expansion takes place following adoptive transfer into the patient, rather than during the *ex vivo* manufacturing process. Notably, this new approach depends on constitutive transgene expression and therefore, strategies that rely upon transiently engineered T lymphocytes (e.g., setting of mRNA transfection) will still require repeated infusions of large numbers of cells.

T cells in CLL

Although in a number of different malignancies T cells are known to be involved in anti-tumor immunity, CLL T lymphocytes do not mount effective endogenous immune responses against CLL cells⁶²⁻⁶⁷. This may be a direct consequence of the microenvironment elicited by leukemic B cells that promotes tumor survival and facilitates escape from T cell

immunosurveillance⁶⁸. In this regard, CAR T cells hold great promise for overcoming tumorigenic tolerance, as long as they are not subjected to the same immunosuppressive factors. Interestingly, endogenous T cells may support the microenvironment responsible for the continued survival of CLL cells, as increased B cell proliferation in this disease appears to occur concomitant with the expansion of T lymphocytes^{69,70}. This may also involve an increase in the absolute numbers of circulating regulatory T cells (T_{reg}), which are of negative prognostic significance in CLL⁷¹⁻⁷⁴ and could contribute to effector T cell dysfunction. In addition to increased T_{reg} frequency, direct tumor-intrinsic mechanisms underlying poor T cell-mediated immunity may include suboptimal antigen presentation by CLL B cells^{65,75}, the elaboration of immunosuppressive cytokines such as transforming growth factor (TGF)- β , Interleukin (IL)-6 and IL-10^{76,77}, downregulation of costimulatory molecules^{64,78} and down-modulation of CD40L on T cells⁷⁹, as well as overexpression of the inhibitory CD200 receptor^{80,81}. Fortunately, genetic modification with CARs bypasses defects associated with endogenous antigen presentation and co-stimulation. However, the extent of direct or indirect immunosuppression by leukemic CLL B cells is likely to influence the effectiveness of anti-tumor responses by genetically-modified T cells, unless they are endowed to specifically overcome these inhibitory influences.

Baseline CLL disease-associated T cell defects could also hamper the clinical efficacy of genetically-redirected lymphocytes by decreasing the efficiency of gene transfer, poor proliferation during manufacturing, and/or poor expansion of engineered T cells following infusion into the patient. In CLL, total *ex vivo* CD8⁺ T cells exhibit hallmarks of exhaustion: overexpression of the inhibitory molecules programmed death-1 (PD1), CD244 and CD160, accompanied by diminished cytotoxic and proliferative capacities³⁶. However, unlike “classical exhaustion,” such as that observed in chronic viral infections, CD8⁺ T cells obtained from CLL patients are able to effectively elicit cytokines upon acute stimulation. In fact, total *ex vivo* CD8⁺ T cells isolated from individuals with CLL show increased production of interferon (IFN)- γ and tumor necrosis factor (TNF)- α when compared to age-matched healthy donors. This paradoxical state of pseudoexhaustion (**Figure 3**) may be attributed to antigen affinity differences in CLL, relative to chronic viral diseases⁸². Thus, CLL patient CD8⁺ T cells may receive persistent, low-affinity self-antigen stimulation in contrast to chronic triggering of the TCR by high-affinity viral peptides. In addition, T cells in CLL exhibit an impaired ability to form immunological synapses with APCs^{83,84}, which might further contribute to low-avidity TCR signaling and account for defective cytolytic activity. The impaired cytotoxicity of these cells has been attributed to the failure of granzyme localization at immunosynaptic clefts³⁶. While constitutive overexpression of non-MHC-restricted CARs on CLL patient T cells may produce effectors with intact cytolytic capacity¹⁰, the repair of immunologic synapse defects has the potential to enhance *ex vivo* expansion of genetically-modified lymphocytes during manufacturing and further potentiate their clinical efficacy upon re-infusion.

