Clinical pharmacology of CAR-T cells: Linking cellular pharmacodynamics to pharmacokinetics and antitumor effects

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Adoptive cell transfer of T cells genetically modified with tumor-reactive chimeric antigen receptors (CARs) is a rapidly emerging field in oncology, which in preliminary clinical trials has already shown striking antitumor efficacy. Despite these premises, there are still a number of open issues related to CAR-T cells, spanning from their exact mechanism of action (pharmacodynamics), to the factors associated with their in vivo persistence (pharmacokinetics), and, finally, to the relative contribution of each of the two in determining the antitumor effects and accompanying toxicities. In light of the unprecedented curative potential of CAR-T cells and of their predicted wide availability in the next few years, in this review we will summarize the current knowledge on the clinical pharmacology aspects of what is anticipated to be a brand new class of biopharmaceuticals to join the therapeutic armamentarium of cancer doctors.

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1. Introduction

For approximately 50 years, from the mid-1940s through the mid-1990s, the pharmacotherapy of cancer has been entirely based on conventional cytotoxic drugs [1]. These drugs consist of pure chemical substances with known structures, well-defined pharmacokinetics (PK) and pharmacodynamics (PD), and a clear correlation between the two. From the 2000s, a better understanding of cancer biology and the availability of advanced technologies, e.g. tissue microarrays and proteomics, have allowed the identification and the detailed characterization of cancer-specific molecular targets, marking the beginnings of targeted therapies [2]. These targets typically include signaling molecules, cell-cycle proteins, modulators of apoptosis and pro-angiogenic factors [3]. The “druggability” of molecular targets relies on the rational design of small molecules that are well adsorbed (A), widely distributed (D), metabolized (M) after a known period of time, and subsequently excreted (E), following the so-called ADME criteria. Moreover, for therapeutic effectiveness, the ADME criteria of candidate small molecules need to be weighted against an acceptable toxicity profile at pharmacologically active concentrations. Although not classified as targeted therapies, monoclonal antibodies (mAbs) fall within the same concept, with the additional complications of biopharmaceuticals with a multifactorial PK, depending on whether they are “naked” [4], potentiated [5] or conjugated with chemotherapeutic agents [6,7] or radioisotopes [8], and a still to be fully-elucidated PD [9].

The idea of transferring ex vivo-manipulated cells of the immune system into cancer patients for therapeutic purposes, also known as adoptive cell therapy (ACT), is an idea that dates back to the mid-1980s, approximately ten years before the debut of targeted therapies, when Steve Rosenberg pioneered the use of lymphokine-activated killer cells in metastatic melanoma [10]. In thirty years, the original concept of ACT has dramatically evolved, now embracing the use of immune effectors as disparate as cytokine-induced killer (CIK) cells [11], tumor-infiltrating lymphocytes or TILs (T cells isolated from tumor sites, expanded and re-infused back into patients [12]), and, more recently, T cells genetically modified to express clonal T-cell receptors (TCRs) or chimeric antigen receptors (CARs). Initially conceived by Zelig Eshhar, CARs are monomeric receptors usually designed by fusing the single-chain fragment variable (scFv) of a tumor-reactive mAb with a transmembrane domain and one or more signaling molecules containing intracellular immunoreceptor tyrosine-based activation motifs (ITAMs) [13]. Upon CAR modification, originally with viral vectors, but more recently also with non-viral systems [14], T cells become cytotoxic against tumor cells expressing the antigen recognized by the mAb of origin. The greatest payback of ACT over targeted therapies and mAbs relies on the nature of effector T cells, which are endowed with unparalleled potency, wide biodistribution and long-term persistence, without the need of...
repetitive administrations. Moreover, at least in the case of CARs, HLA-independent recognition ensures intrinsic resistance to the defects in antigen processing and presentation that tumor cells often put in place to evade T-cell recognition [15,16].

Given the complexities of ACT, it is clear that the classical concepts of ADME, PK and PD as found in textbooks of clinical pharmacology fail at addressing the multiple issues related to this new class of “living” medicines. However, since these therapies are gaining increasing attention from health-care professionals, patients and industrial stakeholders, a careful appraisal of their peculiar pharmacology has become of age. The aim of this review is to briefly unfold the PK and PD aspects of ACT, and in particular of CAR-T cells, by focusing on all information available to date on their mechanism of action and preliminary, yet undoubtedly striking, antitumor efficacy in humans.

2. Pharmacodynamics (PD) of CAR-T cells

2.1. Role of intracellular signaling domains

The most widely adopted ITAM-containing molecule for CAR-mediated recognition is the CD3 zeta-chain of the TCR complex. Although the precise mechanism by which the extracellular scFv sequence of CARs should signal across phospholipid bilayers is unknown, structural cues suggest that the pathways may significantly deviate from those employed by endogenous TCRs. What is known is that some of the physiological TCR-signaling downstream events also take place in the setting of CAR-mediated recognition, including tyrosine phosphorylation of the CD3 zeta-chain [17], recruitment of ZAP-70 [18], initiation of the MAPK cascade and activation of NFAT [19].

Torsion-based models have substantiated the concept that the TCR is an anisotropic mechanosensor, which upon MHC-restricted antigen recognition, undergoes conformational changes allowing productive non-covalent bonding interactions with the CD3 complex [20]. On the contrary, it has been recently proposed that, in CARs, the phosphorylation of the endogenous CD3 complex may happen as a result of the supra-molecular recruitment of Src kinases upon antigen-mediated clustering of the chimeric molecules [21] (Fig. 1). Efficient CAR-mediated signaling indeed requires physical homomultimerization of chimeric molecules and interaction with components of the endogenous TCR complex [22]. This is confirmed by the fact that specific mutations in the CD3 zeta-chain transmembrane region significantly reduce CAR homo-multimerization and non-covalent ionic bonding with other components of the TCR complex. Nonetheless, ITAM-deficient CARs still signal to a certain degree [23] and targeted deletions of their ITAMs do not result in significant impairment in signal transduction [21], suggesting that the formation of membrane-proximal complexes between CARs and endogenous CD3 components play a crucial role through inter-chain phosphorylation events. Accordingly, selective mutations of the transmembrane L9 residue, which is crucial for CAR clustering on the cell membrane, significantly impairs signaling [22], which is conversely maintained by ITAM-containing molecules not classically associated with T cells, such as the Fc-epsilon receptor [24]. Recent evidence also suggests that CARs might have an intrinsic propensity to tonic signaling in an

