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FONDAZIONE GHISLIERI

Progetto "Progressi in Biologia e Medicina"

20° Corso di formazione avanzata

CAR-T cells

4 - 5 - 6 maggio 2022

A cura di CarloAlberto Redi

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Prefazione

Ad oggi, due delle strategie più utilizzate per la cura del cancro sono l'immunoterapia e l'ingegnerizzazione genetica di linfociti T e di cellule Natural Killer, meglio conosciute con il nome di CAR-T e CAR-NK terapie (Chimeric Antigen Receptor T cell therapies o Natural Killer cell therapies).

Il Collegio Ghislieri quest'anno dedica un intero corso a quella che certamente si configura come una delle più potenti innovazioni tecnologiche nella lotta contro i tumori e si rivolge non solo agli addetti ai lavori ma anche a chi entra, per la prima volta, in questo campo.

L'opportunità di complessare variamente ed in diverso grado le proteine chimeriche permette di attrezzare diversi tipi cellulari per trattare efficacemente diverse patologie del sangue (CAR-T cells sono presenti stabilmente anche dopo 10 anni dalla remissione della patologia) e nel prossimo futuro per trattare patologie cardiache e tumori cerebrali. Il concetto biologico di fondo, che i partecipanti al corso faranno proprio per sviluppare con originalità future applicazioni, è che le proteine CAR sono delle guide molecolari (poi complessate con il tipo cellulare di interesse) per raffinare e colpire le cellule bersaglio. La fantasia dei biotecnologi (siano essi biologi, medici o altre figure) è il limite per la produzione delle mitiche chimere, ciascuno di loro saprà trovare il leone, la capra ed il serpente più adatto.

Docenti a livello internazionale presenteranno le attuali e le prossime applicazioni oltre agli aspetti teorici rilevanti a definire le condizioni al contorno dell'immunoterapia CAR.

CarloAlberto Redi

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Il mondo delle molecole e cellule chimeriche

CarloAlberto Redi

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È obbligo dell'organizzatore del corso far sì che l'eterogenea composizione dei partecipanti possa proficuamente godere delle specialistiche presentazioni degli esperti chiamati ad illustrare il campo di studio prescelto: quest'anno il mondo delle molecole e cellule chimeriche quali utili farmaci nelle terapie tumorali innovative.

E dunque per definire i contorni concettuali del nostro corso è bene precisare l'epistemologia genetica sottesa al mondo delle cellule CAR. L'acronimo esplicita "Chimeric Antigen Receptor" per indicare la creatura della mitologia greca creata dalla fusione di diverse parti di un leone, una capra ed un serpente. In completa metafora per noi oggi CAR è un complesso proteico ingegnerizzato utile per corredare la superfice di membrana di diversi tipi di cellule del sistema immunitario. Il più delle volte queste cellule sono linfociti T per cui queste cellule sono chiamate CAR-T e queste sono le più conosciute ma ve ne sono molte altre di cellule modificate con proteine CAR: ad esempio le Natural killer, NK, che divengono CAR-NK.

Le proteine CAR funzionano da guida molecolare per attaccare in modo selettivo particolari e specifiche entità biologiche: in tal modo la cellula del sistema immunitario chimerizzata (la cellula CAR) viene a diretto contatto con l'entità bersaglio che sarà distrutta immunologicamente (Ciceri e Arosio, 2021). Nei recenti anni passati, nel corso dei quali sono state sviluppate le tecnologie CAR, i bersagli elettivi delle terapie cellulari CAR sono stati per la gran parte diversi tipi di cellule leucemiche e quelle dei linfomi. Aspetti questi che i docenti del corso illustreranno ampiamente chiarendo che nell'approntare le strategie cliniche più efficaci sino ad oggi si sono privilegiate le terapie basate su cellule CAR autologhe; in altre parole privilegiando il concetto che le cellule CAR sono veri e propri farmaci viventi preparati su misura dalle cellule del sistema immunitario prelevate dal singolo paziente, ingegnerizzate in laboratorio e poi ritornate al paziente sotto forma di infusione di cellule CAR. Purtroppo spesso si manifestano effetti collaterali e non è infrequente il caso in cui il trattamento scatena una tempesta di citochine con conseguenze difficili da controllare clinicamente e che a volte possono essere fatali. Il presentarsi di effetti collaterali gravi da ragione del fatto che a fronte della grande potenzialità della tecnologia CAR gli impieghi siano approvati solo per un ristretto numero di pazienti, in genere pazienti che presentano specifici tipi di tumori del sangue (alcuni tipi di leucemie e linfomi) che non hanno risposto efficacemente ai

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trattamenti convenzionali. Come risulta intuitivo, solo pochi centri ospedalieri sono in grado di offrire simili terapie stante il complesso tecnologico e di competenze professionali necessario al loro sviluppo. Risulta infatti immediatamente chiaro che simili procedure sono estremamente laboriose, costose e richiedono tempi a volte troppo lunghi durante i quali le condizioni di salute del paziente possono peggiorare o addirittura il paziente può morire. Queste considerazioni hanno spinto la ricerca verso quello che è definito lo sviluppo di nuove tipologie di cellule CAR, le principali delle quali sono tre tipologie conosciute come:

– cellule CAR 2.0-CAR-T,
– cellule CAR-NK,
– cellule CAR allogeniche.

A meglio definire le condizioni al contorno dell'ottimismo che segna il mondo della biologia delle cellule CAR vi è il recentissimo dato scientifico che indica la persistenza e la vitalità terapeutica delle cellule CAR-T anche dopo ben 10 anni dalla loro infusione e dalla remissione della malattia dei pazienti (Melenhorst et al., 2022). Di grande interesse questo dato poiché rivela anche alcune delle dinamiche trasformative *in vivo* delle cellule chimeriche: infuse dieci anni orsono in due pazienti affetti da leucemia linfatica cronica come cellule CD8+ sono oggi presenti e vitali come un clone CD4+ originatosi nelle fasi iniziali della risposta al trattamento ed alla remissione completa dalla malattia.

Tutte le cellule di queste tre nuove tipologie sono cellule che condividono la importante caratteristica di poter essere disponibili immediatamente alla richiesta di intervento e dunque potenzialmente infuse nel momento del bisogno terapeutico. Oltre al vantaggio terapeutico vi è sotteso l'altro grande vantaggio di essere prodotti della gestione di banche del sangue e dunque sono in grado di essere assai meno costose di cellule prodotte su specifica domanda; di essere prodotte in quantità di scala (ed anche, soprattutto, grazie a donatori di sangue); e di avere il grande pregio tecnologico di permettere di utilizzare tutto il tempo necessario alla loro ingegnerizzazione (senza limiti temporali o costrizioni temporali di natura terapeutica) anche con le tecniche di editing genomico più efficaci e recenti, in particolare con la tecnologia CRISPR. Grazie a tutti questi avanzamenti del sapere delle scienze della vita molti oncologi sono convinti che entro breve tempo le cellule CAR potranno essere utilizzate su uno spettro ben più ampio di forme tumorali grazie al fatto che è possibile attrezzare le cellule T con interruttori cellulari (*safety switches*) capaci di spegnere le cellule a comando, se necessario così da ridurre i danni collaterali del trattamento per l'azione citotossica fuori bersaglio. Gli avanzamenti scientifici non si fermano agli aspetti, seppur di rilievo, sulla sicurezza dei trattamenti ma riguardano l'opportunità di impiegare congiuntamente più tecnologie (come nel caso sopracitato dell'editing con CRISPR) per poter trattare patologie in organi solidi. È del 7 gennaio 2022 la pubblicazione su *Science* di un articolo (Rurik et al., 2022) ove si dimostra che la generazione CAR 3.0 può trattare con successo, su un modello murino, patologie cardiache. Di rilievo che il successo del trattamento è essenzialmente dovuto al fatto che le cellule T sono indotte a trasformarsi in cellule CAR *in vivo* grazie alla stessa tecnologia a mRNA impiegata per la produzione dei vaccini a mRNA utili alla prevenzione

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dell'infezione COVID: un bell'esempio di fusione delle tecnologie mRNA con la terapia immunologica.

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Manipolazione cellulare nell'ambito della nuova frontiera del trapianto aploidentico e della terapia con CAR -T cell

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Il trapianto aploidentico, una nuova frontiera

Il trapianto di cellule staminali emopoietiche da donatori HLA compatibili è l'unica terapia curativa nei pazienti affetti da malattie onco-ematologiche o ereditarie ad esempio le talassemie e le anemie falciformi.

La possibilità di identificare un donatore HLA identico familiare non supera il 30% dei pazienti candidati al trapianto. Per quei pazienti per cui non è disponibile la possibilità di identificare un donatore volontario HLA compatibile tipizzato per i loci HLA-A, -B, -C e DRB1 èfortemente legato all'etnia: infatti riguarda circa il 75% dei pazienti di etnia caucasica e circa il 20-50% per le rimanenti etnie. Nel caso in cui un donatore volontario non familiare (MUD) venga identificato, la possibilità di eseguire il trapianto non va oltre il 50%. La progressione di malattia durante l'indaginoso processo di ricerca del donatore è una delle cause principali. Da sottolineare poi che i processi migratori degli ultimi decenni hanno inevitabilmente aumentato lo scacchiere etniconei paesi occidentali, per cui è fondamentale identificare strategie nuove che permettano di identificare fonti alternative di cellule staminali tutte le volte che un donatore MUD non sia prontamente disponibile.

La possibilità di eseguire il trapianto per questo nutrito gruppo di pazienti è legato a tre fonti alternative: donatori familiari aploidentici, (mismatch completo di 3/6 o 4/8 antigeni HLA), sangue placentare da banca, attualmente quasi azzerata come fonte, oppure ai donatori volontari HLA mismatch per almeno un antigene o allele.

L'impiego di tecniche di tipizzazione HLA ad alta risoluzione ha consentito di evidenziare che non tutti i mismatch HLA sono uguali, poichè esistono mismatch allelici "permissivi" o "non permissivi" che, influenzano fortemente la capacità dei linfociti T di riconoscere le differenze nelle sequenze HLA.Tuttoi questo spiega i differenti andamenti del trapianto in presenza di graft apparentemente simili.

Di contro i donatori familiari aploidentici sono ovviamente facilmente contattabili, consentendi quindi di eseguire velocemente il trapianto con la possibilità di richiamare il donatore nel caso fosse necessaria una terapia cellular aggiuntiva post trapianto. Il maggior svantaggio nella scelta del donatore aploidentico è ovviamente la disparità HLA.

I vantaggi del sangue placentare sono rappresentati da: pronta disponibilità, relativo basso rischio di GvHD legato al basso numero di linfociti T contenuti nel graft, Gli svantaggi sono legati al basso contenuto in cellule staminali con un rischio più elevato di mancato attecchimento rispetto alle altre fonti e il lento recupero immunologico post trapianto conaumento importante del rischio infettivo ed una più elevata mortalità. Per tali motivi negli ultimi anni l'impiego del sangue placentare per le procedure trapiantologiche è fortissimamente diminuto.

