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Escape from *ALL-CART*az: Leukemia immunoediting in the age of chimeric antigen receptors

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Abstract

Chimeric antigen receptor (CAR) T-cell therapy has been transformative for the treatment of B-cell malignancies, with CD19- and CD22-directed CARs being prime examples. However, immunoediting and ensuing antigen loss remain the major obstacle to curative therapy in up to 25% of patients. For example, to achieve the CD19-negative phenotype, malignant cells can pick from a broad array of mechanisms, including focal loss-of-function mutations, dysregulated trafficking to the cell surface, alternative splicing, and lineage switching. In other cases, where resistance is mediated by insufficient antigen density, trogocytosis has been proposed as a possible underlying mechanism. To overcome these barriers, compensatory strategies will be needed, which could include the use of combinatorial CARs, harnessing epitope spreading, and targeting tumor neoantigens.

Keywords

Immunotherapy; immunoediting; chimeric antigen receptors; adoptive T-cell therapy; leukemia; lymphoma; therapeutic resistance; epitope loss; antigen escape; alternative splicing

INTRODUCTION

Hematologic malignancies, especially B-cell leukemias and lymphomas, are generally considered to be "cold" tumors, i.e. to have low mutation burden. Not only does this "coldness" result in the paucity of targetable drivers, but it also makes them largely invisible to the adaptive immune system, trained to recognize non-self antigens. Therefore, immunotherapies for these cancers typically target lineage-, rather than cancer-specific markers. So far, among the surface epitopes, CD19 and CD22 are the most promising targets

CONFLICT OF INTEREST STATEMENT

ATT has an interest in intellectual property "Discovery of CD19 Spliced Isoforms Resistant to CART-19." This interest does not meet the definition of a reviewable interest under Children's Hospital of Philadelphia's (CHOP's) conflict of interest policy and is therefore not a financial conflict of interest. Furthermore, this intellectual property is held by CHOP and has not been licensed or otherwise commercialized to date. However, should this technology be commercialized in the future, ATT would be entitled to a share of royalties earned by CHOP per its patent policy.

because of their high expression levels and restricted B-cell expression profile. Successful targeting of these surface markers in the clinic resulted in the recent FDA approval of blinatumomab, a CD19-CD3 bi-specific T-cell engager (BiTE)[1] and inotuzumab ozogomycin, a CD22-directed antibody-drug conjugate[2]. But perhaps the most transformative therapeutics for B-cell malignancies are chimeric antigen receptor (CAR) T-cells, which highlight the promise of this type of immunotherapy in cancer. CAR T-cells (CART) are engineered to express recombinant receptors containing a single-chain variable fragment (scFv) that targets a specific extracellular epitope, an intracellular CD3ζ signaling domain (so-called signal I) and one or more co-stimulatory domains (e.g. 4–1BB or CD28, so-called signals II). scFvs are anchored on the cell surface by short hydrophobic transmembrane domains to ensure formation of proper immunological synapses[3].

In 2010, the first adult patient with chronic lymphocytic leukemia (CLL) was successfully treated with a CD19-directed CAR T-cell therapy (CTL019) at the University of Pennsylvania[4]. Two years later, the first pediatric patient received life-saving CTL019 for B-cell precursor acute lymphoblastic leukemia (B-ALL)[5]. Subsequent clinical trials using various CD19-directed CARs have shown encouraging results for the treatment of relapsed/refractory leukemias and lymphomas. A phase II ZUMA-1 trial of axicabtagene ciloleucel (YESCARTA®, developed by Kite Pharma) demonstrated a complete response rate of 54% in adult B-cell lymphoma patients mostly within the first month after administration[6]. In parallel, a phase II ELIANA trial of tisagenlecluecel (KYMRIAH® also known as CTL019, developed by Novartis) in pediatric patients with B-ALL demonstrated an overall remission rate of 81% within 3 months[7]. Other CD19-directed CAR T-cell trials have yielded similar complete response rates ranging from 60 to 90%[8–10]. In the last three years, these trials led to accelerated FDA approvals of axicabtagene ciloleucel and tisagenlecluecel for relapsed/refractory B-ALL and B-cell lymphoma. Clinical trials using CD19 CAR T-cells for chronic lymphocytic leukemia (CLL) are ongoing, although lower response rates have thus far precluded FDA approval of CAR T-cell therapies for this disease[4,11]. CD22-directed CAR T-cells have also shown clinical promise. A recent NIH-based phase I trial in children/adults with relapsed B-ALL treated with an optimized cell dosage of a CD22-directed CAR demonstrated a 73% complete response rate[12].