Fig. 1. Signaling transduction events following TCR- and CAR-mediated activation. (Left) Torsion-based model of TCR activation: upon recognition of a peptide from an intracellularly processed antigen presented on the surface of a tumor cell in the context of MHC, the TCR undergoes a series of mechanical torsion changes allowing the recruitment of a number of intracellular signaling molecules that ultimately lead to nuclear translocation of transcription factors responsible for T cell activation. This TCR torsion is assisted by the CD4/CD8 co-receptors. (Right) CAR multi-homodimerization: differently form TCRs, CARs recognize mAb epitopes of extracellular antigens clustered on the surface of tumor cells. This leads to the formation of an intracellular supra-molecular complex in CAR-T cells that also leads to nuclear translocation and activation. During this process, CARs exploit “parasitic” interactions with components of the TCR.
antigen-independent context during ex vivo culture of T cells, leading to premature exhaustion [25]. This phenomenon, characterized by altered transcriptional profiles, up-regulation of bona fide exhaustion markers and an early collapse of proliferative potential, is remarkably similar to that of chronically stimulated T cells in the setting of cancer [26] and chronic infections [27].

2.2. Role of CAR design

While it is theoretically possible to start from multiple sources (Fab fragments, natural ligands, peptides, aptamers), most investigated CARs to date use scFvs derived from mouse mAbs. Mouse mAbs are indeed characterized by high affinity, which is one of the major factors determining their antitumor activity. Other key factors are antigen-expression levels of targeted antigens [28], which should be sufficiently high, as well as epitope accessibility [29]. The optimal combination between overall CAR affinity, antigen-expression levels and epitope accessibility is however poorly characterized and most likely distinctive for each individual target. In the case of natural T cells, TCR signaling is ensured by the small size of TCR/peptide–MHC complexes, which result in their physical segregation and protection from the inhibitory activities of tyrosine phosphatases [30]. The number of molecules that are expressed on the tumor-cell surface and available for CAR recognition can vary substantially from target to target, and it is typically much higher than that of peptide–MHC molecules available for TCR binding [31]. Accordingly, the affinity of the scFvs from which the CARs are constructed is one of the major determinants of their antitumor potency, as demonstrated for the target antigen (lg)-like/Frizzled region of ROR1 in xenograft mouse models [32]. Moreover, careful tuning of CAR affinity has been advocated as a way to mitigate the potential toxicities deriving from low-density target antigen expression on normal tissues, as in the case of the epidermal growth factor receptor [33].

2.2.1. Role of spacer elements

The extracellular portion of CARs can be directly linked to intracellular signaling elements or separated by spacer regions allowing the necessary flexibility for contacting poorly accessible epitopes. For instance, membrane-distal epitopes, such as MFE23 on CEA, can be optimally engaged in the absence of extracellular spacers, whereas membrane-proximal epitopes, such as D29 on NCAM, in general require longer spacers [34]. Examples of CAR spacers include the CH2CH3 Fc portion of Iggs or lg-like extracellular portions of CD4 or CD8. In vitro data indicate that CAR-T cells carrying IgG1-derived CH2CH3 spacers may interact with cells of the innate immune system expressing Fc receptors (macrophages, NK cells) regardless of their antigen specificity, resulting in detrimental off-tumor effects [35]. Additionally, the same spacers have been shown to contribute to activation-induced cell death (AICD) and premature clearance of CAR-T cells in xenograft models [36]. Although from human studies performed to date, there is no evidence that these phenomena may result in reduced functionality of CAR-T cells and/or cause adverse events, this possibility has to be necessarily kept in mind for future developments.

2.2.2. Role of costimulatory endodomains

The first CARs to enter clinical investigation, now referred as first-generation (1G) CARs, have been constructed by linking the scFvs of tumor-reactive mouse mAbs to the TCR CD3-zeta-chain, a design that, at least in vitro, results in potent cytotoxicity [13]. In patients, however, the antitumor responses elicited by T cells engineered with 1G CARs have been modest and incomplete, possibly because of their poor in vivo persistence [37–39]. Although the immunogenenicity due to mouse scFvs might have played a role in premature clearance of CAR-T cells in these trials, it was soon recognized that the main reason for limited persistence was the suboptimal signaling provided by 1G designs [40]. A major advance in the field was achieved when investigators started incorporating one (second generation, 2G) or two (third generation, 3G) costimulatory endodomains from CD28 [41,42], 4-1BB [43, 44], and/or OX-40 [45] into the chimeric molecules in order to improve signaling. Since there is an ongoing debate on which is the best costimulatory endodomain for optimal antitumor activity, it is important to analyze the different options.

Together with TCR-mediated signals, the interactions of the costimulatory molecule CD28 on T cells with B7-1 (CD80) and B7-2 (CD86) on antigen-presenting cells (APCs) positively influence many aspects of T-cell biology, including, but not limited to, induction of anti-apoptotic proteins and active transcription/translation of growth factors (cytokines) essential for their proliferation and differentiation. CD28 endodomains of 2G CARs typically contain the YMNFM motif beginning at tyrosine 170, which is known to recruit important Src-homology 2 (SH2)-domain proteins, such as PI3K [46] and Grb2/Gads adapters [47], and to activate NFkB via two proline-rich motifs (Fig. 2). Moreover, the Y170 residue itself induces Bcl-xl via mTOR, while both Y170 and N172 augment IL-2 transcription via PKC-th. Importantly, both phases of IL-2 transcription, i.e. Y170- and PI3K-dependent transcription initiation and PI3K-independent secondary stabilization of IL-2 transcripts, therefore appear to be supported by CD28 endodomains of 2G CARs. These also contain two proline-rich motifs binding other SH2-domain proteins, such as Lck, linking CD28 signaling to the co-receptors CD4/CD8. Compared with T cells engineered with 1G CARs, those engineered with CD28-based CARs are therefore characterized by enhanced cytokine production and proliferative potential [48], resulting in superior antitumor activity, as shown in xenograft models [44].