Le cellule staminali emopoietiche da sangue periferico (PBSC) raccolte mediante leucaferesi dopostimolazione del donatore con fattore di crescita granulocitario rappresentano attualmente la fonte principe per il trapianto sia per la facilità di raccolta (procedura leucaferetica vs espianto di midollo osseo), che per il più elevato numero di cellule staminali CD34+ recuperabili ed il più veloce attecchimento Tuttavia, l'incidenza di graft versus host disease (GvHD) acuta e cronica post trapianto è maggiore rispetto ai pazienti ifusi con midollo osseo a causa dell'alto contenuto (5-10 volte superiore) di linfociti T CD3+. Non solo, il maggior contenuto di linfociti B CD19+ aumenta il rischio di patologia linfoproliferativa associata al virus di Epstein Barr. Fatte queste premesse biologiche nel caso di trapianto aploidentico, l'elevata quantità di linfociti presenti in un graft compatibile solo a metà con il ricevente, impone la manipolazione delle cellule per permetterne l'attecchimento e soprattutto contenere l'insorgenza di GvHD. Già a partire dagli anni ottanta si sono sviluppati programmi di trapianto aploidentico con manipolazione *ex vivo* (selezione immunomagnetica) o *in vivo* (trapianti T repleti mediante somministrazione di anticorpi monoclonali e immunosoppressori) del graft proprio con l'intento di ridurre drasticamente il numero di linfociti T infusi.

Manipolazione della raccolta

Immunoselezione positiva

Storicamente i primi tentativi di trapianto aploidentico non manipolato risalgono agli anni '80 e furono gravati da un'elevata incidenza di GvHD, malattia del trapianto contro l'ospite, di grado severo. Successivamente il regime di condizionamento venne modificato con l'impiego di nuovi farmaci mieloablativi ed anticorpi monoclonali anti linfociti T (ATG) per limitare la risposta immunitaria residua dell'ospite. I trapianti estensivamente T depleti furono però gravati da un'elevata incidenza di rigetto. Per limitare il rigetto, superando la barriera HLA ed eludendo la residua attività dei linfociti T citotossici del ricevente contro il donatore, all'inizio degli anni 90' il gruppo di Perugia adottò la tecnica di infondere dosi molto grandi di cellule staminali CD34+ (*stem cell megadose*) ottenute dopo una immunoselezione positiva di cellule staminali emopoietiche e una estensiva T deplezione nel tentativo di ridurre il rischio di mancato attecchimento/rigetto. La media di cellule infuse era di 13,8x10*6/kg.

L'immunoselezione positiva si basa sull'impiego di anticorpi anti-CD34 legati a biglie di ferrodestrano che, si legano alle cellule esprimenti l'antigene CD34, vengono bloccati da un magnete e infine deviati in una sacca apposita. In tal modo si ottiene un prodotto estremamente puro (>95%) in cellule staminali e si depletano

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estensivamente i linfociti T (mediamente 3-4 log) e più moderatamente i linfociti B (mediamente 2-3 log). Le PBSC (Peripheral Blood Stem Cells) sono ritenute la miglior fonte di cellule staminali per l'immunoselezione poichè, considerando una perdita media prevista dalla manipolazione pari a circa il 50% si può programmare adeguatamente la procedura di raccolta aferetica assicurando un elevato carico di celluleda manipolare. Questo approccio di manipolazione evidenzia come l'attecchimento fosse promosso dall'elevato numero di cellule staminali CD34+, in grado di sopprimere i precursori dei linfociti T citotossici diretti contro i propri antigeni (effetto veto). Inoltre, un importante effetto anti-leucemia (graft versus leukemia-GvL) in assenza di GvHD veniva indotto dalla generazione di cellule Natural Killer (NK) alloreattive del donatore verso il ricevente. Le cellule NK rappresentano circa il 10% dei linfociti circolanti e sono regolate dai recettori inibitori chiamati "killer cells immunoglobulin like receptors" (KIRs) i quali riconoscono i gruppi allelici HLA di classe I (KIR ligands). Solo le cellule NK che esprimono i KIRs inibitori per i ligandi HLA propri diventano funzionali. Quando le cellule NK del donatore sono presenti nel contesto di un trapianto aploidentico, "avvertono" la mancata espressione del ligando KIR e mediano l'alloreattività (mancanza del riconoscimento del self). La scelta del donatore NK alloreattivo (potenzialmente disponibile per circa il 50% dei pazienti) ha dimostrato di ridurre significativamente il rischio di recidiva (3% vs 47%) in pazienti affetti da leucemia acuta mieloide (LAM) adulti e pediatrici.

Immunoselezione negativa TCR αβ/CD19

Numerosi studi hanno dimostrato la grande importanza delle cellule accessorie contenute nel graft nel mediare l'insorgenza della GvHD, aumentare l'effetto GvL e diminuire il rischio infettivo.

Il gruppo di Tuebingen ha messo a punto un protocollo di deplezione dei linfociti esprimenti il T cell receptor (TCR) α/β e l'antigene CD19 mediante selezione immunomagnetica ex-vivo (Clinimacs Miltenyi). Questo approccio è volto a mantenere le cellule effettrici NK e i linfociti T TCRγ/δ$^+$ oltre naturalmente alle cellule staminali CD34+.

Le cellule Tγ/δ$^+$ rappresentano un subset di linfociti T che esprimono una variante del TCR composta da catene gamma e delta. Il γ/δ TCR è presente in circa il 2-10% delle cellule T circolanti. Al contrario, la maggioranza dei linfociti T esprime il TCRα/β, risiede nei linfonodi e nel timo ed è deputata al riconoscimento degli antigeni presentati dal sistema maggiore di istocompatibilità (MHC). I linfociti Tα/β$^+$ sono quindi direttamente coinvolti nello sviluppo della GvHD.

I linfociti γ/δ$^+$ chiamati anche "innate like T cells", appartengono alla parte adattativa del sistema immune e svolgono molteplici funzioni effettrici compresa la rapida secrezione di chemochine e citochine in risposta ad uno stimolo e sono in grado di riconoscere l'antigene indipendentemente dal sistema MHC; questo fa sì che i linfociti γ/δ$^+$ non siano coinvolti nello sviluppo della GvHD. I linfociti Tγ/δ$^+$ svolgono un ruolo importante anche nella protezione contro I patogeni intra ed extra cellulari, nello sviluppo di tumori (in particolare quelli oncoematologici), modulano la risposta immune e mantengono l'omeostasi tissutale.

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Nel contesto del trapianto allogenico partecipano inoltre alla risposta anti-CMV, che è molto importante nelle primissime fasi post trapianto, quando la risposta immune è assente o gravemente ridotta. Infine, dai numerosi studi condotti finora, emerge che i linfociti Tγ/δ$^+$ nel contesto del trapianto allogenico, sarebbero in grado di reclutare i linfociti Tα/β nei siti di invasione degli organismi patogeni, mediante la secrezione di citochine specifiche. Queste proprità indurrebbero una ulteriore amplificazione dei linfociti Tα/β esprimenti i recettori specifici per i patogeni presenti. I dati disponibili circa la cinetica delle cellule NK mostrano come occorrano molte settimane dopo il trapianto perchè dalle cellule staminali CD34+ si generino cellule NK pienamente funzionanti in grado di esercitare una durevole risposta immunitaria anche di tipo antivirale, oltre a contribuire alla prevenzione della GvHD attraverso l'eliminazione delle cellule dendritiche del ricevente. Nell'ambito di un trapianto TCRγ/δ$^+$ /CD19+ la possibilità di infondere un elevato numero di cellule NK alloreattive mature del donatore eserciterebbe un effetto GvL nelle fasi immediatamente post trapianto e contribuirebbe a cooperare nella protezione contro le infezioni. Inoltre, la deplezione dei linfociti B CD19+ è volta a prevenire lo sviluppo di patologie linfoproliferative EBV correlate.

Analogamente, la deplezione B avviene attraverso il legame con un anticorpo anti-CD19 legato ad una microbiglia il tutto in un sistema sterile completamente chiuso. ll recupero medio delle cellule CD34+ è attualmente molto soddisfanete e si aggira intorno al 75/80%. Mentre la deplezione dei linfociti αβ, CD19 è pressochè totale.

I risultati clinici ad oggi disponibili sono estremamente incoraggianti per il rapido attecchimento, la bassissima incidenza di GvHD acuta di grado I e II, e l'assenza di GvHD con una rapida ricostituzione immunologica.

Il trapianto aploidentico rappresenta ormai una reale alternativa per i pazienti che non dispongono di un donatore familiare HLA identico e l'immunoselezione negativa TCRαβ/CD19 rappresenta la più avanzata tecnica di manipolazione *ex vivo* del graft.

Raccolta di linfociti per la terapia con CAR-T cell

La Chimeric antigen receptor (CAR) T cell therapy ha di fatto cambiato il panorama di cura per i pazienti con malattia refrattaria o ricaduti da linfoma B e quindi ritunuti incurabili. Non solo questo approccio terapeutico si sta allargando anche ad altre patologie oncoematologiche (leucemie pediatriche e tumori solidi dell'adulto e del pediatrico).

Le cellule CAR-T sono generate grazie all'uso di vettori virali, di solito lentivirus o retrovirus, con la transduzione delle cellule T autologhe del paziente stesso in modo tale che esprimano un recettore antigenico specifico diretto contro un antigene cellulare di superfice della neoplasia ematologica o solida da eradicare.

Una volta completata la fase di "montaggio" del recettore specifico inizia la fase di espansione cellulare per ottenere la dose cellulare desiderata e ritunuta efficace per eradicare la malattia e da ultimo la reinfusione al paziente delle cellule T espanse.

Il presupposto fondamentale affinchè tutti questi delicati passaggi di manipolazione ed espansione avvengano senza problemi è insito nella qualità del prodotto raccolto pre manipolazione.

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L'impiego di separatori cellulari di ultima generazione, dotati di programmi di gestione dedicati, permette di raccogliere in sicurezza, in tempi brevi (circa 2 ore) e con una elevatissima efficienza (> del 65/70%) cellule T autologhe da inviare al laboratorio di manipolazione cellulare che provvederà alla trasduzione e alla amplificazione delle stesse.

La contaminazione del prodotto con globuli rossi e soprattutto con piastrine complica le successive fasi manipolative riducendo l'efficienza di trasduzione e l'amplificazione delle cellule T raccolte.

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3

Approaches to increase the therapeutic index of CAR-T cells in solid tumors

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T cell-based therapies for the treatment of cancer: promises and challenges

Cancer immunotherapies are currently one of the most promising areas of cancer research, thanks to the remarkable clinical results of immune checkpoint inhibitors (ICI) (1) and CAR-T cells. These therapies rely on the ability of T cells to eliminate tumours. The mechanism of action of ICI is to reinvigorate endogenous tumour-specific T cells by blocking the interaction between inhibitory molecules on T cells (including PD-1 and CTLA-4) and their ligands in tumour cells (PD-L1). Therapy with ICI can mediate long-term responses, especially in a subset of tumours with numerous genetic mutations that are infiltrated with neoantigen-specific T cells. ICI therapy has revolutionized cancer treatment, highlighting the power of T cells in controlling solid tumours. However, a majority of patients fail to respond to ICI therapy (2, 3), or relapse after a certain period of time (4, 5). Mechanisms of resistance include lack of appropriate neoantigens and/or defects on the process or presentation of tumour antigens by MHC-I molecules (6). An alternative approach, that can address some of these challenges, is to genetically modify T-cells, obtained from patient's blood, to express CARs (7).

CAR-T cells for the treatment of B-cell malignancies

CAR-T cells have produced unprecedented results in the clinic, including durable and complete responses in patients with certain types of hematologic malignancies (8-13). The year 2017 resulted in a historical milestone when tisagenlecleucel, the CAR against CD19 developed at the University of Pennsylvania and Novartis became the first gene therapy to be approved by The Food and Drug Administration (FDA), closely followed by the approval of Kite Pharma's axicabtagene ciloleucel. There are now five CAR-T cell therapies approved for the treatment of B cell malignancies targeting CD19 and BCMA. CAR-T cells for B-cell malignancies represent a true bench-to-bedside clinical translation. The question now is: Can we make CAR-T cells work in the context in solid tumours?