In contrast to CAR T-cell therapies for lymphoid malignancies, CAR T-cell options for the treatment of acute myeloid leukemias have been more limited. Early trials have sought to target CD123 and CD33 but further progress in targeting myeloid markers has been hindered by high toxicity in the myeloid and hematopoietic stem cell compartments[13–15].Hence, the subsequent discussion of acquired resistance to CAR T-cell therapies will focus on B-lymphoid malignancies and how selective pressures of potent immunotherapeutics shape their genomes, transcriptomes, and proteomes.

IMMUNOEDITING BY CAR T-CELLS

The concept of immunoediting was first formulated almost 20 years ago to explain how the host's immune system controls the spread of pre-malignant cells and how these cells evolve to escape these controls[16]. It postulated the existence of three distinct 'E' phases: *elimination* (when the immune system surveys for and clears transformed cells), *equilibrium*

(when transformed cells that had evaded the first phase are primed with further editing), and *escape* (when clone variants shaped by immune pressures expand and become clinically dominant). Recent iterations of the 3Es theory hold that both neoplastic and immune cells are shaped by the process of immunoediting[17]. And although the original concept dealt primarily with endogenous immunosurveillance, it is now being applied to immunotherapy settings, first and foremost checkpoint inhibition[18]. However, it stands to reason that CAR T-cell therapy is no exception and could be viewed through the lens of immunoediting as well, in particular during the 'escape' phase.

Indeed, despite the impressive rates of initial complete responses to current CAR T-cell therapies for B-cell malignancies, longer term follow-up has demonstrated that a significant proportion of patients do relapse after treatment and often do not respond robustly to re-infusions of the same immunotherapeutic. For example, in the ELIANA trial, the rate of relapse-free survival among patients with an initial response dropped from 80% at 6 months to 59% at 12 months[7] In order to design the next generation of immunotherapies with better response rates, more durable remissions, and expanded utility beyond B-cell malignancies, it is imperative to understand how cancer cells evade CAR T-cell immunotherapies, develop resistance, and fuel relapses.

One major paradigm borne out across multiple trials using different CD19 CAR constructs is that escape mechanisms could be either leukemia- or T-cell-intrinsic. In the case of CD19-directed CAR T-cells, patient relapses are often binned into CD19-positive and CD19-negative diseases. In the case of CD19-positive relapses, disease recurrence most often results from the loss of CAR T-cell persistence or T-cell dysfunction[19,20]. In these scenarios, CAR T-cell failures could be attributed to either intrinsic properties of the engineered T-cells (e.g. the paucity of naïve T-cells for in vitro expansion, the lack of central memory T-cells for lasting antitumor effects, etc.) or factors extrinsic to T-cells (immunologic rejection of CAR T-cells, unfavorable microenvironment, etc.)

As important as these mechanisms are, some trials suggest that the majority of relapsed B-ALLs fall into the CD19-negative category. In the ELIANA trial, out of 22 relapse samples assessed for CD19 status by flow cytometry, at least 15 were CD19-negative. This corresponds to an overall CD19 negative relapse rate of 25% (15 relapses out of 61 initial responders)[7]. Other B-ALL trials have yielded CD19-negative relapse rates between 7 and 24%[8,21,22]. However, these rates likely underestimate the true incidence, as lengths of follow-up were highly variable, with a good proportion of patients proceeding to transplant shortly after CAR T-cell administration. CD19-negative clones were also seen in lymphoma patients at relapses following CD19-directed CAR T-cell therapy, albeit with lower prevalence than in B-ALL[6]. Moreover, at least one CLL patient has been reported to relapse with an aggressive CD19-negative plasmoblastic lymphoma after CAR T-cell therapy[23]. Finally, blinatumomab therapy is also yielding CD19-negative relapses at rates comparable to CD19-directed CAR T-cells, indicating that antigen escape is not a CAR-dependent mechanism of relapse[24,25].