Following initial TCR/peptide–MHC and CD28 signaling, a number of additional receptor-ligand pairs are up-regulated on T cells and APCs, respectively. After productive interactions with their ligands, members of the tumor necrosis factor (TNF) receptor family, such as 4-1BB, OX40 and CD27, in particular, appear to be crucial for supporting the complex series of events that sustain T-cell activation (Fig. 2) [49]. In vitro, 4-1BB signaling has been shown to promote Th1/Tc1 polarization [50] and prevent AICD [51] independently from CD28 [52], while in vivo it is known to play a non-redundant role during both primary [53] and secondary immune responses [51]. Like other members of the TNF receptor family, however, 4-1BB does not possess an intrinsic kinase activity, but requires binding to TRAF2. Accordingly, important downstream effectors of 4-1BB signaling are NF-kb and the MAPKs p38 and JNK [54]. Results in xenograft models suggest that, at least for the CD19 target, T cells engineered with 4-1BB-based CARs may possess superior antitumor activity compared with those engineered with the CD28-based counterparts [44]. Nevertheless, given the number of confounding variables in ongoing clinical trials (Table 1), at least for the time being, it would be incautious to claim the absolute superiority of 4-1BB over CD28-based CAR designs.

Although not yet under clinical investigation, endodomains from other costimulatory molecules have been evaluated forameliorating CAR-mediated signaling, including those from OX40 [45], CD27 [55] and ICOS [56]. Similarly to 4-1BB, OX40 is dependent on TRAF-2 for active signaling, which in turn results in potent and CD28-independent activation of NFkB. Accordingly, 3G CARs incorporating both OX40 and CD28 endodomains showed synergistic activation of NFkB compared with CARs having either one or the other [45]. Moreover, OX40-mediated co-signaling in 3G CARs already incorporating a CD28 endodomain has been shown to effectively and specifically repress IL-10 secretion, without interfering with cytotoxicity and pro-inflammatory cytokine production [57]. CD27 is another member of the TNF receptor family having an important role in promoting T-cell survival [58] and immunological memory [59]. In xenograft models, T cells engineered with CD27-based CARs were characterized by enhanced persistence and antitumor activity similar to T cells engineered with 4-1BB-based CARs, but somewhat superior to
those engineered with CD28-based CARs [55]. Contrary to CD28-based CARs, CD27-based CARs, however, appear not to mediate the production of IL-2, a cytokine known to promote Tregs outgrowth [56], and might therefore have beneficial effects in restraining potential immune regulation. The last newcomers in the family of CAR endocostimulatory domains are derived from ICOS. Differently from 4-1BB and OX40, ICOS belongs to the CD28 family and has been shown to be specifically required for optimal expansion and function of human Th17 cells [60].

2.3. Effectors other than alpha-beta T lymphocytes

Most studies on CARs so far have focused their attention on conventional alpha-beta T cells. However, other lymphocyte subsets, e.g. natural killer (NK) cells, invariant NKT (iNKT) cells, gamma-delta T cells and cytokine-induced killer (CIK) cells, may possess advantageous characteristics over alpha-beta T cells and are currently under intense scrutiny by different investigators.

Although CAR-mediated recognition is HLA-independent, and therefore does not require HLA matching between the patient and the T cells, this technology is usually employed in the autologous setting, because of the potential risks of graft-versus-host (GVH) reactions due to alloantigen recognition by native TCRs. Being TCR-negative, NK cells provide a valuable alternative to alpha-beta T cells, as they can be harvested from HLA-disparate donors, redirected with CARs and infused into patients without incurring the adverse immunological reactions caused by alloreactivity [61]. Moreover, clinical trials with adoptively transferred allogeneic NK cells have demonstrated that these cells can survive in vivo for several weeks to months [62]. Importantly, studies with the NK-92 cell line and primary NK cells suggest that, when incorporated into CARs, ITAMs from the NK-specific ITAM-containing molecule DAP12 may perform better than the CD3zeta chain [63]. Although very rare (0.1% of circulating T cells), CD1d-restricted Valpha24-invariant (type-I) natural killer T (NKT) cells are another promising cell type for CAR redirection, because of their low toxicity profile (CD1d-expression is restricted to APCs) and their marked tumor tropism [64]. Based on these assumptions, iNKT cells optimally redirected with 3G CARs incorporating both CD28 and 4-1BB endodomains have been recently shown to be capable of mediating potent antitumor effects in xenograft mouse models of neuroblastoma, without causing GVHD [65]. Conversely, while sharing with iNKT the lack of alloreactivity and an intrinsic antitumor potential [66], gamma-delta T cells represent a much higher fraction of circulating T cells (1–5%), which can be further expanded with clinical-grade aminobiphosphonates [67]. Zoledronic acid, for example, selectively promotes the proliferation of Vgamma9/Vdelta2 cells [68] and has been used to effectively redirect them with CD28-based CARs [69]. Finally, cytokine-induced killer (CIK) cells are a heterogeneous population enriched for Vgamma9/Vdelta2 cells, which can be generated after stimulation of peripheral blood mononuclear cells with anti-CD3 mAbs and supra-physiological concentrations of IL-2. Similarly to iNKT and gamma-delta T cells, CIK cells display some antitumor functions, yet display negligible alloreactivity [70]. Interestingly, it has been found that over-costimulation by 3G CARs paradoxically diminishes the antitumor activity of CIK cells, possibly because of accelerated terminal differentiation [71].