The solid tumour challenge

The success shown by CAR-T cells in hematologic cancers supports the translation of this technology to the more challenging setting of solid tumours. This

type of tumours contributes to more than three quarters of cancer-related deaths in humans, and therefore represents the largest medical challenge. Compared to hematologic malignancies, solid tumours pose extra barriers of complexity that could limit the benefits of engineered T cell therapies (14). First, T cells must traffic and infiltrate into the solid mass. On arrival at the tumour, T cells encounter an immunosuppressive environment that, together with chronic antigen exposure, can lead to T-cell hypofunction (15, 16). Finally, intra-tumoral heterogeneity and tumour cell plasticity can contribute to primary or acquired therapy resistance, including, but not limited to, loss or downregulation of the tumour antigen.

More than one hundred clinical trials testing CAR-T cells for the treatment of solid tumours are ongoing worldwide. However, broader clinical application of CAR-T cells to solid tumours is still in its infancy. In most trials, CAR-T cell efficacy has been disappointing, although recent reports in patients with difficult to treat tumours have provided evidence for feasibility and for transient activity (including complete responses) in the absence of serious adverse events (17-20). Some of the lessons learned by initial clinical trials that will drive the design of future CAR-T cell therapies include:

1) Despite CAR-T cells trafficking to the tumour, initially proliferating in situ and exerting some direct anti-target activity, substantial anti-tumour responses are rarely observed (21-24);
2) significant decrease or loss of antigen-expression has been observed following CAR-T cell administration, suggesting some level of transient CAR-T cell activity and pointing at antigen heterogeneity and antigen loss as major barriers to overcome (21, 22);
3) CAR-T cells have demonstrated significant toxicities (25, 26), so great caution should be taken when implementing novel CAR-T cell therapies to mitigate toxicities.

Overcoming tumour escape due to heterogeneity of antigen expression: Armoured CAR-T cells

One of the major obstacles of CAR-T cells in solid tumors is the heterogeneity of antigen expression, which makes it challenging to identify a universal target expressed throughout the whole tumor. Moreover, immune pressure by CAR-T cells can drive cancers to escape due to downregulation or loss of the target antigen (21, 22, 27). Simultaneous targeting of different tumour antigens could be a promising solution to address tumour escape (28, 29); however, it may significantly increase the chances of toxicity.

One possibility to address tumour heterogeneity and to avoid tumour escape is to find strategies to promote an endogenous T cell response against other tumour-associated antigens (14). Studies suggest that, during CAR-T cell killing, tumour neoantigens are released and cross-presented to endogenous T cells, in a process known as epitope spreading (24, 30, 31). Epitope spreading would ensure a polyclonal, polyfunctional immune response that could lead to the elimination of CAR-targeted antigen negative tumour cells. Whether CAR-T cells can induce this

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kind of response in patients is not well understood. While epitope spreading may not be a relevant phenomenon in the context of B-cell malignancies (due to the low mutational burden and fewer potential neoantigens compared to solid tumours), an effective CAR-T cell treatment in the context of solid tumour may require the activation of an endogenous immune anti-tumour response.

One potential strategy to promote an endogenous T cell response against the tumour would be to further modify CAR-T cells to release (a) cytokines with the potential to promote epitope spreading, such as IL-12 or IL-18 (32, 33) (b) blocking antibodies that inhibit mechanisms of resistance of tumour cells to T cell killing. IL-12 expression by tumor-specific T cells has been shown to recruit and activate an innate immune cell response towards cancer cells. However, a first clinical trial infusing tumor specific T cells modified to express IL-12 was promptly terminated due to unexpected toxicities, likely attributable to secreted IL-12 (34). Alternatively, IL-18 has been proposed as a safer strategy to armor T cells due to its capacity to enhance T cell responses. Both IL-12 and IL-18 have been implicated in promoting epitope spreading in cancer and auto-immune diseases, and therefore could be ideal candidates to be expressed from CAR-T cells. However, it may be important to limit their expression at the tumor site. Developing system to regulate transgene expression selectively in the tumor site would be a vertical advance in the field.

T-cell dysfunction in the tumor microenvironment

T cell dysfunction has been extensively studied in virus- and tumor-specific T cells in chronic infections and cancer, respectively, but little is known about the behavior of engineered T cells after the introduction of synthetically derived receptors (35, 36). When antigen persists, CD8+ T cells progressively lose their effector functions, entering in a dysfunctional state. This state can be aggravated by the lack of costimulation during antigen encounter, suboptimal CD4+ help, or the presence of an inhibitory tumor microenvironment. Various states of T-cell dysfunction have been described as a consequence of altered activation and differentiation processes, and, depending on the experimental or clinical settings and phenotypic and functional features of the T cells, terms such as exhaustion, anergy and senescence have been used to describe this dysfunctional state. The detailed molecular signals that drive T-cell exhaustion remain undefined, but it has been proposed that both extrinsic negative regulatory pathways found within established tumors (such as immunoregulatory cytokines) and T cell-intrinsic negative regulatory pathways (such as PD-1, LAG-3, TIM-3 and CTLA-4) have key roles in exhaustion.

One possible strategy to revert T-cell exhaustion in the tumor is immune checkpoint blockade (14). CAR-T cells undergo activation-induced upregulation of coinhibitory receptors, including PD-1, TIM-3 and LAG-3, although the role of these markers is not fully understood yet. On the other side, tumor cells can augment the expression of coinhibitory ligands such as PD-1 ligand (PD-L1), upon T cell activation-mediated release of Th-1 cytokines. Strategies that combine CAR-T cells with checkpoint blockade, most of them focusing on the PD-1/PD-L1 interaction, are being investigated. PD-1/PD-L1 pathway interference through antibody check-

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point blockade, shRNA blockade or PD-1 dominant negative receptor or chimeric switch-receptors have been shown to augment the efficacy of CAR-T cells in both solid tumors and hematologic malignancies (37-40). However, the results from different groups also highlighted the ambiguous role of PD-1 in defining efficient or ineffective immune T cell responses (41). A better understanding on how these inhibitory receptors affect CAR-T cell dysfunction together with the identification of novel pathways implicated in T cell dysfunction, will be required to further enhance the effector functions and persistence of CAR-T cell in the tumor microenvironment.

Avoiding CAR-T cell toxicity

CAR-T cells have demonstrated significant and unique toxicities. In the context of solid tumors, main adverse events have been "on-target off-tumor" toxicities that occurs when healthy tissues expressing low levels of the target antigen are damaged. The most obvious strategy to prevent toxicities is to target antigens that are expressed in tumor cells but not in normal tissue. However, finding suitable tumor antigens to target solid tumors has proven challenging. One alternative to target shared antigens is to tune the affinity of the scFv so that it can differentiate between tumors and normal tissues based on different levels of antigen expression (42).

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4

Fuga dall'immuno-sorveglianza e dall'immunoterapia delle cellule di Leucemia Mieloide Acuta e della nicchia ematopoietica

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L'idea che le cellule immuno-competenti di un donatore di cellule staminali ematopoietiche (CSE) possano eradicare le cellule leucemiche di un paziente con leucemia mieloide acuta (LAM) sottoposto a trapianto allogenico venne per la prima volta ipotizzata negli anni '60 gettando le basi dell'immunoterapia adottiva. Da allora l'efficacia terapeutica di questa reazione immunologica nota come "Graft versus Leukemia" (GVL) e responsabile della potenzialità curativa del trapianto allogenico è stata più e più volte confermata e proprio per questo motivo la GVL è stata il fondamento di tutti quei protocolli di immunoterapia adottiva oggigiorno impiegati per colpire bersagli immunologici espressi sulla superficie o all'interno delle cellule neoplastiche. Tuttavia, nonostante quest'enorme potenzialità curativa l'efficacia terapeutica dell'immunoterapia adottiva può essere sopraffatta dal cattivo funzionamento dei meccanismi di immuno-sorveglianza che in tal modo permettono lo sviluppo e la progressione della malattia o dalla capacità della cellula leucemica di manipolare il proprio microambiente rendendolo più adatto alla propria sopravvivenza.

Meccanismi di evasione immunologica attivati dai blasti leucemici

Le cellule leucemiche possono eludere la sorveglianza immunologica riducendo l'espressione di particolari antigeni, esprimendo in modo aberrante ligandi di "checkpoint" immunologici, alterando la formazione di sinapsi immunologiche con cellule T, secernendo molecole solubili ad azione inibitoria nei confronti delle risposte immuni di tipo T, eludendo il proprio riconoscimento da parte delle cellule "natural killer" (NK), facendo aumentare il numero di cellule mieloidi soppressorie ed il numero di macrofagi tumore-associati.

Ridotta espressione di particolari antigeni

Il successo del trapianto allogenico dipende dalla capacità delle cellule T e delle cellule NK di riconoscere ed eliminare i blasti leucemici, ma questi ultimi possono eludere questo meccanismo d'immuno-sorveglianza perdendo dopo trapianto da donatore aplo-identico l'espressione di molecole HLA o attivando dopo vari tipi di

trapianto allogenico meccanismi epigenetici che consentono di ridurre l'espressione di molecole HLA di classe II.

Espressione aberrante di ligandi di checkpoint immunologici (CI)

I CI sono molecole regolatorie espresse dalle cellule T che hanno lo scopo di creare uno stato di tolleranza verso il "self" e di evitare quindi reazioni auto-immuni. Pertanto, per sfuggire questo meccanismo d'immuno-sorveglianza i blasti leucemici esprimono i ligandi per i CI. Tra questi ultimi il meglio studiato è il ligando 1 (L-1) del recettore "Programmed-cell-death" (PD-1) che nelle LAM induce l'esaurimento delle cellule T ed un'espansione delle cellule T regolatorie (Treg) con un blocco della funzione effettrice delle cellule T CD8 positive. Un altro ligando espresso dai blasti leucemici è la galectina 9 specifico per un altro IC, specifico per il recettore "T-cell Immunoglobulin and mucin domain 3" (TIM3) espresso dai linfociti T effettori e dalle NK. Questo legame promuove il "self-renewal" attraverso la via di segnale della β-catenina e del NFkB, riduce il rilascio di citokine pro-infiammatorie e ha un ruolo fondamentale nel mantenimento delle "leukemic stem cells" (LSC). Nei modelli murini e nei pazienti alti livelli di cellule T TIM3 e PD1 positive si associano a prognosi sfavorevole. Altri recettori inibitori degli IC con ben definito ruolo prognostico nelle LAM sono il "T-cell immunoglobulin and ITIM domain" (TIGIT) che lega i ligandi DNAM-1, CD155 e CD47, il "Cytotoxic T-lymphocyte associated protein 4" (CTLA4) ed il "Lymphocyte activating-3" (LAG-3). Un'elevata espressione di ligandi di TIGIT o di CTLA4, o di LAG3 sui blasti leucemici si accompagna a prognosi sfavorevole.

Alterazione della sinapsi immune T

I soggetti affetti da LAM presentano valori di cellule T nel sangue periferico superiori a quelli dei soggetti sani, ma questi linfociti non sono capaci di formare sinapsi immunologiche per un'anomala "signature" di attivazione al profilo di espressione genica. Questo risultato era in accordo con osservazioni precedenti che avevano indicato un fenotipo citotossico effettore, l'espressione di marcatori di attivazione ed una capacità citotossica alterata per i linfociti dei pazienti con LAM.