These relapse phenotypes, associated with insufficient levels of cognate epitopes on the surface of escaping cells, are the main subject of this review. We will demonstrate that to

achieve the CD19-negative phenotype, malignant B-cells utilize a broad array of mechanisms, including focal mutations, dysregulated trafficking to cell surface, alternative splicing, lineage switching, etc. In contrast, relapses after CD22-directed CAR T-cells occur via downregulation of CD22 rather than total CD22 loss. Thus, understanding the strategies which cells employ to achieve antigen loss or low antigen density is crucial to optimizing future immunotherapy targets and regimens[26].

MECHANISMS OF CART-DRIVEN IMMUNOEDITING

Mutational mechanisms of antigen loss

Nonsense/frameshift mutations.—Frameshift mutations are a powerful tool of gene inactivation, especially when premature STOP-codons are inserted close to 5' ends of open reading frames. These mutations typically result in loss of functional protein either through nonsense-mediated mRNA decay or through significantly truncated polypeptide chains. Thus, when our group began examining CD19 negative B-ALL relapses following CTL019 treatment, it was considered a default mechanism of antigen loss[27]. Interestingly, only one patient out of four (CHOP133) demonstrated a loss of one copy of chromosome 16 (on which CD19 resides) and a frameshift mutation in exon 2 of CD19 on the other allele. Since the variant allele frequency (VAF) was 100%, this classical two-hit event fully explained the observed total loss CD19 expression by flow cytometry.

Subsequently, Orlando et al analyzed 12 patients treated with CTL019 who developed surface CD19 (sCD19)-negative relapses[28]. De novo frameshift mutations mapping to exons 2–5 were found in all 12 relapsed samples, and copy number analysis confirmed loss of heterozygosity (LOH) in 8 of the 9 patients evaluated. As the transmembrane domain of CD19 is encoded in exon 5, none of the resultant short polypeptides could localize to cell surface, even if translated, consistent with the CD19 negativity by flow cytometry.

Missense mutations affecting protein trafficking.—In Sotillo et al, we also described another sCD19-negative relapse (CHOP105) with segmental chromosome loss spanning the CD19 locus on one allele and an in-frame insertion (with 100% VAF) of 3 codons in exon 2 on the other allele. It was not immediately clear why this gain of amino acids would cause loss of sCD19 expression, as measured by surface staining with the FMC63 antibody recognizing the CTL019 epitope[29], especially in light of recent studies implicating exon 4-encoded peptides in recognition by CTL019[30]. Subsequently, we were able to show that the CHOP105-CD19 exon 2 variant cannot be recognized by FMC63 even in permeabilized cells[31], attesting to the importance of exon 2-derived amino acid sequences for recognition by CTL019. Of broader significance, however, was the finding that CHOP105-CD19 was not folded properly and thus was retained in the endoplasmic reticulum. Additionally, it has lost its ability to bind to the tetraspanin CD81. As CD81 is critical for proper trafficking of CD19 from the Golgi complex to the plasma membrane[32], even counteracting ER retention would be insufficient to deliver CHOP105-CD19 to cell surface for detection by the cognate CAR T-cell[31].

Noncoding mutations in splice sites.—In addition to mutations in the CD19 open reading frame, there are at least two instances where DNA alterations affect splice sites.

Such mutations, which are likely to cause intron retention, were identified by Orlando et al in the Novartis B-ALL cohort ([28], patient #8) and by Delage et al in diffuse large B-cell lymphoma[33].

In summary, focal mutations can mediate antigen escape by preventing the full-length protein from being expressed, by conformationally disrupting the epitope region, by interfering with trafficking to the cell surface, and also by deregulating splicing. Based on existing CTL019 studies, the first mechanism (gene inactivation) appears to be more prevalent, at least in the cohorts studied thus far. However, as the following paragraph will show, both deregulated splicing and impaired trafficking can occur in the absence of identifiable cis-acting mutations, highlighting the role of non-mutational mechanisms.