3. Pharmacokinetics of CAR-T cells

Differently from conventional drugs, the pharmacological notions of adsorption, distribution, metabolism and excretion (ADME) cannot be used to model the pharmacokinetics of CAR-T cells. Since these cells are usually infused intravenously, adsorption is clearly not an issue. Conversely, distribution, metabolism and excretion also apply, but need to be thoroughly reconsidered. Similarly to natural T cells, it is assumed that CAR-T cells distribute widely in the tissues and, after exerting their antitumor effects, die and are eliminated through yet poorly understood mechanisms. Theoretically, CAR-T cells dying due to lack of survival factors (death by neglect) or to excessive AICD are
### Table 1
Characteristics and clinical outcome of anti-CD19 CAR-T cell trials published so far.

<table>
<thead>
<tr>
<th>Indication</th>
<th>Pts (n)</th>
<th>Conditioning</th>
<th>Construct&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Vector</th>
<th>Culture (type/duration)</th>
<th>Cell dose&lt;sup&gt;2&lt;/sup&gt; (×10E6)</th>
<th>CD4/CD8 (average)</th>
<th>Persistence (up to)</th>
<th>Toxicities</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jensen (2010)</td>
<td>FL 2</td>
<td>Flu</td>
<td>FMC63 CH2CH3 zeta</td>
<td>Electro</td>
<td>OKT3/IL-2/LCL 14 days</td>
<td>100–2000/m²</td>
<td>Not specified</td>
<td>1 week</td>
<td>None</td>
<td>2 NR</td>
</tr>
<tr>
<td>Kochenderfer (2010)</td>
<td>FL 1</td>
<td>CTX + Flu</td>
<td>FMC63 CD28z</td>
<td>RV</td>
<td>OKT3/IL-2 12 days</td>
<td>400 total IL-2 iv</td>
<td>60% CD8 34% CD4</td>
<td>27 weeks</td>
<td>Fever</td>
<td>CR</td>
</tr>
<tr>
<td>Savoldo (2011)</td>
<td>NHL 6</td>
<td>None</td>
<td>FMC63 CH2CH3 CD28z</td>
<td>RV</td>
<td>OKT3/IL-2 6–18 days</td>
<td>20–400/m²</td>
<td>50% CD8 50% CD4</td>
<td>2G 6 weeks</td>
<td>None</td>
<td>2 SD</td>
</tr>
<tr>
<td>Kalos (2011) Porter(2011)</td>
<td>CLL 3</td>
<td>Ben or Pnt + CTX</td>
<td>FMC63 4-1BBz</td>
<td>LV</td>
<td>Beads/IL-2 10–12 days</td>
<td>0.1–16/kg</td>
<td>27% CD8 73% CD4</td>
<td>25 weeks</td>
<td>BCA TLS CRS</td>
<td>2 CR 1 PR</td>
</tr>
<tr>
<td>Brentjens (2011)</td>
<td>8 CLL 2 ALL 10</td>
<td>None</td>
<td>SJ25C1 CD28z</td>
<td>RV</td>
<td>Beads/IL-2 11–19 days</td>
<td>3–30/kg</td>
<td>12% CD8 88% CD4</td>
<td>6 weeks 1 death Fever</td>
<td>1 CR 1 PR 2 SD</td>
<td></td>
</tr>
<tr>
<td>Kochenderfer (2012)</td>
<td>3 FL 4 CLL 1 SMZL 8</td>
<td>CTX + Flu</td>
<td>FMC63 CD28z</td>
<td>RV</td>
<td>OKT3/IL-2 24 days</td>
<td>3–30/kg L2 iv</td>
<td>53% CD8 47% CD4</td>
<td>27 weeks</td>
<td>BCA CRS</td>
<td>1 CR 5 PR 1 SD</td>
</tr>
<tr>
<td>Brentjens (2013)</td>
<td>ALL 5</td>
<td>CTX</td>
<td>SJ25C1 CD28z</td>
<td>RV</td>
<td>Beads/IL-2 10–12 days</td>
<td>1.5–3/kg</td>
<td>37% CD8 63% CD4</td>
<td>8 weeks</td>
<td>CRS</td>
<td>4 allo-SCT 1 relapse</td>
</tr>
<tr>
<td>Grupp (2013) Cruz (2013)</td>
<td>ALL 2 ALL 4 CLL 8</td>
<td>None or Eto + CTX</td>
<td>FMC63 4-1BBz</td>
<td>LV</td>
<td>Beads/IL-2 10–12 days</td>
<td>1.4–12/kg</td>
<td>Not specified</td>
<td>27 weeks</td>
<td>CRS Neurotox</td>
<td>1 CR 1 relapse 3 CR 1 PR 1 SD</td>
</tr>
<tr>
<td>Low (2013)</td>
<td>ALL 4 ALL 4</td>
<td>None</td>
<td>FMC63 CH2CH3 CD28z</td>
<td>RV</td>
<td>LCL/IL-2 25–52 days</td>
<td>5.1–41/m²</td>
<td>5 pts. 90% CD8 3 pts. 90% CD4</td>
<td>12 weeks</td>
<td>None</td>
<td>3 CR 1 PR 1 SD</td>
</tr>
<tr>
<td>Koehler (2013)</td>
<td>4 CLL 2 DLCL 4 MCL 10</td>
<td>None</td>
<td>FMC63 CD28z</td>
<td>RV</td>
<td>OKT3/IL-2 8 days</td>
<td>0.4–7.8/kg</td>
<td>52% CD8 48% CD4 5 weeks</td>
<td>TLR CRS</td>
<td>1 CR 1 PR 6 SD</td>
<td></td>
</tr>
<tr>
<td>Davila (2014)</td>
<td>ALL 16</td>
<td>CTX</td>
<td>SJ25C1 CD28z</td>
<td>RV</td>
<td>Beads/IL-2 11–19 days</td>
<td>3.0/kg</td>
<td>32% CD8 68% CD4</td>
<td>4 weeks</td>
<td>CRS Neurotox</td>
<td>14 CR</td>
</tr>
<tr>
<td>Maude (2014)</td>
<td>ALL 30</td>
<td>None or CTX or CTX + Flu</td>
<td>FMC63 4-1BBz</td>
<td>LV</td>
<td>Beads/IL-2 10–12 days</td>
<td>0.8–17.4/kg</td>
<td>Not specified</td>
<td>104 weeks</td>
<td>BCA CRS Neurotox</td>
<td>27 CR</td>
</tr>
<tr>
<td>Lee (2015)</td>
<td>ALL 21</td>
<td>CTX + Flu</td>
<td>FMC63 CD28z</td>
<td>RV</td>
<td>OKT3/IL-2 11 days</td>
<td>1–3/kg</td>
<td>Not specified</td>
<td>6 weeks</td>
<td>CRS Neurotox</td>
<td>14 CR 4 PD 3 SD</td>
</tr>
<tr>
<td>Koehler (2015) Porter (2015)</td>
<td>9 DLCL 2 IL 4 CLL 15</td>
<td>CTX + Flu</td>
<td>FMC63 CD28z</td>
<td>RV</td>
<td>OKT3/IL-10 days</td>
<td>1–5/kg</td>
<td>90% CD8 10% CD4</td>
<td>10 weeks</td>
<td>CRS Neurotox</td>
<td>8 CR 4 PR 1 SD</td>
</tr>
<tr>
<td>Porter (2015)</td>
<td>CLL 14</td>
<td>CTX + Flu or Pnt</td>
<td>FMC63 4-1BBz</td>
<td>LV</td>
<td>Beads/IL-2 10–12 days</td>
<td>262 total (median)</td>
<td>20% CD8 80% CD4</td>
<td>196 weeks</td>
<td>BCA CRS Neurotox</td>
<td>4 CR 4 PR</td>
</tr>
</tbody>
</table>