Secrezione di fattori solubili ad azione inibitoria nei confronti della cellula T

Nelle LAM le cellule Treg sono aumentate ed il loro valore si correla con una prognosi sfavorevole. Inoltre, la dimostrazione che la nicchia ematopoietica contiene diverse sottopopolazioni cellulari a diversa potenzialità immunosoppressiva è sostenuta dal fatto che le cellule Treg del midollo osseo hanno una capacità immunosoppressiva superiore a quella delle cellule Treg da sangue periferico. Questa maggiore attività immuno-soppressoria delle cellule Treg contenute nella nicchia dipende dal fatto che i blasti leucemici secernono fattori che inibiscono la risposta immune come l'interleuchina 10, il "tansforming growth factor beta" (TGFβ), e l'indolamina 2,3-diossigenasi 1 (IDO1) che inducono la polarizzazione dei T linfociti verso un fenotipo Treg con progressione della LAM. L'IDO1 in particolar modo altera il metabolismo del triptofano e con accumulo di suoi metaboliti tossici come la l-kinurenina che induce l'apoptosi delle cellule T. Inoltre la riduzione della

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concentrazione di citokine pro-infiammatorie come l'interleukina 15 e l'interferone γ altera ulteriormente la funzione delle cellule T effettrici. Altri fattori che hanno un'azione immunomodulante sul microambiente della cellula leucemica sono l'arginasi II che polarizza i monociti verso un fenotipo M2, la sovra-regolazione della ossido nitrico sintetasi (iNOS) e la capacità dei blasti leucemici di orientare il metabolismo del glucosio e degli acidi grassi fornito dagli adipociti verso la produzione di acetil-coenzima A necessario alla produzione di ATP attraverso il ciclo di Krebs e la fosforilazione ossidativa (OXPHOS).

Mancato riconoscimento da parte delle cellule NK

Può avvenire per un meccanismo epigenetico che consiste nel silenziamento di geni codificanti per i recettori di attivazione della cellula NK. Inoltre, l'attività citotossica delle cellule NK può essere alterata anche attraverso altri meccanismi rappresentati dalla capacità dei blasti leucemici di secernere la forma solubile del ligando del recettore attivatorio NKG2D (sNKG2DL), di esprimere alti livelli di CD12 e CD155 con riduzione dei livelli di espressione del loro recettore DNAM-1 espresso dalle NK, d'indurre recettori di co-inibizione come TIGIT nelle cellule NK con ridotto rilascio di γ-IFN.

Aumento delle cellule mieloidi soppressorie e di macrofagi tumore-associati

Avviene perché i blasti leucemici possono rilasciar vescicole extracellulari che contengono l'oncoproteina MUC1 che determina a sua volta la sovra-espressione di c-myc nelle vescicole extracellulari attraverso il microRNA miR34a con proliferazione delle cellule mieloidi soppressorie.

Meccanismi di evasione immunologica attivati dalla nicchia ematopoietica

La sorveglianza immunologica può essere evasa anche dal microambiente tumorale attraverso un aumento dell'ipossia, dell'infiammazione ed una riprogrammazione metabolica.

Capacità immuno-soppressoria delle cellule stromali mesenchimali (CSM)

Le CSM possiedono la capacità di attivare indurre la proliferazione e la funzionalità delle cellule dell'immunità innata ed adattatoria. È stato riportato che la coltura delle cellule leucemiche con CSM rende le prime meno suscettibili al "killing" da parte delle NK grazie ad un meccanismo attivato dal contatto cellula-cellula causato dall'espressione di "Toll-like receptor 4" (TLR 4) da parte della CSM. Inoltre queste ultime hanno la capacità di indurre cellule Treg, di far aumentare l'espressione di IDO, d'inibire le funzioni delle cellule dendritiche (CD) con la produzione di TGFβ e *in vitro* hanno un effetto immuno-soppressorio sui linfociti superiore a quello svolto dalle CSM di soggetti sani ed una minore capacità di secernere interleukina 10, e possiedono profili diversi a seconda del sottogruppo clinico/citogenetico di LAM. La capacità immunosoppressiva della CSM è potenziata dalla capacità del blasto leucemico di crear un microambiente infiammatorio. La tempesta citokinica causata dalle CAR T potrebbe stimolare l'attività immuno-soppressoria

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delle CSM e provocarne la morte, ma l'osservazione che le CSM inibiscano la funzionalità T lasciando intatta l'attività CD19 specifica delle CAR T suggerisce che la funzione effettrice di queste ultime possa vincere l'effetto inibitorio delle MSC.

Modifiche dell'accasamento delle cellule preposte all'immunità

Nelle LAM vi è un'alterazione dell'asse CXL12/CXCR4, necessario all'accasamento non solo della CSE ma anche delle cellule effettrici e delle CAR T. I blasti leucemici esprimono alti livelli di CXCr4 mentre le CSM bassi livelli di CXCL12. La sovra-espressione di CXCR4 determina una prognosi sfavorevole mentre la sotto-espressione di CXCL12 promuove la migrazione nella nicchia delle cellule staminali leucemiche sulla migrazione delle CSE e riduce la quantità di CSM nella nicchia.

Alterazioni metaboliche

Le cellule leucemiche con la competizione per il glucosio ed amminoacidi e con il rilascio di fattori inibitori non solo rimodellano la nicchia ematopoietica, ma bloccano anche la funzionalità delle sottopopolazioni di cellule immuni. Questa capacità dei blasti leucemici può essere ulteriormente esacerbata dalle CSM che nelle LAM tendono a differenziarsi in adipociti. I blasti leucemici inducono una lipasi ormone-sensibile negli adipociti con attivazione di lipolisi e successivo trasferimento di acidi grassi alla popolazione leucemica, L'abbondanza di questi ultimi altera le funzioni effettrici delle cellule T attraverso la sovra-espressione di PD-1 e il blocco della secrezione di interferone γ che promuovono la maturazione dei Treg e l'acquisizione di un fenotipo M2 da parte dei macrofagi.

Le CSM possono trasferire mitocondri nella cellula leucemica per vie intracitoplasmatiche o nanotubuli tunnellizati, processo promosso dalla chemioterapia che fa aumentare la fosforilazione ossidativa nei blasti leucemici. Questo trasferimento è guidato dalla produzione di superossido generato dalla nicotinamide adenina dinucleotide fosfatasi ossidasi-2 (NOX2) prodotta dalla popolazione leucemica. Recentemente, nella nicchia ematopoietica sono state osservate delle "gap-junciton" tra CSM e cellula leucemica che regolano il metabolismo di quest'ultima. La grande quantità di "Reactive Oxigen Species" (ROS) che si osserva nelle LAM a citotipo M4-M5 deriva dall'attivazione costitutiva di NOX e dalla produzione mitocondriale di OXPHOS. Grazie alla produzione di ROS i blasti leucemici possono sfuggire all'azione effettrice dei T linfociti e delle cellule NK perché queste ultime vengono inattivate dai radicali liberi che ne inducono l'apoptosi.

Rimodellamento della nicchia vascolare

Questo processo avviene con la progressione della LAM per il verificarsi di un rimodellamento dell'endotelio vascolare ad opera dell'ossido nitrico (NO). Si crea così una nicchia leucemica ipossica che presenta una perdita di vasi a livello della regione endostale del midollo osseo, un aumentata permeabilità vascolare ed un flusso ematico ridotto. Queste alterazioni fanno sì che alcune aree del midollo osseo siano poco perfuse con compromissione della distribuzione della chemioterapia e del traffico di cellule immunocompetenti. Inoltre, l'infiammazione causata

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dai blasti leucemici attraverso la produzione di E-selectina altera l'adesione delle cellule immunocompetenti all'endotelio.

Strategie per superare la resistenza della LAM all'immunoterapia

Le alterazioni sopra riportate creano un complesso sistema di immunosoppressione che può rendere inefficaci anche i più protocolli di immunoterapia. Tuttavia, questo problema potrebbe essere superato non solo dall'uso combinato di specifici protocolli d'immunoterapia ma anche da terapie che abbiano come bersaglio componenti non neoplastici del microambiente tumorale.

Approcci combinati

Attività antileucemica di BITE e DART

I BITE (CD3xCD33, AMG330) sono molecole bi-specifiche formate da due domini ciascuno costituito da un frammento variabile a singola catena (scFv) derivato da un anticorpo, uno specifico per un antigene tumorale e l'altro specifico per CD3ε, montati su una catena polipetidica. AMG330 si lega simultaneamente alle cellule bersaglio (blasti CD33+) e a cellule effettrici (cellule T CD3+), facilita il reclutamento e l'espansione di cellule effettrici T causando l'eliminazione della popolazione leucemica anche quando il rapporto tra effettore verso bersaglio è basso (lisi in vitro sino a 1:80). Tuttavia, le cellule T memoria CD4+ e CD8+ che legano AMG330 sovra-esprimono PD-1, TIM-3 e LAG-3 rimanendo suscettibili all'inibizione dei "checkpoint" ed il blocco dell'asse PD-1/PDL-1 aumenta la lisi delle cellule neoplastiche, la proliferazione delle cellule T e la secrezione di interferone γ. La risposta al BITE AMG330 dipende dal carico tumorale e dalla quantità di cellule T endogene.

I DART hanno una maggiore stabilità e vita media dei BITE. Il flotetuzumab, un DART bispecifico CD123/CD3, ha dato risultati incoraggianti.

Cellule CAR T

Queste cellule potrebbero essere più suscettibili all'inibizione dei checkpoint nelle neoplasie mieloidi rispetto alle neoplasie linfoidi B e la combinazione delle CAR T con inibitori dei checkpoint sembra aumentarne l'efficacia terapeutica. Anche i farmaci epigenetici possono essere impiegati per aumentare la funzionalità delle cellule T e di altre sotto-popolazioni importanti nella risosta immune. In particolare, la metilazione del DNA può impedire l'espressione di NKG2D durante lo sviluppo di una LAM, ma il trattamento con inibitori delle de-acetilasi istoniche e con DNA metil-transferasi può causare un'ipo-metilazione di TIM3 con ripristino dell'espressione del ligando di NKG2D. Inoltre la decitabina, un farmaco ipometilante, può aumentare significativamente l'attività antileucemica di una CAR T CD123 specifica sia in vitro che in vivo. Recentemente, sono state disegnate nuove CAR T la cui attività potrebbe essere potenziata dall'uso di agonisti immunologici, di citokine co-stimolatorie e dal tentativo di orientare il fenotipo delle cellule T verso lo stato di cellula staminale "central memory". È stato però dimostrato che cellule CLL-1 CAR T che esprimevano interleukina 15 causavano una CRS asso-

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ciata ad alti livelli di "Tumor Necrosis Factor α", complicanza superata dal blocco di quest'ultimo e dalla rimozione delle CAR T.

La nicchia come bersaglio

Sono state proposte diverse molecole per bersagliare l'attività immunosoppressiva della nicchia leucemica: piccole molecole ad azione inibitoria (inibitori dell'IDO, dell'eme-ossigenasi-1, del fattore di crescita epatocitario, dell'arginasi I e II, della PGE2 e del TGFβ) e l'amminofosfonato zolendronato per colpire l'attività immunomodulante della CSM. Anche l'asse CXCL12/CXCR4 è un buon bersaglio perché la sua inibizione con piccoli inibitori o con anticorpi monoclonali permetterebbe il passaggio dei blasti leucemici nel sangue periferico dove sarebbero più facilmente colpiti non solo dalla chemioterapia ma anche da una immunoterapia. Un altro possibile bersaglio terapeutico potrebbe essere rappresentato dall'alterato metabolismo della cellula leucemica specialmente quello degli acidi grassi. La sua alterazione non aumenterebbe solo l'efficacia della chemioterapia ma accrescerebbe anche la funzionalità di cellule T impiegate per l'immunoterapia.