Analysis of T cell differentiation in CLL indicates that the peripheral T cell compartment is skewed toward an increased proportion of antigen-experienced lymphocytes³⁶, which poses a challenge to obtaining a less differentiated seed population with enhanced plasticity for CAR-based redirection and subsequent adoptive transfer. It has been suggested that the

expansion of circulating CD8+ and CD4+ T cells described in CLL ^{85,86} could be driven by cytomegalovirus (CMV) infection, as elevated absolute numbers of these lymphocytes were found mainly in CMV-seropositive patients ⁸⁷. However, reactivations of latent virus infections such as CMV are uncommon in untreated CLL patients, and the CMV-specific T cell response in these individuals was recently shown to be functionally intact ⁸². Therefore, the shift to increased frequencies of antigen-experienced T cells with the phenotypic and functional characteristics of exhaustion appears to be independent of cytomegalovirus (CMV) infection ³⁶.

Because the survival of B-CLL cells is dependent on a particular threshold of B cell receptor (BCR) signaling, there has been debate over the identity of antigens that drive this disease ⁸⁸. The discovery of antigen-independent constitutive BCR signaling in CLL ⁸⁹ implicates autonomously active leukemic cells as a source of chronic stimulation and, in turn, the T lymphocyte pseudo-exhaustion. Collectively, these reports underscore heterogeneity in the T cell compartment in CLL and suggest that a thorough understanding of the mechanisms underlying the divergent functionality of particular T cell subsets is required to further develop and improve CAR-based adoptive T cell therapy platforms.

Building CARs out of T cells from CLL patients

The therapeutic efficacy of CAR T cells likely depends on many factors, including the quality of the infusion product, the state of the host immune system, tumor burden, aggressiveness of disease and the balance between growth/survival factors and inhibitory signals that influence T cells. Upon infusion into the patient, genetically-redirected cells must traffic to tumor cells, avoid immunosuppression, interact with their cognate antigen, proliferate, kill target cells, and persist as sentinels to guard against any residual malignancy. Roadblocks in the context of CAR T cell-based therapy for CLL appear to be associated with the poor proliferative capacity and function of T cells, which in many cases hamper clinical manufacturing, as well as anti-tumor efficacy and persistence following infusion.

Several methods have been implemented to improve the expansion and survival of T cells for ACT platforms by focusing on stimulatory pathways known to improve T cell function. The common γ -chain cytokines IL-2, IL-7, IL-15 and IL-21 have been incorporated into T cell expansion protocols, either alone or in combination with engagement of costimulatory molecules ^{90,91}. Our group has shown that CAR T cells expanded with anti-CD3/CD28 antibody-coated beads exhibit superior anti-tumor activity and persistence, relative to CAR T cells that were driven to proliferate with soluble anti-human CD3 antibody and high dose IL-2 ⁹². In addition, T cells that are less-differentiated and/or those expanded in the presence of IL-12 or IL-15 tend to have enhanced proliferative ability ^{93,94}. The use of IL-15, IL-7 and IL-21 alone or in combination with bead-based stimulation has been shown to facilitate the *ex vivo* differentiation of gene-modified T cells with increased plasticity and superior anti-tumor efficacy ^{90,95-97}. In the context of CAR-based genetic modification of autologous T cells for the treatment of CLL, culture conditions, as well as the duration of *ex vivo* expansion will require optimization. Furthermore, incorporation of agents that could repair the immunological synapse, such as lenalidomide ⁸⁴ may enhance the proliferative capacity

of CLL patient T cells in GMP-compliant procedures that rely upon APCs for CAR T cell expansion.

Additional strategies to enhance the persistence of adoptively-transferred CAR-bearing T cells may include transient or permanent manipulation of apoptosis machinery. To this end, it has been demonstrated that overexpression of anti-apoptotic Bcl-xL enhances the survival of T cells in a pro-apoptotic environment ⁹⁸. We have previously demonstrated that targeted silencing of Bcl-2 homologous antagonist killer (Bak), a major gate-keeper of the intrinsic programmed cellular death pathway, inhibits the apoptosis sensitivity of primary CD4+ and CD8+ T cells obtained from a chronically exhausted disease state ⁹⁹. Another possible approach to prolong the survival and persistence of CAR T cells, could be overexpression of the telomerase gene *hTERT* ¹⁰⁰. Finally, because TGF- β is overexpressed in CLL ¹⁰¹ and represents yet another immunosuppressive barrier to the potential success of ACT approaches, blockade of TGF- β signaling specifically in transferred T cells may enhance their proliferative and cytotoxic abilities ¹⁰². It should be noted that each of the above strategies must be carefully and thoroughly evaluated in the most sensitive pre-clinical models available prior to translation, as they could potentially induce autoimmunity (e.g., T cell lymphoproliferative disorders).