Non-mutational mechanisms of escape

Despite the prevalence of loss-of-function mutations in sCD19-negative CTL019 relapses, there is also experimental evidence that mutations alone do not account for CD19 negativity in all cases. Indeed, Sotillo et al reported that in patient CHOP101, frameshift mutations in CD19 collectively accounted for no more than 50% of CD19 alleles, yet still led to complete loss of sCD19 expression by flow cytometry[27]. Similarly, in the Novartis cohort of sCD19-negative relapses, quite a few of the reported mutations were subclonal, even after adjusting for tumor content. For example, in patient #5, the VAF of the indel mutation in exon 2 of CD19 was estimated to be 36%. Collectively, these data suggest that other, post-transcriptional or post- translational mechanisms may be at play.

Alternative Splicing.—The subclonal nature of mutations in some sCD19-negative patients informed initial investigation into post-transcriptional regulation of CD19. RNA sequencing followed by the application of the splicing analysis algorithm called MAJIQ[34] revealed that in patient CHOP101 skipping of exon 2 led to the emergence at relapse of the alternatively spliced isoform, Δex2 CD19, which lacked the cognate FMC63 epitope[27]. In fact, this event could essentially bypass frameshift mutations in exon 2, rescuing the transcript from nonsense-mediated decay in patient CHOP133. Of note, while it is possible that the Δex2 CD19 isoform retains some signaling capabilities of CD19, most of it is sequestered in the ER, away from active B-cell receptor complexes where CD19 normally functions.

Additional studies have demonstrated that CD19 splice isoforms are present in leukemia at the time of diagnosis, suggesting that pressures from CD19-directed therapy can simply select for preexisting treatment-resistant subclones[35] rather than require de novo mutations described by other groups[28,33]. Consistent with this notion, our recent work has identified global dysregulation of splicing as a hallmark of B-ALL, when compared to normal pro-B-cells[36]. Of high relevance, the list of deregulated splice factors included SRSF3, previously shown to regulate CD19 mRNA splicing and increased inclusion of its exon 2[27].

Exon 2 is not the only non-constitutive cassette exon in CD19, nor the only one potentially important for immunotherapy. Also increased at relapse was the isoform with skipping of exon 5 and 6. This isoform is predicted to yield a truncated protein without the

transmembrane domain, but with a signal peptide and thus with a potential to be secreted. Whether it could act as a soluble decoy for CTL019 remains to be determined.

Epitope Shielding.—While the existence of soluble CD19 isoforms has not been documented, one unusual case of epitope shielding has been described in the literature. In one unique case of post-CTL019 CD19-negative relapse, investigators found that a single leukemic blast had slipped through the T-cell manufacturing process and become positive for both endogenous CD19 and the anti-CD19 CAR[37]. This allowed the CD19 CAR to bind in *cis* to the CD19 protein on the same cell, thus shielding the epitope from CD19 CAR T-cells. While this event might be exceedingly rare, it illustrates the power of immunoediting and shows that clonal expansion of a single leukemic cell can underlie therapeutic resistance and subsequent clinical relapses.

Decrease in antigen density.—Loss of the CD19-CD81 interaction described by Bagashev et al[31] appears to be a common theme in resistance to immunotherapy. Indeed, Braig et al described a B-ALL patient who developed the sCD19-negative relapse after blinatumomab therapy but had not acquired mutations in either CD19 or CD81. Nevertheless, this leukemia somehow became CD81-negative, as determined by flow cytometry[38]. This observation led the authors to conclude that loss of sCD19 was due to impaired trafficking to the cells surface.

While the requirement for a chaperone makes CD19 somewhat unique among B-cell differentiation markers, diminished antigen density is emerging as an important immunoediting mechanism, affecting CD19 and CD22 as well as other surface markers. Yu et al have described sequential loss of surface expression of multiple B-cell markers following rounds of CD20, CD30, and finally CD19-directed immunotherapy for primary mediastinal large B-cell lymphoma[39]. The same group documented significant heterogeneity of surface CD19 and CD22 expression in B-ALL[40].