Farmaci come la daunorubicina, la citosina arabinoside promuovono il trasferimento di mitocondri nella cellula leucemica con aumento del metabolismo ossidativo ed inattivazione di cellule T e NK, processo che potrebbe bloccato con il blocco dei nanotubuli, dell'endocitosi e del superossido. L'antigene di superficie CD38 ha un ruolo chiave nel trasferimento dei mitocondri ed il suo blocco con il daratumumab, anticorpo monoclonale specifico, blocca questo trasferimento, riduce il consumo di ossigeno ed inibisce la crescita leucemica. Altri possibili bersagli connessi con l'attività mitocondriale potrebbero essere il "proliferator-activated receptor-gamma coactivator *1alpha"* (PGC-1α) e NOX2, mentre gli inibitori di NOS potrebbero normalizzare l'alterata permeabilità vascolare della nicchia.

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5

Engineering CAR T cells for access to solid tumors

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Cancer immunotherapy has entered clinical application more than 10 years ago (Kobold et al., 2015). T cell disinhibition using antibodies targeting check point molecules on T cells, thereby unleashing T cell activation against cancer, represent a paradigm shift in oncology (Ribas and Wolchok, 2018). Importantly, such advance demonstrates with clinical efficacy the potency of T cells in oncology. A direct consequence of addressing endogeneous T cells for therapy is their direct therapeutic use, also known as T cell therapy (Rosenberg and Restifo, 2015). In principle T cell therapy can be performed by two main avenues: use of T cells with endogeneous cancer specificity, found most abundantly in cancer tissue, so called tumor infiltrating lymphocytes (TIL) or by engineering T cells for specificity (Rosenberg and Restifo, 2015).

The so far only clinically approved strategy are chimeric antigen receptor (CAR) modified T cells (Stoiber et al., 2019). CAR are fully synthetic receptors not found in nature constituted extracellularly of the antigen binding domain of an antigen fused to T cell activating and T cell costimulatory domains (Tokarew et al., 2019). CAR T cells targeting the B cell antigen CD19, have induced unprecedented complete response rates in patients with refractory B cell lymphoma or leukemia (Maude et al., 2018; Neelapu et al., 2017). Importantly these responses rates also translated in bettered overall survival and likely cures for a significant number of patients (Neelapu et al., 2017; Raje et al., 2019). More recently, CAR targeting BCMA, a plasma cell antigen found on multiple myeloma were approved for treatment based on remission rates in refractory myeloma patients (Raje et al., 2019). More approvals are likely to come in the next years in hematologic oncology.

In the far more frequent solid tumors, which add up to more than 90% of all cancers, the use of CAR T cells have been disappointing, in spite of excellent preclinical data (Lesch et al., 2020). CAR T cells for example targeting mesothelin, an antigen broadly found on many cancer such pancreatic cancer, did not induce remissions in clinical trials (Beatty et al., 2018). A major difference, might come in the biology of solid cancer which differ from the one of hematological neoplasm to form an organized tumor microenvironment, difficult to access in many situations (Lesch et al., 2020). As a consequence CAR T cells face obstacles not found in hematology. We and others have found that CAR T cells are limited in their activity by three distinct mechanisms:

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1. access of T cells to the tumor site: if CAR T cell as active principle cannot reach tumor tissue, they cannot possibly exert their activity;
2. tumor heterogeneity with antigen heterogeneity which either does not allow efficient targeting with single antigens or select for negative variants and
3. local and systemic immune suppression (Lesch et al., 2020).

Ultimately all of these aspects will need to be addressed to deliver the benefits of cellular therapies to patients suffering from solid cancers.

Entry of T cells in tissue is a tightly regulated process involving sensing of mediators, so called chemokines by their matching chemokine receptors expressed by said T cells and integrins to mediate adhesion (von Andrian and Mackay, 2000). As part of their immunosuppressive strategy, cancer tend to produce such chemokines preferring recruitment of suppressive cells rather than cytotoxic ones. In other words, there is a frequent mismatch between chemokine receptor expression by the T cell and the chemokines expressed in the TME. To overcome this major limitation, my team thought to restore homing ability of therapeutic T cells to tumor tissue by genetic engineering of matching chemokine receptors (Zhang et al., 2019). Analyzing the TME of tumors, we found that subsets of T cells were enriched that expressed C-X-C-motive receptor 6 (CXCR6) on their surface (Di Pilato et al., 2021). We discovered that this receptor mediates T cell recruitment to dendritic cells producing CXCL16, which endows T cells with survival and proliferation signals via IL-15 presentation (Di Pilato et al., 2021). We sought to utilize this discovery to restore CXCR6 expression on therapeutic CAR T cells through genetic engineering (Lesch et al., 2021). We can indeed express CXCR6 in CAR T cells, which enables recruitment to CXCL16 gradients. In murine and xenograft tumor models of pancreatic cancer, this enables efficient CAR T cell recruitment and subsequently tumor control and rejection in a large proportion of animal treated. Similarly, CXCR6 enables recruitment of T cells and therapeutic activity in patient-derived models in vitro and in vivo. CXCR6 might thus serve as a CAR attractant to better T cell therapy in solid cancer entities expressing CXCL16 and enable cellular therapies in such otherwise resistant entities.

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6

CAR-T: sfide e opportunità per la ricerca indipendente

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Negli ultimi 10 anni i settori della terapia cellulare, della terapia genica e della medicina rigenerativa sono stati in rapida espansione. Alcune terapie cellulari hanno già raggiunto la commercializzazione, mentre molti altri prodotti medicinali per terapia cellulare sono in fase di ricerca e sviluppo.

Le cellule CAR-T fanno parte dei cosiddetti prodotti medicinali per terapia avanzata (*advanced therapy medical products, ATMP*). Oltre alle cellule CAR-T, gli ATMP includono molte altre tipologie di prodotti:

- *Prodotti medicinali per terapia genica:* sono prodotti medicinali biologici che contengono o che sono costituiti da acidi nucleici ricombinanti, per correzione di difetti congeniti o per riprogrammare le cellule effettrici immunocompetenti. Rientrano, a esempio, in questo gruppo, le cellule CAR-T oppure le cellule CD34+ modificate con geni per correzione difetti congeniti monogenici (ad esempio beta-thalassemia).
- *Prodotti medicinali per terapia cellulare somatica:* sono prodotti medicinali biologici composti da cellule o tessuti, manipolati *in vitro* al fine di cambiarne le proprietà biologiche, oppure somministrati per finalità funzionali diverse da quelle esercitate nell'organismo del donatore. Ad esempio, sono prodotti medicinali per terapia cellulare somatica le cellule stromali mesenchimali, i linfociti T naturali virus-specifici o tumore-specifici, le cellule CD34+ utilizzate per riparo tissutale.
- *Prodotti medicinali da ingegneria tissutale:* sono prodotti medicinali biologici composti da cellule o tessuti ad impiego rigenerativo o riparativo dei tessuti umani. Un esempio di questa tipologia di ATMP è costituito dalle cellule staminali corneali autologhe.
- *Prodotti combinati per terapie avanzate:* sono prodotti composti da cellule o tessuti combinati ad un dispositivo medico, che è parte integrante del farmaco. Un esempio è rappresentato dai progenitori di cellule delle insule pancreatiche caricate su device immunoprotettivi.

La maggior parte degli ATMP sono stati sviluppati, e continuano ad essere sviluppati, attraverso la ricerca accademica, condotta negli ospedali e nelle università. Tuttavia, la percentuale di prodotti per terapie avanzate che raggiungono l'approvazione da parte delle autorità regolatorie per l'impiego clinico e la commercializzazione è estremamente basso. Cerchiamo di capirne le motivazioni.

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I principali punti di forza della ricerca accademica sono:

1. Il focus dei centri di riferimento terziari su patologie complesse, con la presenza nei grossi ospedali di ricerca di competenze ed *expertise* multidisciplinari.
2. La cosiddetta ricerca *curiosity-driven*, vale a dire l'interesse nella ricerca di base e nella traslazione delle nuove scoperte alla ricerca clinica; l'interesse nel disegno di studi clinici per testare terapie innovative e nuovi farmacia.
3. La possibilità di avere un accesso diretto a cellule e tessuti dei pazienti: questo permette di svolgere ricerca sui meccanismi fisiopatologici delle malate rare e di creare modelli in vitro ed in vivo di malattia.
4. La possibilità di facile accesso al materiale di partenza di origine umana, grazie alla presenza negli ospedali dei centri di trapianto di cellule staminali emopoietiche e di trapianto d'organo solido.
5. L'expertise nella logistica di raccolta e validazione del materiale biologico di partenza, sempre grazie alla presenza dei centri di trapianto di cellule staminali emopoietiche e di organo solido.
6. La capacità da parte dei centri di riferimento di arruolare anche di coorti numerose di pazienti affetti da malattie rare.

Le principali difficoltà ed ostacoli dei centri accademici nel completare tutte le fasi di sviluppo delle ATMP fino alla loro commercializzazione sono invece rappresentati dai seguenti fattori:

1. La ricerca accademica è fortemente focalizzata sull'output scientifico (pubblicazioni) ed è finanziata con fondi destinati soprattutto alla ricerca a scopo traslazionale/esplorativo.
2. Vi è una scarsa abitudine ad un disegno del prodotto per traguardi a lungo termine e per il miglioramento continuo del prodotto stesso, dopo le fasi iniziali di sperimentazione, ai fini di una sua diffusione su larga scala e commercializzazione.
3. Vi è la mancanza di personale da dedicare agli aspetti regolatori, sempre più complessi e articolari, ma necessari per il *technology transfer* e l'immissione in commercio di un nuovo prodotto per terapia cellulare.
4. I finanziamenti alla ricerca sono frequentemente limitati nel tempo. Lo sviluppo iniziale di sperimentazioni cliniche di terapie cellulari negli istituti accademici è supportato da diverse fonti di finanziamento. Tuttavia, poiché questi finanziamenti sono frequentemente concessi per un periodo di tempo limitato, la continuità del sostegno finanziario è ostacolata. Tuttavia, ciò è necessario per lo sviluppo di nuovi farmaci.

Il trasferimento tecnologico dalla ricerca accademica allo sviluppo commerciale, con investimenti a scopo di lucro, può portare alla commercializzazione di terapie cellulari altamente sicure ed efficaci. Purtroppo, solo le terapie cellulari commercialmente più attraenti ed appetibili saranno trasferite allo sviluppo commerciale, mentre la maggior parte delle terapie cellulari potenzialmente utili per le malattie rare o ultra-rare potrebbero non diventare mai disponibili, o disponibili solo a un prezzo che è percepito come eccessivo dalla società. Sfortunatamente, i

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finanziamenti per lo sviluppo di prodotti senza scopo di lucro verso il trasferimento a basso costo a entità commerciali o verso la fornitura accademica per la cura standard dei pazienti sono ancora raramente disponibili.

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7

Real-life CAR-T cell treatment in Lymphomas

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The outcome of relapsed/refractory large B-cell lymphomas, not eligible or cured by high dose chemotherapy due to persistent disease, is very unsatisfactory. The introduction of anti-CD19 chimeric antigen receptor T cells (CAR-T) in this setting, showed impressive long-term results in registrative trials. Axicabtagene ciloleucel (axi-cel) and tisagenlecleucel (tisa-cel) are registered and reimbursed in Italy by Agenzia Italiana del Farmaco (AIFA) for the treatment of relapsed/refractory (R/R) diffuse large B-cell lymphoma (DLBCL) and primary mediastinal B-cell lymphoma (PMBCL) patients after at least 2 lines, with an ECOG 0-1, and an age lower than 71 years. To evaluate the real-life patients treated in Italy with CAR-T cells, the Italian Society of Haematology (SIE) designed an observational study.