The combination of CAR T cell therapy with targeted agents for the treatment of CLL is still in early stages, but is a likely next step for clinical investigators. A number of different combinations are possible, such as co-administration of agents that will activate the CD40/CD40L pathway ¹⁰³, and/or antibodies that will engage additional co-stimulatory molecules. Blockade of negative regulatory pathways has recently demonstrated great promise in restoring defective T cell function in other malignancies ^{104,105}. To date, there is a paucity of clinical data showing that blockade of immune checkpoints (e.g., via anti-PD-1 antibodies) can augment CAR T cell function, but this likely represents an exciting area of future study. Identification of other regulatory checkpoints of reversible T cell impairment in CLL may also provide a platform for targeted combinatorial intervention and reinvigorating exhausted T lymphocytes that dominate a state of persistent stimulation by leukemic B cells.

Small molecule inhibitors such as ibrutinib ³ and idelalisib ^{4,106} that target aberrant signaling in leukemic cells and trigger rapid tumor clearance could give kinetically slower, but long-lived CAR T cells a much needed head-start in mediating anti-tumor immunity ¹⁸. Such targeted agents that do not negatively affect T cell function ¹⁰⁷ are likely to be superior for combinatorial approaches. Finally, induction of tumor egress from bulky lymph nodes with pharmacologic agents and/or improving trafficking of transferred lymphocytes to areas of proliferating tumor load may produce additive or synergistic effects with CAR T cell therapy.

Potential Toxicities of CAR T cell Therapy for CLL

In initial clinical studies of CTL019 cells infused into CLL patients, some on-target toxicities were anticipated, including the potential for infusional toxicity. The first CLL patients treated with CTL019 received fractionated doses of cells to prevent acute infusion-related reactions ¹¹. However, we have found that infusion toxicity is a relatively infrequent

event and have therefore started to administer a total cell dose in a single administration ^{11,108}. On-target toxicities associated with CTL019 therapy include CRS, in which inflammatory cytokines are produced as a consequence of T cell activation and expansion, tumor lysis syndrome, and B cell aplasia ¹⁰⁹. The CRS resulting from CTL019 administration has been observed in both children and adults, but the most severe cases tend to be observed in pediatric patients with B-ALL. In ALL, there appears to be a direct correlation between tumor burden and the severity of CRS; however this correlation does not appear to be true for CLL. Severe CRS is characterized by clinical deterioration and high systemic levels of IL-6, and sometimes triggers macrophage activation syndrome ^{13,29}. Treatment with anti-IL-6 receptor blocking antibodies (tocilizumab) in combination with high-dose steroids typically leads to rapid abrogation of CRS ²⁹. However, the optimal management of CRS in the context of CAR T cell therapy remains an open question. Finally, B cell aplasia is a biomarker of CTL019 function and can be managed with the regular administration of immunoglobulin.

Potential on-target, off-tumor or off-target toxicities are also possible with the use of other forms of CAR T cells for CLL therapy. In the case of CD19, expression is restricted to normal B cells and CLL cells. Compared to CD19 and CD20, relatively little is known about the range of expression of other potential targets. Current CAR targets in development for CLL include CD20 ¹¹⁰ and ROR1 ^{111,112}. As these and other novel antigens are proposed for CAR-based approaches to treat CLL, strategies used to limit the persistence of the transferred T cells may be particularly useful. Current approaches include the engineering of self-limiting transgene expression, such as the use of mRNA-transfected “biodegradable” CAR T cells, introduction of a suicide gene, or the inclusion of a moiety on the surface of gene-modified T cells that will facilitate antibody-based depletion ¹¹³⁻¹¹⁵. The fact that most CARs are currently derived from murine antibodies presents a risk of developing human anti-mouse antibody responses, which could limit the possibility of CAR T cell re-infusion ¹⁰⁸. In this regard, CAR humanization methods or scFv derivation from human antibodies will likely mitigate these problems. Finally, all gene transfer approaches that result in integration of the transgene into the host genome carry a risk of malignant transformation. Fortunately, the chance of insertional mutagenesis occurring in T cells, at least in a retroviral-based transgene delivery system appears to be very low ¹¹⁶; and there have not been reports of transformation resulting from CAR gene transfer into mature T cells.