**Table notes:**
- ¶FMC63 or SJ25C1, name of mAbs from which the CAR was derived; CH2CH3, extracellular spacer containing an Ig CH2CH3 domain; zeta, intracellular signaling by the CD3 zeta-chain only; CD28z, intracellular signaling by the CD3 zeta-chain and a CD28 endodomain; 4-1BBz, intracellular signaling by the CD3 zeta-chain and a 4-1BB endodomain.
- §Range.

**Table abbreviations:**
- FL, follicular lymphoma; NHL, non-Hodgkin lymphoma; CLL, chronic lymphocytic leukemia; ALL, acute lymphoid leukemia; SMZL, spleen marginal zone lymphoma; DLCL, diffuse large B-cell lymphoma; IL, indolent lymphoma; Flu, fludarabine; CTX, cyclophosphamide; Ben, bendamustin; Pnt, pentostatin; Eto, etoposide; Electro, electroporated; RV, retroviral vector; LV lentiviral vector; LCL, lymphoblastoid cell line; BCA, B-cell aplasia; TLS, tumor-lysis syndrome; CRS, cytokine release syndrome; Neurotox, neurotoxicity; NR, no response; PR, partial response; CR, complete response; SD, stable disease. Best responses are listed.
removed from the circulation by the reticulo-endothelial system of the spleen and the liver.

### 3.1. Biodistribution

A prerequisite for the antitumor efficacy of CAR-T cells is their specific migration and retention of full effector functions at tumor sites. It is widely accepted that malignant cells, especially those of epithelial origin, profoundly remodel their microenvironment making it hostile for incoming T cells. Accordingly, up to now, the best clinical results with CAR-T cells have been attained in hematological cancers, which are admittedly characterized by a microenvironment better accessible to T cells and less immunosuppressive compared with that of solid tumors. While earlier trials with 1G CARs failed to document significant accumulation of CAR-T cells at tumor sites [72,73], more recently 2G CAR-T cells have been found inside regressing tumors of both hematological and non-hematological origin, confirming that efficient migration is a key factor for antitumor efficacy [74]. In fact, CAR-T cells must traffic to and accumulate at tumor sites. These properties involve a complex sequence of events (Figure 1): beginning with the adhesion of T cells to endothelial cells followed by chemokine-driven extravasation into antigen-rich tissues. During this process, T cells actively degrade the main components of the subendothelial basal membrane and of the extracellular matrix (ECM). It was recently demonstrated that the poor infiltration of CAR-T cells into solid tumors may be due, at least in part, to the down-regulation of the ECM-degrading enzyme heparanase [75]. Accordingly, genetically complementing CAR-T cells with heparanase has been shown to dramatically increase their penetration into solid tumor masses and therefore their activity in xenograft mouse models. On the other hand, the immunosuppressive culprits typical of some tumors can be specifically exploited to readdress adoptively transferred T cells, as demonstrated by CCR4 co-expression in CAR-T-cells for enhancing their infiltration in the Th2-polarized microenvironment of Hodgkin lymphoma [76]. Moreover, CAR-T cells accumulating in solid cancers may transiently up-regulate exhaustion markers and reversibly lose their functionality, as recently shown in xenograft mouse models of mesothelioma [77]. Since TGF-beta is a major immunosuppressive cytokine found in the microenvironment of solid tumors and potentially responsible for these effects, investigators have proposed equipping adoptively transferred T cells with a dominant-negative form of the TGF-beta receptor, rendering them insensitive to the detrimental effects of this cytokine [78].