The CART-SIE is an ongoing prospective and retrospective observational trial with the following aims:

1. consecutively register all DLBCL and PMBCL treated in the Italian authorized centers;
2. evaluate the intention to treat overall response rate (ORR, complete [CR] and partial response [PR]), duration of response (DOR), progression free survival (PFS) and overall survival (OS);
3. evaluate safety in terms of cytokine release syndrome (CRS), immune effector cell-associated neurotoxicity syndrome (ICANS) and long-term cytopenia;
4. evaluate the two different CAR-T products. Primary endpoint was to evaluate the overall response and survival at one year of the patients receiving CAR-T cells.

Since March 2019 to June 2021, 208 patients were enrolled and leukapheresed; 191 patients were infused (92%) with CAR-T cells.

In CART-SIE study, CAR-T cells treatment showed an outcome similar to those of the registrative trials. No differences across histotypes and commercial CAR-T products (axi-cel and tisa-cel) were observed. CRS and ICANS in real world are manageable with adequate risk management plan. Cytopenias are an emerging problem in real-life setting.

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8

Approved and emerging CAR T cells in MM

Hermann Einsele

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What are the current treatment schedules and what are the unmet clinical needs in MM? Patients suitable for stem cell transplantation receive a 4-drug regimen, high dose chemotherapy followed by autologous stem cell transplantation, consolidation and maintenance.

Patients ineligible for stem cell transplantation receive VRD or Dara-Rd as first line therapy. Treatment results have considerably improved by the most recent strategies 3-years progression-free survival for transplant-eligible patients is nearly 90% and in patients not eligible for transplantation progression free survival with Dara-Rd is > 5 years. But there is clearly an unmet medical need, esp. for patients with high and ultra-high risk MM, - with a median progression-free survival of only 15.4 months and in addition, patients with early relapse after front-line therapy with a progression-free survival of only 27 months.

Furthermore, due to the fact, that we increasingly use most of the anti-MM agents during the first 2 lines of therapy, we see an increasing number of patients that are refractory to PI/IMiDs and CD38 antibodies and for these patients the median progression-free survival is less than 4 months.

So, how can CAR T cell therapy address these unmet medical needs? First of all one CAR T cell product, Ide-Cel or Abecma, was approved by the FDA and EMA based on the KarMMa trial - in which patients with a median of 6 lines of prior therapy, 84% triple refractory and 39% of patients with extramedullary disease received 450 million CAR T cells with an overall response rate of 81%, a complete remission rate of 35% and a progression-free survival of 11.3%.

Therapy was associated with quite a low grade III CRS and neurotoxicity of 3 and 5%. In a subgroup of patients with a complete remission after CAR T cell therapy, the progression-free survival was >20 months. In addition, in the KarMMa trial - patients beyond the age of 65 and also 70 were successfully and safely treated with Ide-Cel.

At the last ASH meeting there was an update on another BCMA-directed CAR T cell product that is targeting BCMA with two target domains - Ciltacel. Again heavily pretreated patients with 6 lines of prior therapy, 42% penta-refractory and 13% of the patients with extramedullary disease underwent this CAR T cell therapy. The overall response rate was 97.9%, the complete remission rate higher than 80% and the median progression-free survival was not reached after 2 years.

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Especially patients with a maintained MRD-negativity had a 100% progression-free survival at 2 years. But with both BCMA-directed CAR T cell products, there are patients that do less favorable, - these are high risk patients, patients with ISS III and patients with extramedullary disease.

One strategy to improve CAR T cell therapy is to improve persistence and reduce T cell exhaustion. One of the strategies here is to obtain T cells for CAR T cell therapy production at earlier lines of therapy, - which were shown to have a better fitness and proliferative capacity.

In the CARTITUDE-2 trial, patients with early relapse after initial therapy received Ciltacel and, as you can see, these patients were less heavily pretreated and showed thus, less severe cytopenias and fortunately, in spite of fitter T cells no higher rate of CRS or neurotoxicity. – and the overall response rate and the rate of complete remission were again very impressive. A trial is starting soon like in lymphoma in which in the first-line therapy CAR T cell therapy is challenging HD-therapy and ASCT.

Another strategy to improve the persistence of CAR T cells is to go for a CAR T cell product with long-lived CAR T cells and a better proliferative capacity achieved by adding a PI3k inhibitor bb007 to the culture medium to enrich for T cell displaying a memory-like phenotype.

A further strategy to improve CAR T cell efficacy for Multiple Myeloma is to increase target antigen expression. And this can be done by using gamma secretase inhibitors which was shown by Damian Greens group to increase BCMA expression by at least 15 fold.

Bibliografia essenziale

1. Hermann Einsele, MD, FRCP, is Full Professor of Internal Medicine and has been Director of the Department of Internal Medicine II of the University Hospital Würzburg, Germany, since 2004.
2. Following his medical training at the Universities of Tübingen, Manchester, and London, Hermann Einsele became a research fellow in the Department of Haematology, Oncology, Rheumatology, and Immunology at the University of Tübingen, Germany. Hermann Einsele was board certified in Internal Medicine in 1991 and in Haematology/Oncology in 1996. In 1999, he was promoted as an Associate Professor. He was Visiting Professor at the City of Hope Hospital, Duarte, CA and the Fred Hutchinson Cancer Research Center Seattle, USA.
3. From 2011-2015 and since 2022 Hermann Einsele was Vice Dean of the Faculty of Medicine of the University of Würzburg, 2015 -2021 Vice President of the University of Würzburg. Since 2018, he is the chair of the scientific working group on immunotherapy for hematological malignancies of the European Hematology Association. Since 2022, Prof. Einsele is Executive Chairman of the German Society of Hematology and Oncology.
4. In 2003, he received the van Bekkum Award, the highest Annual European award for research in the field of stem cell transplantation. In 2011, he was elected as an Honorary Fellow of the Royal College of Pathologists (London)

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and in 2012 Nobel Lecturer Stem Cell Biology/ Transplantation, Nobel Forum Karolinska Institute. Since 2014, he was elected as a member of the Academy of Sciences and Literature, Mainz and 2017/2018/2019/2020/2021 as an ISI "Highly Cited Researcher" in the category Clinical Medicine.

5. Hermann Einsele is expert in the field of multiple myeloma with focus on CAR T cells, bi-specific antibodies, adoptive immunotherapy and stem cell transplantation.

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9

Innate immunity and inflammation in neoplastic progression

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Inflammation is a manifestation of innate immunity and has emerged as a metanarrative of modern medicine (Mantovani et al., *Immunity*, 2019; Furman et al., *Nature Medicine*, 2019), a component of diverse disease ranging from cancer to COVID-19 (Mantovani et al., *Nature* 2008; Mantovani and Netea, *New England Journal of Medicine*, 2020). Dissection of the diversity and complexity of regulatory pathways of innate immunity has taken advantage of hypothesis driven and non-hypothesis driven approaches.

IL-1 is the prototypic member of a complex family of cytokines and receptors which play a central role in innate immunity and in the activation and regulation of adaptive immune responses (Mantovani et al., *Immunity*, 2019). Based on structure, we originally hypothesized that IL-1R2 should behave as a decoy receptor, a tenet confirmed by extensive experimental data. In the same line, we cloned IL-1R8 and focused on it based on the hypothesis that it would behave as a negative regulator. IL-1R8 was then found to be a negative regulator of signaling downstream of members of the IL-1 and Toll like receptor (TLR) family and a component of the receptor complex recognized by the anti-inflammatory immunosuppression cytokine IL-37.

As a result of a fishing expedition we originally cloned PTX3 as a gene induced by IL-1 (Garlanda et al., *Physiol Rev*, 2020). This distant relative of C reactive protein (CRP) was then found to represent an essential component of the humoral of innate immunity playing a role in antimicrobial resistance and in the regulation of inflammation. The latter includes selected solid tumors and hematological malignancies (Garlanda et al., *Physiol Rev*, 2020). We also cloned in silico PTX4, but its function remains to be defined. Interestingly, PTX3 was recently found to be highly expressed in monocytes and lung macrophages at population and single cell level in COVID-19 and to represent a candidate novel biomarker of disease severity (Brunetta et al., *Nature Immunol* 2021).

Stemming from our interest in macrophage diversity and polarization (Locati, Curtale, Mantovani, *Ann Rev Pathol* 2020; Mantovani et al., *Nature Rev. Clin. Oncol*. 2017; Jaillon et al., *Nature Rev Cancer* 2020), we identified the tetraspan MS4A4A, a gene of unknown function, as a gene associated with M2-like macrophage polarization (Mattiola et al., *Nat Immunol*. 2019). We found that MS4A4A partner with Dectin-1 and is essential for full syk signalling downstream of this pattern recognition receptors.

The examples discussed above, selected from our previous and ongoing research efforts, highlight the complexity of innate immunity and its regulation. The dissec-

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tion of the daunting complexity and diversity of cellular and human innate immunity requires the use of unbiased non-hypothesis-driven approaches complemented by hypothesis-driven, biased experimental and clinical testing.

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10

Cell therapy with cytotoxic T lymphocytes (CTLs) for the control of leukemia relapse

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Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is an effective treatment for many hematologic malignancies, and remains the therapy with the highest chance of long-term remission for acute leukemia patients, especially for those transplanted in first complete remission. Unfortunately, recurrence of original neoplastic disease is still one of the major causes of failure of this therapy, even considering the occurrence of the graft-versus-leukemia (GVL) effect mediated by donor T cells and, when disease relapse occur after allo-HSCT, the prognosis of patients is poor. A second allo-HSCT it may be offered only a minority of patients, whose relapse occur late after the transplant (>6 months), while the majority of patients do not tolerate the severe toxicity of a second conditioning regimen (1, 2). Several immunologic strategies have been proposed for restoring or enhancing anti-tumor immunity in patients with leukemia after allo-HSCT and, besides CAR-T cell therapy, also somatic cell therapy may offer a new tool to prevent or treat relapse (3).

Unmanipulated donor lymphocytes infusions (DLI) can be considered the first kind of immunotherapy able to induce durable remission by enhancing GVL effect. DLI, introduced for the treatment of leukemia relapse in the early 1990s, has been used for prophylactic or therapeutic purposes and was shown to be more effective in chronic myeloid leukemia (CML) and in acute myeloid leukemia (AML) than in acute lymphatic leukemia. However, the prognosis of these patients remains dismal even after DLI infusion (2-year overall survival, ~25%), and the efficacy is achieved at the cost of toxicities such as graft-versus-host (GVH) disease (4-6). With the aim of separating GVL effect from GVHD, T cells have been genetically modified to express a suicide gene, for their selective elimination in the case of emergence of GVHD. Phase I-II clinical trials using HSV-TK cells documented that suicide gene therapy is safe and effective in controlling GVHD (7, 8).

Cell therapy based on the transfer of cytokine-induced killer cells (CIK) cells have also been considered in relapsed patients. CIK cells are CD8 T lymphocytes that have acquired NK-like cytotoxicity in culture by stimulation with anti-CD3 antibody, IFNγ and IL-2. CIK cells mediated a strong non-HLA restricted cytotoxicity and pre-clinical evidences suggest that donor-derived CIK cells are unable to mediate the emergence of graft-versus-host disease (GVHD). Clinical trials based on infusion of CIK cells in the setting of allogeneic HSCT, documented low GVHD

toxicity (grade I-II) and anti-leukemia effect slightly superior to the unmanipulated donor leukocyte infusion (DLI). These observations, together with the success of CAR strategies in ALL, prompted the development of molecules specific for AML targeting. In particular, in the last years it has been investigated the specific targeting of CD123 AML antigen exploiting CAR-redirected CIK cells (3, 9, 10).