Diminished sCD22 expression has particularly important clinical implications, since CD22-directed CAR T-cells have become another viable treatment option for many patients with CD19-negative relapses. For example, in the initial phase I trials conducted at the NIH by Fry et al, 8 out of 12 patients relapsed within 1 year after receiving CD22 CARs[26]. Interestingly, 7 out of these 8 patients did not sustain complete antigen loss but instead demonstrated downregulation of CD22 surface expression. This occurred in the absence of any acquired CD22 mutations or changes in mRNA levels, suggesting that surface expression could be regulated at the post-transcriptional level or through generation of alternative isoforms.

Most recently, Hamieh et al published an interesting report illustrating the mechanism by which antigen site density may be decreased[41]. Using in vivo models to mimic CAR T-cell therapy relapse, they showed that CD19 downregulation can happen through CAR-mediated trogocytosis, a process through which lymphocytes extract surface molecules from the antigen-presenting cells conjugated through the immunological synapse[42]. Of note, this phenomenon was observed with multiple anti-CD19, anti-CD22, anti-B-cell maturation antigen (BCMA), and anti-mesothelin CARs, demonstrating a pervasive role of trogocytosis

in downregulating surface expression even in CAR T-cell therapies that ultimately develop complete antigen loss as a major form of resistance. Interestingly, trogocytosis brought about not only diminished antigen density, but also “fratricide” T-cell killing and hastened T-cell exhaustion. While T-cell-specific inhibitory effects could be partially ameliorated by increasing the ratio of CAR T-cell-to-leukemia cells to facilitate cooperative killing, trogocytosis could be an important connection between tumor cell- and T-cell-intrinsic mechanisms of resistance.

Lineage switching.—Another important mechanism of antigen loss occurs through lineage switching, in which the selective pressure of B-cell marker-directed immunotherapy causes B-ALL to undergo re-programming into a myeloid leukemia expressing none of the B-lineage markers. In humans, lineage switching is most commonly associated with B-ALL or mixed phenotype acute leukemias (MPAL) harboring MLL rearrangements (MLL-r). Prior to the advent of CAR T-cell therapies, it was thought to be a very rare event associated with chemotherapy[43,44]. However, in a Seattle-based CD19-directed CAR T-cell trial, 2 out of 7 patients with MLL-r B-ALL demonstrated lineage switching at the time of relapse[21]; similar results were reported by Jacoby et al[45]. At the cellular level, lineage switching in CD19 CAR T-cell treated patients could be due to either outgrowth of pre-existing CD19-negative myeloid subclones or active reprogramming of differentiated lymphoid cells into myeloid blasts at time of relapse. While the underlying mechanism remains to be elucidated, the existence of well-documented mouse models of lineage infidelity[46,47] argues in favor of the second scenario.

THERAPEUTIC IMPLICATIONS AND FUTURE PERSPECTIVES

Surface antigen loss and/or decreased expression have emerged as major mechanisms whereby B-cell malignancies evade CAR T-cell detection (Figure 1). Since immunoedited malignant cells fuel clinical relapses, antigen escape clearly poses a major obstacle to latter-day immunotherapies. There is still much that is poorly understood about resistance to CAR T-cell therapies. On the one hand, some anticipated mechanisms of resistance thus far have not borne out. One salient example is the apparent lack of epigenetic silencing of the target gene, be that CD19 or CD22. Furthermore, there has been no evidence so far for inherent or acquired cross-resistance to immunotherapy. For example, prior exposure to CD19 CAR T therapy appears to have no effect on sensitivity to CD22 CAR T-cells[26]. One the other hand, new resistance mechanisms continue to be discovered and undoubtedly will be more fully elucidated over time.

In the future, there is also hope that certain patients might be good candidates for receiving CAR T-cells as an upfront therapy, thereby decreasing toxicities associated with extended chemotherapy regimens and potentially eliminating the need for hematopoietic stem cell transplants. Biomarkers that predict for decreased risk of CAR T-cell relapse would be useful in selecting such candidates. At present, it is still unclear what can be done to anticipate, circumvent, or better yet prevent antigen escapes.