### 3.2. Role of intrinsic factors

#### 3.2.1. Role of the memory-differentiation phenotype

It is recognized that one of the most important factors determining the persistence of adoptively transferred T cells is their memory-differentiation state at the end of the culture period and before infusion into patients. Accordingly with the recently revised linear model of memory T-cell differentiation, there is consensus on the fact that increasing levels of antigen stimulation determine the progressive transition of naïve T cells first into stem-cell memory T (T_{SCM}) cells, then into central-memory T (T_{CM}) and finally into effector-memory T cells (T_{EM}) cells [79]. While in vitro T_{EM} cells are superior to T_{CM} cells in terms of effector functions, in mouse models, T_{CM} cells have been shown to display greater therapeutic effects, most likely because of their longer persistence compared with T_{EM} cells [80]. The long-term persistence of T_{CM}-derived antigen-specific T cells in vivo is possibly a common feature of mammals, as confirmed in non-human primates [81]. The molecular mechanisms underlying this inverse correlation may include the inability of terminally differentiated T cells to produce IL-2, a decreased expression of receptors required for homeostatic cytokines and an unbalance between pro-apoptotic and anti-apoptotic molecules. Ex vivo manipulation of T cells, as it is performed for clinical use since several years, has been shown to induce a progressive differentiation towards the T_{EM} phenotype [82], resulting in functional changes that make them less “fit” to mediate antitumor responses [83]. Retrospective analyses of the results from clinical trials using earlier protocols of ex vivo manipulation have indeed demonstrated that the long-term persistence of CAR-T cells in vivo (up to 192 weeks) strongly correlates with the percentage of T_{EM} cells [84] or of T_{SCM} cells [85] in the infused products. In another experience, the long-term in vivo persistence (up to 6 months) of anti-CD19 CAR-T cells incorporating a 4-1BB endomain associated with their T_{EM} phenotype and with the expression of CCR7 and CD127, and lack of CD27 and PD-1 [86]. These data strongly support the assumption that ex vivo manipulation protocols specifically designed for preserving an early differentiation phenotype may result in enhanced antitumor activity.

#### 3.2.2. Role of costimulatory endodomains

While it is well accepted that the introduction of next-generation CAR designs has been the major determinant behind the latest successes in clinical trials, it remains unclear whether any particular costimulatory endodomain results in superior persistence of CAR-T cells. Notably, 4-1BB endodomains were shown to allow stronger in vitro proliferation compared with CD28 endodomains and to enhance the persistence of anti-CD19 CAR-T cells in mouse models [44]. Recent findings indicate that the incorporation of 4-1BB endodomains may mitigate the exhaustion phenotype deriving from tonic signaling in an antigen-independent context during ex vivo culturing of T cells [25], and provide an explanation for their extended in vivo survival. Conversely, other preclinical studies using anti-PSMA CAR-T cells failed to demonstrate any significant difference between CD28 and 4-1BB endodomains [87]. In clinical trials, the in vivo persistence of anti-CD19 CAR-T cells incorporating CD28 endodomains was limited to approximately 30 days [88,89]. Conversely, the experience with anti-CD19 CAR-T cells encompassing 4-1BB endodomains has shown persistence in the range of months [90,91]. These trials, however, vary significantly with respect to underlying disease, tumor burden, conditioning regimen, viral vector type and cell dose, making a comparative analysis of the results particularly challenging (Table 1).

#### 3.2.3. Improving in vivo long-term persistence

The first requisite to satisfy in order to perform successful ACT is to obtain an adequate number of effectors to infuse using clinical-grade manufacturing protocols. This aim can be achieved with protocols that significantly differ one from the other in terms of activation signals, culturing conditions and vector used for gene transfer. The first expansion protocol for cytotoxic T lymphocytes (CTL) to be used in cancer patients was described by the group of Steve Rosenberg and relied on anti-CD3 mAb (OKT3) activation and culture with feeder cells and high dose IL-2. The resulting population was intentionally biased towards T cells with ready and potent effector functions, which in turn mediated substantial antitumor effects when transferred into patients [12]. In the meantime, studies in the field of ACT for viral diseases proposed to add costimulation with anti-CD28 mAbs in order to circumvent the need of feeder cells during the culturing period [92]. Anti-CD28 mAbs allowed obtaining higher numbers of lymphocytes, while maintaining hypervariable TCR-beta repertoires, possibly because of a stronger signal capable of recruiting T_{EM} in the cell cycle. Subsequently, magnetic beads coupled with anti-CD3 and anti-CD28 mAb (CD3/CD28-beads) were implemented as effective and reproducible tools for expanding patient-derived T cells ex vivo [93] and for enhancing the antitumor effects of donor lymphocyte infusions after allogeneic transplantation [94]. The same protocols have been used to genetically modify T cells while preserving their T_{CM} phenotype and long term in vivo persistence in preclinical models [82].

Another active area of research relates to the cytokine requirements during ex vivo culturing for preserving an early memory-differentiation phenotype. Several studies have shown that, despite being an effective
T-cell growth factor, IL-2 has undesirable effects, including the promotion of terminal differentiation, AICD, and loss of the expression of lymph-node addressins. Moreover, IL-2 has been implicated in the generation and in the functional activity of CD4+ /CD25+ /FoxP3 + natural Tregs [95]. Since in vivo the maintenance of early-differentiated T cells in the absence of antigen depends on the disposal of homeostatic gamma-chain cytokines [96], these interleukins are currently being evaluated for the ex vivo generation of genetically modified T cells with clinically compliant protocols. We have shown that IL-7 and IL-15 are required for the expansion of human alloreactive gene-modified T cells capable of extensive self-renewal and responsible for GVHD [97] and for the graft-versus-leukemia (GVL) effect [83] in xenograft mouse models. The same cytokines are indeed required for the generation of CAR-T cells with an early-differentiation memory phenotype that display optimal antitumor efficacy in xenograft mouse models [98]. Alternative approaches promoting the selective expansion of TSCM are also being evaluated. Manipulation of the Wnt/β-catenin pathway represents one interesting possibility [99], despite the fact that beta-catenin accumulation often leads to a reduced T-cell expansion and to a differentiation arrest [100].

3.3. Role of extrinsic factors

3.3.1. Role of host conditioning

The notion that the lymphopenic state enhances the antitumor efficacy of adoptively transferred T cells has been known for more than 35 years, albeit for a long time the mechanisms were not fully understood. In the early 1980s, it was demonstrated that ACT with tumor-sensitized T cells was effective only if the recipient was previously made lymphopenic by thymectomy and irradiation [101]. In a pivotal clinical trial in metastatic melanoma, immunosuppressive conditioning with fludarabine and cyclophosphamide before the adoptive transfer of TILs resulted in a 50% response rate and robust long-term persistence [12]. Experimental findings have later shed a light on the mechanism by which lymphodepletion enhances the antitumor activity of tumor-specific T lymphocytes. Lymphodepletion indeed acts by eliminating most resident T cells, crucially including Tregs [102], which may compete with the adoptively transferred T cells for homeostatic cytokines [103]. Accordingly, the profound lymphopenic state following T-cell depleted allotransplantation has been shown to make available high amounts of IL-7 and IL-15 [104], which in turn promote the expansion of TCM and TSCM cells [105].