Another potentially successful approach for controlling leukemia relapse after allo-HSCT is represented by infusion of donor-derived anti-leukemia cytotoxic T lymphocytes (CTLs), even their applicability has some restrictions. Limitations include the identification of tumor-associated antigens with broad specificity, the ability of transferred cells to reach the tumor site, to display effector functions and to persist over time. Cell therapy with CTLs directed against minor histocompatibility antigens or against BCR-ABL peptides, have been used to treat relapsed leukemia after allo-HSCT in adults and represent a proof of principle of the potential efficacy of this approach (11-14).

In recent years, we have optimized a procedure for generating and expanding CTLs directed against different types of tumor cells, including acute leukemia blasts (LB), through stimulation of peripheral blood mononuclear cell (PBMC) with dendritic cells (DC) pulsed with apoptotic patients' neoplastic cells in the presence of opportune cytokines. Donor-derived anti-leukemia CTLs displayed high levels of cytotoxicity against patients LB and negligible or low activity against patient-derived non-malignant cells, employed as an *in vitro* control to evaluate their potential alloreactivity capacity (14-16). Anti-leukemia CTLs, generated using the whole tumor cells as source of leukemia-associated antigens (LAA), are likely to recognize a broader range of LAA, potentially reducing the risk of selecting variant leukemic subclones and include both effector and memory T-cells, suggesting the presence of lymphocytes able to exert, not only an immediate cytotoxic effector activity, but also to maintain long-term immune surveillance (17, 18). Anti-leukemia CTLs, produced in compliance with GMP requirements in the Cell Factory of the IRCCS Policlinico San Matteo, were employed to prevent or treat leukemia relapse in pediatric patients receiving haploidentical HSCT (haplo-HSCT).

T-cell depleted, haplo-HSCT from partially matched family donor, offers an immediate transplant treatment, virtually to any patient in need of an allograft and lacking a suitable matched donor. One of the major advantages of using a family related donor is the possibility to collect additional cellular products from the same immediate available donor and, for this reason, haplo-HSCT represents an ideal platform for post-transplant cellular therapy. Fifteen pediatric patients were enrolled so far and were treated on a compassionate base or in a Phase I/II prospective, non-randomized clinical trial. The aim of this trial is to evaluate the safety and preliminary efficacy of infusions of escalating doses of donor leukemia-directed CTLs for the prevention of disease relapse in pediatric patients with high-risk acute leukemia given haplo-HSCT. No severe adverse reactions, no grade 2-4 toxicities, including emergence of severe GVHD were recorded during follow-up in all patients evaluated. Moreover, data obtained so far confirm that donor-derived anti-leukemia CTL have a role in both prevention and treatment of post-haplo-HSCT recurrence, also leading to long-term remission.

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11

CAR-T cells in hematopoietic stem cell transplantation

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Dramatic progress in the outcomes of allogeneic hematopoietic stem cell transplantation (allo-HSCT) from alternative sources, including mismatched unrelated donors, umbilical cord blood, and haploidentical related donors, has been registered over the past decade in pediatric patients, providing a chance to cure the children and adolescents with hematologic disorders in need of a transplant but lacking a compatible donor (1-2).

After transplantation, recovery of donor-derived T cells facilitates engraftment, protection from opportunistic infections, and, in patients with malignancies, from relapse of the underlying disease. However, T cells transferred with the stem cell graft may also recognize foreign histocompatibility antigens on patient tissues and induce the graft-versus-host disease (GVHD), a significant cause of morbidity and mortality after allo-HSCT (3-4). The continuous development of graft engineering and pharmacologic GVHD prevention strategies, together with better supportive care and optimal conditioning regimens, have significantly improved the outcomes of allo-HSCT from alternative sources (2,5). In particular, transplantation from a full HLA-haplotype mismatched family member (haplo-HSCT), in addition to ensuring a donor for the large majority of patients, offers several other advantages, including prompt availability of the stem cell source, the possibility to select the best donor from a pool of family candidates, and immediate access to donor-derived cellular therapies either for the prevention of relapse or the treatment of infections after HSCT (6).

Despite encouraging results, disease relapse is the main cause of failure in pediatric patients receiving HSCT for acute leukemia (6). Leukemia relapse is likely related to the delayed immune reconstitution, and, in order to overcome its development, different means to boost immune surveillance have been implemented.

Proof of principle studies had demonstrated the feasibility to administer unmanipulated donor lymphocytes (DLI) to treat leukemia relapse after T-cell depleted HSCT (7,8). The rate of acute GVHD developing after the procedure, however, prompted manipulation of donor lymphocytes to reduce alloreactivity while maintaining immune surveillance potency.

Two strategies have been explored to reduce the risks derived from alloreactivity associated with DLI. The first approach was based on transduction of non-

specific T cells with a retroviral construct containing suicide genes, to induce susceptibility to drug-mediated lysis in case of development of alloreactive response (9,10). Although transfer of suicide genes have provided a safety switch to T cells, initial triggering of GVHD may still be a problem. Therefore, reconstitution of leukemia-specific immunity by transfer of donor-derived leukemia-directed T cells, that should contain lower number of alloreactive T cells compared to DLI, is an appealing strategy to rapidly restore virus-specific immunity to prevent or treat viral diseases in this setting (11).

Attempts have been made to boost tumor-specific responses and control leukemia relapse by post-transplant add-backs of donor cytotoxic T cells (CTLs) directed towards patients blasts (12), minor histocompatibility antigens (13), or leukemia-related antigens (14,15). One of the main limitations is that CTL antigen recognition is major histocompatibility complex (MHC)- restricted, unless leukemia blasts from the patient are employed. Moreover, in many cases, tumor-specific antigens able to elicit protective immune responses have not been identified.

To extend the recognition specificity of T lymphocytes beyond their classical MHC-peptide complexes, a gene-therapeutic strategy has been developed that allows redirecting T cells to defined tumor cell surface antigens, by the transfer of an antigen-binding moiety, most commonly a single chain variable fragment derived from a monoclonal antibody, together with an activating T-cell receptor (chimeric antigen receptors, CARs). Recently, CARs directed to the CD19 molecule, expressed on B-cell malignancies, have been employed in pediatric and adult patients with refractory ALL and proven highly efficient, with CR rates of 70% to 90% (16-19). These studies included patients with a prior history of allogeneic HSCT, and no GVHD was recorded.

So far, autologous CAR-T targeting CD19 have been mostly employed in patients with refractory ALL to induce pre-transplant remission, or in patients with overt relapse after HSCT. A few studies have considered the use of donor-derived CAR-T cells after HSCT, and they were mostly employed in adult patients with overt relapse (20). A phase I/II trial in pediatric and adult B-ALL patients relapsed after HSCT used donor-derived CD19 CAR T cells generated with the Sleeping Beauty (SB) transposon and differentiated into cytokine-induced killer (CIK) cells, reporting 61% CR and 30% long lasting remissions (15% without additional treatments) (21).

Only in few adult patients, treatment with haplo donor-derived anti-CD19 CAR-T cells were employed to treat minimal residual disease after T-replete haplo-HSCT (22), with very limited toxicity (2/8 skin GVHD, no CRS) and good efficacy (75% DFS at 1 year). It has been described that clinical efficacy of CAR-T cell therapy depends on in vivo cellular expansion, that is maximal in the presence of overt disease, and is paralleled by the potential to induce severe adverse events, such as cytokine release syndrome, neurological toxicity, and on-target off-tumor toxicities (23).

We hypothesize that the best option for high-risk pediatric ALL patients receving Tαβ/B cell depleted haplo-HSCT is to reduce the rate of overt post-transplant relapse by preventive/preemptive early post-HSCT infusions of low escalating dos-

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es of donor anti-CD19 CAR-T cells. This strategy would also allow to minimize adverse events, as cells are not expected to massively release cytokines in the presence of minimal residual disease.

The majority of subjects treated to date have provided and received autologous or allogeneic dedicated T/NK cells, but this approach may not be best suited for widespread cost-effective delivery of cellular therapy, since dedicated CAR-T cells are personalized medicines that are produced on-demand through a complex and costly supply chain, thus implying some delay in manufacturing the final product and the risk that the disease will evolve and be fatal for the candidate patients. Allogeneic CAR-T cells could be employed as off-the-shelf products, although they carry the risk of immune rejection by the host, and their short persistence may require additional therapies to consolidate CAR-T Cell induced responses. Gene editing offers the prospect of addressing human leukocyte (HLA) barriers and the development of universal T cell therapies (24). Recently, "off the shelf" T cells modified using transcription activator-like effector nucleases (TALENs) and expressing CD19 CAR have been used to treat refractory relapsed B-ALL in infants (25).

Finally, the challenge is now to increase availability of these T cell therapies, through new and sustainable reimbursement modalities or support to producing academic centers, thus allowing their use also in low-income countries.

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Enhancing Chimeric Antigen Receptor T-Cell Efficacy in Solid Tumors

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Abstract

Chimeric antigen receptor (CAR) T-cell therapy has been acclaimed as a revolution in cancer treatment following the impressive results in hematologic malignancies. Unfortunately, in patients with solid tumors, objectives responses to CAR T cells are still anecdotal, and important issues are driven by on-target but off-tumor activity of CAR T cells and by the extremely complex biology of solid tumors. Here, we will review the recent attempts to challenge the therapeutic impediments to CAR T-cell therapy in solid tumors. We will focus on the most promising strategies of antigen targeting to improve tumor specificity and address the tumor heterogeneity, efforts to circumvent the physical barriers of the tumor architecture such as subverted tumor vasculature, impediments of CAR T-cell trafficking and immune suppressive microenvironment.

Introduction

Chimeric antigen receptors (CAR) are antigen receptors resulting from the fusion of a single-chain variable fragment (scFv) of an mAb with the z-chain of the T-cell receptor complex and one or two costimulatory moieties (1-3). Upon expression in T cells, CARs allow MHC-unrestricted recognition of surface antigens expressed by tumor cells, T-cell activation, proliferation, and tumor cytolysis. MHC-unrestricted antigen recognition of CAR T cells overcomes the down-regulation of human leucocyte antigen molecules frequently orches-trated by cancer cells to escape immune recognition, whereas the scFv-mediated antigen recognition enables T cells to target also nonprotein epitopes, widening the repertoire of actionable targets in cancer immunotherapy, and expanding the potentials of adoptive cell therapy in solid tumors pioneered by the use of tumor-infiltrating lymphocytes (TIL) in melanoma (4). As briefly summarized in Table 1, CAR T cells may have several strengths as compared with TILs for the treatment of solid

Table 1. Key features of TILs versus CAR T cells for adoptive cell therapy in solid tumors.