The apparent preservation of cognate antigen expression in at least some patients raises hopes that mutant CD19 isoforms could be targeted with alternative CAR T-cells. However,

at least in the case of CD19, this would require additional effort to rescue the mutated or mis-spliced proteins from ER retention and to facilitate their intracellular trafficking. The downregulation of CD22 as a mechanism of resistance also suggests that B-cells may not need complete epitope loss to resist CAR T-cell killing. Rather, there might be a threshold of antigen density below which CARs are ineffective at recognizing the cognate epitope. This has critical implications for CAR T-cell trial design. If targeted antigens are not adequately expressed on the cell surface, inhibition of trogocytosis (by yet to be identified means) or promoting trafficking to the plasma membrane (via the use of chemical chaperones) could be considered as potential adjuvant therapies.

Another idea to overcome antigen escape that has gained considerable traction is the design of combinatorial CARs, which concurrently target multiple antigens. Several phase I trials are underway investigating the combinations of CD19- and CD22-directed CAR T-cell therapy. The therapies can be combined at the level of CAR specificity (“tandem CAR”), vector design (“bicistronic CAR”), CAR T-cell manufacturing (“co-transduction”), or infusion (“co-administration”)[48]. In addition to CD19 and CD22, other studies are examining various combinations of CD19, CD20, CD22, and CD123 for B-ALL. Combinatorial CARs targeting both CD19 and CD123 may be especially well-suited for MLL-r leukemias as CD123 is expressed on both lymphoid and myeloid blasts[49]. Further combinations include a trivalent CAR to target CD19, CD20, and CD22[50]. However, targeting multiple antigens simultaneously still entails the theoretical risk of relapse with multiple antigen-negative disease, which could quickly exhaust other antigen-directed therapy options.

In light of that risk, exploiting “epitope spreading” has been gaining momentum as an alternative strategy to minimizing resistance. Epitope spreading is the phenomenon whereby initial epitope-directed immune responses prime T-cells to induce recognition of closely related but a more diversified set of epitopes, some of which may even reside on other proteins[51]. This concept has already been shown to aid responses to immunotherapy in melanoma and pancreatic cancer[52,53], but thus far, investigators have not been successful at harnessing the power of epitope spreading to induce wider anti-tumor immune responses in B-ALL. This could reflect the overall lower immunogenicity of B-ALL or the immunosuppressive state from the lymphodepletion prior to CAR T-cell administration. However, it is possible that by combining CAR T-cell therapies with other immunotherapies one would boost the anti-tumor immune response to the points where multiple antigens can be recognized[54].

As promising new CAR T-cell therapies are translated beyond the realm of hematologic malignancies, the paradigm of antigen escape is likely to persist. Solid tumors have a marked degree of heterogeneity in tumor antigen expression and lack highly expressed and highly conserved cell markers, such as CD19 and CD22. Therefore, the threshold for developing antigen escape is presumably much lower for solid tumors. Indeed, antigen loss has also been demonstrated using CAR T-cell therapies against surface epitopes such has BCMA for multiple myeloma and EGFRvIII for glioblastoma multiforme[55,56]. Thus, lessons learned from studying resistance mechanisms in hematologic malignancies should benefit CAR T-cell therapies for cancers beyond leukemias and lymphomas. Additionally, given the

prevalence of antigen escape, there is a pressing need to maintain robust pipelines for discovery and validation of new immunotherapy targets.