Compelling data exist to support the idea that memory cells depend on IL-7 for survival in the absence of antigen stimulation. Upon TCR engagement, IL-7-Ralpha is down-regulated in effector T cells, but selectively retained in long-lasting memory T cells [106]. In ACT trials in melanoma patients, despite low levels of IL-7RALpha expression on the TIL product before infusion, this was observed to be massively up-regulated in vivo [107], suggesting a causative link between IL-7 signaling and long-term persistence. By analogy, in xenograft mouse models, the self-renewal potential of alloreactive suicide-gene modified T cells was tightly related with IL-7RALpha expression [83]. In most CAR-T cell trials, lymphodepletion is therefore nowadays standardly performed to ensure the functional engraftment of genetically modified T cells, at least in the autologous setting (Table 1).

3.3.2. Role of anti-transgene immune responses

The majority of CARs under clinical investigation are based on scFv derived from mouse mAbs, raising justifying concerns on their potential rejection by the host immune system, either by humoral or cellular responses. Moreover, since CARs are synthetic biology products, the joining regions between the different portions may give rise to newly generated immunogenic peptides. In an early clinical experience, patients with heterogeneous lymphoid malignancies were infused with CAR-T cells expressing highly immunogenic resistance genes necessary for ex vivo selection [108]. The in vivo detection of CAR-T cells was very short (24 h–7 days), and cellular immune responses against the infused product could be measured. A more detailed analysis revealed that these responses were directed against the resistance genes, rather than against the CAR construct. In non-human primate models, however, cellular immune responses against the rodent scFv CAR component were documented [36], suggesting that xenogeneic immune barriers could represent a problem when using non-humanized scFv. Accordingly, in a recent clinical trial, T-cell proliferative responses against autologous anti-CD19 CAR-T cells have been observed in some patients, albeit they appeared not to interfere with anti-leukemic effects [11].

4. Clinical results with anti-CD19 CAR-T cells

The most investigated CAR target to date is CD19, and B-cell malignancies were the first cancers to be treated with CAR-T cells, paving the way for several clinical trials aiming at other targets and disease indications. Early clinical studies with anti-CD19 CAR-T cells have been instrumental to the proof-of-concept of antitumor activity in diseases such as chronic lymphocytic leukemia (CLL) and acute lymphoid leukemia (ALL).

4.1. Antitumor effects

4.1.1. Results in chronic lymphocytic leukemia

In 2011, the group at the University of Pennsylvania (U. Penn) reported preliminary, yet striking clinical results with anti-CD19 CAR-T cells in three patients suffering from heavily pre-treated and advanced CLL, including two complete responses (CR) [86,109]. In these initial patients, anti-CD19 CAR-T cells incorporating a 4-1BB endodomain obtained with LV transduction were showed to undergo dramatic in vivo proliferation, eliminating high tumor burdens and persisting up to three years without loss of functionality. Since then, in the cancer immunotherapy jargon, CAR-T cells are often referred as “serial killers”, as it was calculated that one CAR-T cell can kill as many as 1000 tumor cells, and “mass murderers”, as they can eliminate large tumor masses. Very recently, an update from the same group in 14 patients consolidated these encouraging results, with 4 ongoing CRs and 4 partial responses (PRs), for an overall response rate of 57% [91]. In the meantime, quite similar results were observed with anti-CD19 CAR-T cells incorporating CD28 endodomains obtained with RV transduction at both the Memorial Sloan Kettering Cancer Institute (MSKCC) [110] and at the National Cancer Institute (NCI) [111]. Regardless of the differences between the CAR-T cell products investigated in these three trials, it is worth emphasizing that the antitumor effects correlated with the degree of in vivo persistence of genetically modified T cells in each individual patient, confirming once again the importance of PK issues in determining antitumor efficacy.

4.1.2. Results in acute lymphoid leukemia

The same groups performed larger studies in the ALL indication, with results somewhat even more impressive than that obtained in CLL. In a group of 30 pediatric and adult patients suffering from relapsed/refractory ALL, the group at U. Penn recently reported a CR rate of 90%, with ongoing clinical responses up to 4 years after anti-CD19 CAR-T cell infusion [90]. In 16 ALL adult patients, the group at MSKCC scored a very near 88% CR rate, while in an intent-to-treat analysis in 21 children, the NCI topped a 70% [11]. All three studies included patients with a prior history of allogeneic HSCT, in which CAR-T cells were generated from cells harvested after transplant and added back without causing GVHD. If one wants to draw a comparison with the experience in CLL, it is clear that the kinetics of tumor elimination by CAR-T cells in ALL is much more rapid, possibly reflecting very different tumor burdens and accessibility to infiltration by immune effectors between the two tumor types. Additionally, the considerable number of patients treated so far in the ALL indication, which comprises a rather more homogeneous population compared with CLL, also allows some
interesting observations. Although across the different trials the antitumor effects measured at two months were quite comparable, the contribution of CAR-T cells to the long-term outcome was likely quite different. While in the MSKCC and NCI cohorts most patients entering CR subsequently underwent allogeneic HSCT, in the U. Penn cohort the majority of patients achieving durable remissions (15/19, 78%) did not receive any further treatment [90]. This discrepancy is possibly biased by the fact that U. Penn investigators observed a significantly superior in vivo persistence of CAR-T cells with a 4-1BB endodomain (up to two years) [90] compared with that of CAR-T cells with CD28 endodomains observed by MSKCC/NCI investigators (approximately 30 days) [11,88], which might have biased the decision towards a watchful waiting instead of consolidating the results with allogeneic transplantation.