Potentiating CAR T cells for CLL by Managing the Host

Lymphodepletion with chemotherapy and/or radiation generally enhances the engraftment of adoptively-transferred T cells in both solid and liquid tumors ^{117,118}. The use of chemotherapy and/or radiation to pre-condition the host drives transferred T cell persistence by creating homeostatic “space” that would otherwise be occupied by other cells that could compete for available growth and survival factors (i.e., “cytokine sinks”). Inflammatory processes that accompany certain lymphodepletion regimens may also result in the production of homeostatic cytokines that could facilitate the proliferation and maintenance of redirected T cells ¹¹⁹. In addition, host conditioning may reduce the population of immunosuppressive T_{reg} cells and reduce tumor burden such that an optimal ratio of effector

T cells to tumor targets is achieved. While systemic administration of cytokines such as IL-2, IL-7 and IL-15 could also potentiate T cell growth and homeostatic proliferation, timing of administration, appropriate dosing, and toxicity may limit their use. We and others have shown that modulation of post-transcriptional regulators such as micro-RNAs increases the proliferation and effector function of adoptively-transferred CD8⁺ T cells ^{120,121}. In the context of host conditioning, overexpression of micro-RNA 155 in infused CD8⁺ T cells has recently been shown to increase responsiveness to homeostatic cytokines and mediate profound anti-tumor responses ¹²².

While CAR-redirectioned T cells infused in the absence of host conditioning have demonstrated clinical effects ²⁹, most protocols include a lymphodepletion step both for disease control and based on historical experience with T cell therapies. Based on studies of adoptive transfer of non-gene-modified T cells, enhanced engraftment and persistence could occur with infusion early on after high-intensity conditioning regimens ^{123,124}. In one study involving CAR T cells redirectioned to CD19, transfer of genetically-modified T cells was preceded by cyclophosphamide treatment in one cohort, while the other group did not receive prior conditioning ¹².

Conclusion

In conclusion, CAR T cell therapy is a promising new treatment for patients with CLL. The impressive clinical responses effected by CAR T cells directed to CD19 for CLL and other refractory B cell malignancies has led to United States Food and Drug Administration “breakthrough” designation of several versions of CAR T cells as personalized cellular therapy for cancer. Unlike conventional approaches used to manage CLL disease, CAR T cell therapy is unique in that it is the true embodiment of a patient-specific, “living” and self-replicating drug. For this kind of therapy to become widely available, the clinical activity of CAR T cell products manufactured under standardized, industry-grade conditions will need to be confirmed to have a significant clinical benefit for patients with CLL.

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Figure 1. Structure of First, Second and Third Generation Chimeric Antigen Receptors (CARs)
 CARs are composed of antigen-binding moieties (i.e., scFv consisting of an interconnected variable heavy chain (V_H) and variable light chain (V_L)) fused to a transmembrane (TM) region of a protein such as CD8 that is, in turn, linked to a signal transduction domain. First generation CARs contained only the ζ chain of the T cell receptor (TCR). Second generation CARs incorporate a co-stimulatory endodomain fused to CD3 ζ (e.g., CD28 or 4-1BB), and third generation antigen receptors of this type possess a second intracellular signaling module linked in tandem (e.g., both 4-1BB and CD28). Upon binding of the scFv to a cognate antigen, second and third generation CAR T cells receive a primary activation signal, as well as concomitant co-stimulation.

Figure 2. Manufacturing scheme for CTL019 cells

T lymphocytes are initially isolated from the peripheral blood of CLL patients and enriched, as well as activated with anti-human CD3/CD28-coated polystyrene beads. Gene transfer is accomplished by lentiviral transduction and following a ten day expansion period, beads are removed and cells are frozen in an infusible media for adoptive transfer into patients.

Figure 3. Features of T cell pseudoexhaustion and immunosuppression in CLL that could hamper the clinical efficacy of manufactured CAR T cells

T cells isolated from CLL patients exhibit some functional and phenotypic hallmarks of chronic exhaustion, such as high levels of inhibitory molecules (e.g., PD-1, CD244 and CD160), impaired proliferative capacity and cytolytic defects. Soluble immunosuppressive factors elaborated by CLL cells may further contribute to inhibition of T cell function in the setting of adoptive cell therapy.