In order to mitigate antigen loss and on-target/off-tumor effects, a guiding principle in target selection is finding an antigen that is highly expressed and ubiquitous across cancer cells, near- absent in normal tissues, and functionally important to cancer maintenance to minimize easy disposal or immunoediting of the antigen[11]. In reality, various trade-offs are made to accommodate these principles, and antigen escape remains a recurrent problem. A potential long-term solution to this problem is the targeting of tumor neoantigens. Unlike traditional lineage markers like CD19 or CD22, neoantigens hold the promise of minimizing toxicity given their absence, by definition, in normal tissues. Although there has always been significant interest in mutation-driven neoantigens, another useful source of neoantigens might be alternative splicing prevalent in many tumor types[57]. While most effort in that area has been directed toward intracellular neoantigens, the requirement for MHC-restricted presentation sharply reduces the repertoire of suitable epitopes[58]. Thus, one might consider focusing on proteins with alternatively spliced extracellular domains which could be targeted by CAR T-cells and antibody-drug conjugates with the selectivity current immunotherapeutics do not possess.

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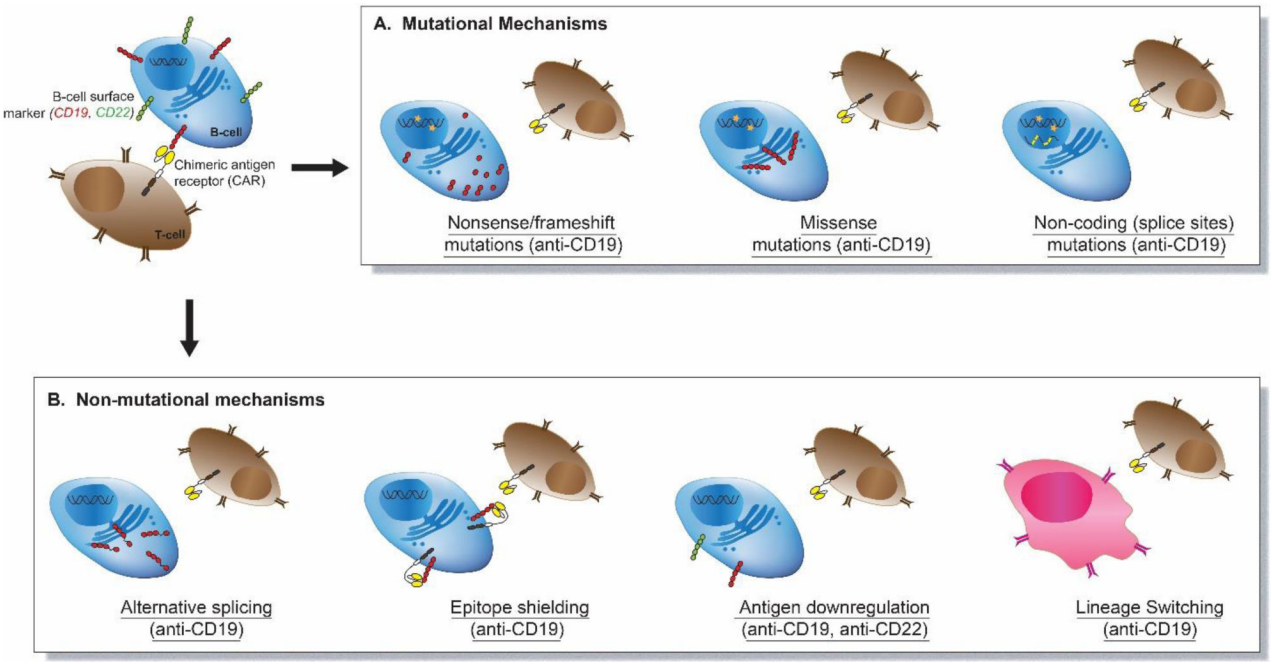

Figure 1. Mechanisms of CAR T cell -driven immunoediting in B-cell leukemia.

CAR T-cell therapies involve the use of genetically engineered T-cells expressing chimeric antigen receptor (CAR) which target B-cells surface markers such as CD19 (red) or CD22 (green). **A**. Focal mutations in the CD19 gene (orange stars) are known to result in antigen loss. Nonsense mutations lead to expression of non-functional truncated protein; missense mutations mediate antigen escape via retention of the misfolded protein in endoplasmic reticulum; splice site mutations cause deleterious alterations in splicing such as intron retention. **B**. Non-mutational mechanisms such as alternative splicing, epitope shielding, antigen downregulation, and lineage switching have also been shown to cause acquired resistance to CAR T-cells.