4.2. Toxicities of CAR-T cells

Before definitively establishing the validity of any therapeutic strategy, a number of regulatory issues need to be carefully addressed, such as identifying unambiguous release criteria defining the minimal effective and the maximal tolerated dose, exploring potential side effects etc. ACT products, however, have peculiarities that prevent the application of standard criteria for regulatory approval. At the current stage of clinical CAR-T cell development, a thorough assessment of all these issues is certainly premature. Nonetheless, it is clear that the therapeutic effects of CAR-T cells are often accompanied with significant toxicities. For example, in an earlier clinical trial using a 3G CAR targeting HER2, a patient died five days after T-cell infusion due to pulmonary toxicity. This severe toxicity was attributed to the on-target, off-tumor recognition of HER-2, which was later found to be expressed at low levels on lung epithelia [112]. Nevertheless, later studies in sarcomas have demonstrated that anti-HER2 CAR-T cells may be relatively safe, especially when using earlier-generation CARs and a more cautious dose-escalation design [74]. Given the relatively advanced stage of development of anti-CD19 CAR-T cells, this review will focus on the description of the toxicities observed in this experience.

4.2.1. B-cell aplasia

Profound and long-lasting B-cell aplasia, and the resulting hypogammaglobulinemia, was the first and most obvious toxicity of anti-CD19 CAR-T cell therapy. Notably, the extent of B-cell depletion and its kinetics is now considered as a highly accurate PD biomarker of CAR-T cell functionality and persistence. Theoretically, persistent B-cell aplasia could result in an increased risk of infection. For this reason, patients receiving anti-CD19 CAR-T cells have been prophylactically infused with intravenous immunoglobulins and did not develop opportunistic infections. Of course, in an ideal setting, it would be necessary to design strategies by which, after attaining the desired anti-tumor effects, anti-CD19 CAR-T cells can be ablated, allowing reconstitution of normal B cells. For example, anti-CD19 CAR-T cells could be removed by means of a co-expressed suicide gene, an approach already investigated in the setting of GVHD after allogeneic HSCT [113].

4.2.2. The cytokine release syndrome

Another common and potentially severe toxicity related to CAR-T cell therapy is the cytokine release syndrome (CRS), a clinical complication characterized by high fevers and hypotension that, if untreated, often leads to end-stage multi-organ insufficiency [86,88,111]. Massive production of pro-inflammatory cytokines (IFN-gamma, IL-6, TNF-alpha) upon on-target recognition by CAR-T cells is the key mechanism of the CRS and associates with pathological signs of systemic macrophage activation resembling hemophagocytic lymphohistiocytosis. The major risk factor for CRS development was identified being a high tumor burden at the time of CAR-T cell infusion, although also patients with minimal tumor loads suffered from this complication. Early attempts at controlling the CRS included anti-TNF-alpha mAbs and corticosteroids administration. The administration of anti-TNFalpha mAbs, aimed at modulating the detrimental effects of excessive activation, rather than eliminating CAR-T cells, has been largely ineffective,
suggesting a redundant role for this cytokine in the CRS. Contrariwise, while clearly effective, corticosteroids also resulted in the premature disappearance of CAR-T cells from the circulation of infused patients. Differently from TNF-alpha blocking strategies, interfering with IL-6-induced pathways by using the anti-IL-6 receptor mAb tocilizumab successfully mitigated the CRS, without preventing the expansion of CAR-T cells and potentially their therapeutic effects. These observations suggest a pathophysiology of the CRS comprising three different steps: i) on-target on-tumor recognition by CAR-T cells, with ensuing IFN-gamma release; ii) activation of monocytes/macrophages, which in turn produce high levels of IL-6 and iii) systemic effects of IL-6 on organs such as the liver, brain and kidneys (Fig. 3). The key role of myeloid cells in the CRS is also suggested by recent data in our lab showing that compared with IL-6 production by monocyte/macrophages, that of CAR-T cells is negligible (Norelli and Bondanza, *unpublished observations*).

4.2.3. Neurotoxicities

Central nervous system (CNS) toxicities are often reported during the course of immune interventions targeted against CD19, i.e. administration of the anti-CD3/CD19 bi-specific mAb blinatumumab. Similarly, acute and reversible neurotoxicities have been frequently observed after CAR-T cell infusion [88,90], both in comitance with the CRS, but also after its resolution. In the initial U. Penn experience, a considerable fraction of all patients infused with anti-CD19 CAR-T cells (13/30, 43%), developed neurological manifestations (confusion, seizures, aphasia, hallucinations, delirium) during the CRS, which were more severe in adults compared with children. Importantly, however, a sizeable fraction (6/30, 20%) experienced delayed-onset neurotoxicities, which were also reported in a later clinical trial [11]. The pathophysiology of these delayed neurotoxicities is at present unknown, but their kinetics clearly suggests mechanisms that are only partially overlapping with those of the CRS. For example CAR-T cells have been found in the CSF of patients, but their presence did not correlate with the severity of neurotoxicities. Anecdotally, the *post-mortem* analysis of some cases revealed diffuse encephalopathy with CAR-T cell infiltration of the brain parenchyma. Some hypothesise a role for IL-6 in the brain, as suggested by the finding of high concentrations of the cytokine in the CSF, which cannot be antagonized by tocilizumab, as this mAb poorly crosses the blood–brain barrier.

5. Conclusions

The results achieved so far with CAR-T cells in indolent, as well as in aggressive hematological tumors resistant/refractory to conventional treatments clearly suggest that this form of ACT has the potential, over the next few years, to completely revolutionize the way cancer is treated. While waiting for more consolidated clinical data, not only in hematological, but hopefully also in solid tumors, it is clear that additional basic research on CAR-T cells is urgently needed, as well as it is needed an entirely new set of concepts on the complex clinical pharmacology of this exciting new form of cancer therapy.

Transparency document

The Transparency document associated with this article can be found, in online version.

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