TILs	CAR T cells
Difficult manufacturing (insufficient availability of tumor tissue or modest T-cell tumor infiltration)	Easy manufacturing regardless of tumor localization and tumor burden
Invasive surgical procedures to obtain tumor tissue for T-cell isolation	Noninvasive procedures (leukapheresis) for T-cell isolation
Long selection and expansion process that can lead to T-cell exhaustion	Relatively short manufacturing process
Lack of T-cell costimulation	T-cell costimulation is engineered within the CAR in second- and third-generation molecules
Autologous use only	Possible engineering strategies to produce "off-the-shelf" T-cell products
HLA-restricted recognition of the tumor cells that can lead to tumor escape (e.g., alteration in antigen processing and presenting mechanisms in tumor cells, HLA downregulation)	Recognize antigens in HLA-unrestricted manner
Can target both surface and intracellular antigens	Can only target surface antigens, even if scFvs targeting peptide presented in the MHC have been developed
Can provide simultaneously multiple target specificity	Up-to-now it is not feasible to target more than two antigens simultaneously
No on-target toxicity	Possible on-target but off-tumor toxicity

Note: Green background indicates the strengths whereas red background indicates the weaknesses of the two immunotherapy approaches.

tumors. Although CAR T cells have been a clinical breakthrough in some hematologic malignancies (5-7), the development of such strategy for solid tumors is still in its infancy. As illustrated in Table 2, objective responses in patients with solid tumors treated with CAR T cells are scant, whereas on-target but off-tumor toxicities remain a great concern (8-32). The modest activity of CAR T cells in solid tumors as compared with B-cell malignancies can be largely attributed to the intrinsic biologic differences between the two cancerous entities. Extreme heterogeneity

Table 2. Summary of the safety and activity data from published studies on CAR T-cell therapy in solid tumors.

Author (year)	Type of study	Patients treated	Target	Tumor type(s)	Costimulation	Route	Lymphodepletion	On-target off-tumor toxicity	Objective responses	ORR *n* (%)
Morgan et al. (10)	Case report	1	HER2	Colorectal cancer	CD28+4-1BB		Yes	ARDS (fatal)	Not applicable	Not applicable
Ahmed et al. (14)	Phase I/II	19	HER2	Sarcoma	CD28	i.v.	No	No	Not observed	0/19 (0)
Ahmed et al. (25)	Phase I	17	HER2	Glioblastoma	Virus-specific T cells+CD28	i.v.	No	No	1 PR	1/17 (6)
Feng et al. (27)	Phase I	11	HER2	Biliary tract cancer Pancreatic cancer	4-1BB	i.v.	Yes	Liver enzymes increase	1 PR	1/11 (9)
Katz et al. (15)	Phase I	6	CEA	Liver metastases	CD28	Hepatic artery infusion	No	No	Not observed	0/6 (0)
Zhang et al. (24)	Phase I	10	CEA	Colorectal cancer	CD28	i.v.	Yes	No	2 PR	2/10 (20)
Thistlethwaite et al. (26)	Phase I	14	CEA	Colorectal cancer Gastro-esophageal cancer Pseudomyxoma peritonei Pancreatic cancer	—	i.v.	Yes	Transient acute respiratory toxicity	Not observed	0/14 (0)
Katz et al. (32)	Phase I	6	CEA	Liver metastases	CD28+use of selective internal radiation therapy (SIRT)	Hepatic artery infusion	No	No	1 metabolic CR	1/6 (17)
Louis et al. (11)	Phase I	19	GD2	Neuroblastoma	Virus-specific T cells	i.v.	No	No	3 CR	3/19 (16)
Gargett et al. (17)	Case report	4	GD2	Melanoma	CD28+OX40	i.v.	No/Yes	No	Not observed	Not applicable
Beatty et al. (13)	Case report	2	Mesothelin	Pleural mesothelioma Pancreatic cancer	4-1BB	i.v./intratumoral	No	No	1 PR	Not applicable
Beatty et al. (30)	Phase I	6	Mesothelin	Pancreatic cancer	4-1BB	i.v.	No	No	Not observed	0/6 (0)
Brown et al. (16)	Pilot study	3	IL13Rα2	Glioblastoma	—	Intracranial	No	Neurologic event	Not observed	0/3 (0)
Brown et al. (19)	Case report	1	IL13Rα2	Glioblastoma	4-1BB	Intracranial	No	No	1 CR	Not applicable
Feng et al. (21)	Phase I	11	EGFR	Non–small cell lung cancer	4-1BB	i.v.	No/Yes	Serum lipase increase	2 PR	2/11 (18)
Feng et al. (22)	Case report	1	EGFR/CD133	Cholangiocarcinoma	4-1BB	i.v.	No	Liver enzymes increase (aEGFR), rash and mucositis (aCD133)	1 PR	Not applicable
O'Rourke et al. (28)	Case report	10	EGFRvIII	Glioblastoma	4-1BB	i.v.	No	No	Not observed	Not applicable
Goff et al. (31)	Phase I	18	EGFRvIII	Glioblastoma	CD28+4-1BB	i.v.	Yes	ARDS (one treatment-related death)	Not observed	0/18 (0)
Kershaw et al. (8)	Phase I	14	α-folate receptor	Ovarian cancer	—	i.v.	No	No	Not observed	0/14 (0)
Park et al. (9)	Phase I	6	CD171	Neuroblastoma	—	i.v.	No	Lymphopenia, neutropenia, anemia, pneumonitis	1 PR	1/6 (17)
Lamers et al. (12)	Phase I	12	CAIX	Renal cell carcinoma	—	i.v.	No	Liver enzymes increase	Not observed	0/12 (0)
Junghans et al. (18)	Phase I	5	PSMA	Prostate cancer	—		Yes	No	2 PR	2/5 (40)
You et al. (20)	Case report	1	MUC1	Seminal vesicle cancer	CD28+4-1BB	Intratumoral	No	No	Not observed	Not applicable
Hege et al. (23)	Phase I	16	TAG-72	Colorectal cancer	—	i.v./hepatic artery infusion	No	Retinal artery occlusion	Not observed	0/16 (0)
Tchou et al. (29)	Phase 0	6	c-MET	Breast cancer	4-1BB	Intratumoral	No	No	Not observed	0/6 (0)

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of antigen expression and antigen-sharing with vital organs, presence of physical barriers for immune cell trafficking and penetration, together with the development of an immune suppressive microenvironment are general features of most of the nonhematologic malignancies. In this article, we provide a concise overview of the approaches that have been implemented to adapt CAR T cells to the complex pathophysiology of solid tumors taking particular attention to the strategies that are reaching the clinical investigation.

Identification of Targetable Antigens in Solid Tumors

On-target but off-tumor activity

The success of CAR T cells targeting the pan-B cell marker CD19 in B-cell malignancies was predicated on the tolerability of the protracted B-cell aplasia caused by the sustained persistence of CAR T cells (7). In sharp contrast, the development of CAR T cells targeting solid tumors has been hampered by the anticipated and intolerable toxicity that can be caused by CAR T cells targeting antigens expressed by tumor cells but shared with normal tissues. Severe toxicities caused by on-target but off-tumor antigen recognition by CAR T cells have been reported in clinical studies. In a phase I study of anti-CAIX CAR T cells in metastatic renal cell carcinoma, the expression of CAIX on the bile duct epithelial cells caused dose-limiting liver toxicity (12). In a second study, the infusion of CAR T cells targeting HER2 caused fatal acute respiratory distress syndrome due to recognition of lung epithelia cells expressing low levels of HER2 (10). It remains an open question if antigens overexpressed on the cell surface of tumor cells in solid tumors and at low levels in some normal tissues can be safely targeted by CAR T cells. Some clinical data and preclinical models suggest that a therapeutic window may be achievable taking in consideration which antigen epitopes are targeted by CAR T cells, the density of antigen expression and the affinity of the scFvs used to generate CARs. For example, HER2-specific CAR T cells targeting different epitopes can significantly reduce the activity of CAR T cells in normal tissues without compromising tumor recognition likely due to different accessibility to the epitope by the scFv in normal tissue as compared with cancerous cells (14). Preclinical observations suggest that higher antigen density expression in the tumor as compared with normal tissues can be exploited to effectively eliminate tumor cells, while sparing normal cells (33, 34). Similarly, tuning the affinity of scFvs may also preferentially trigger CARs upon binding to tumor cells expres-sing high levels of the antigen, sparing normal cells expressing physiologic levels of the antigen, as preclinically showed for both HER2 and EGFR (35, 36). Solid tumors can express aberrant splicing isoforms and molecules characterized by subverted protein glyco-sylation, thus targeting tumor-specific splicing variants or tumor-specific glycosylation sites represents another strategy to overcome on-target but off-tumor activity in solid tumors (28, 37, 38). To increase the tumor specificity, CAR T cells can also be engineered to express combinatorial antigen-sensing systems that trigger T-cell activation only when two tumor-specific antigens are simultaneous-ly present on the cell surface, as in the case of the integration of combinatorial targeting and splitting signal (38) or ON-switch strategies (39, 40).

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Heterogeneity of antigen expression in solid tumors

The second critical aspect of the identification of targetable antigens in solid tumors concerns the high heterogeneity of antigen expression at both intra- and intertumoral level leading to tumor escape due to both antigen loss and clonal evolution. Targeting more than one antigen already represents the next-generation CAR T cells in B-cell malignancies upon the evidence that both leukemia and lymphoma cells can lose the expression of the CD19 targeted epitope after treatment with CD19-specific CAR T cells (41). Consistently, CAR T cells engineered to recognize multiple antigens in solid tumors such as EGFR, HER2, and IL13Ra2 have been tested in preclinical models (42). Alternatively, CAR T cells can be combined with epige-netic drugs that can upregulate the expression of target antigens, as reported for GD2 upregulation in Ewing Sarcoma upon pharmaco-logic inhibition of EZH2 (43).

Overcome Physical Barriers in Solid Tumors

Immune cell trafficking and infiltration of peripheral tissues is regulated by a complex signaling network and physical processes, which are significantly subverted in tumors. Tumor blood vessels are characterized by abnormalities in endothelial junctions and expression of adhesion and extravasation molecules that limit an efficient T-cell recruitment (44). Moreover, the chemokine network orchestrating immune cell migration and the stroma including fibroblasts and myeloid cells are highly dysregulated in solid tumors further impairing the access of T cells within the tumor bed (45).

Local delivery of CAR T cells

Local drug delivery is one of the most exploited strategy to overcome biological barriers in solid tumors especially in malignancies charac-terized by locoregional aggressiveness (Fig. 1A). Local CAR T-cell delivery in solid tumors in addition to maximizing the accumulation of CAR T cells at the tumor site, may also improve the safety profile, limiting their systemic biodistribution and, consequently, the access to vital organs sharing the expression of targeted antigens. Brain tumors are a typical example where local CAR T-cell delivery has high clinical potential. Complete tumor regression was reported in a patient with highly aggressive recurrent glioblastoma with multifocal leptomenin-geal disease upon infusion of anti-IL13Ra2 CAR T cells into the resected tumor cavity and into the ventricular system (19). Intracranial delivery of CAR T cells was effective also in a mouse model of HER2þ breast cancer with brain metastasis (46).

Malignancies with a pleural or peritoneal spreading are also par-ticularly attractive for the local delivery of CAR T cells. As a proof of concept, intravenous injection of anti-MUC1 CAR T cells caused their redistribution mainly to the liver and spleen, with a trafficking circuit comprising axillary, retroperitoneal, and popliteal lymph nodes, but with modest localization in the peritoneal cavity (47). In contrast, CAR T cells infused intraperitoneally localized and persisted throughout the peritoneal cavity with limited extraperitoneal distribution causing improved antitumor

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activity in mouse models of colorectal cancer peritoneal carcinomatosis and ovarian cancer as compared with systemic administration (33, 48). Liver-limited metastatic disease is another interesting setting for local delivery of CAR T cells. In a phase I clinical trial, anti-CEA CAR T cells delivered through percutaneous hepatic artery infusion in 5 patients with CEA-positive colorectal cancer liver metastases were safe and promoted sustained stabilization of disease in one patient (15). Other proof-of-concept clinical studies with promising data of antitumor activity were conducted with locally delivered anti-cMET CAR T cells in patients with metastatic breast cancer and with anti-mesothelin CAR T cells administered intrapleu-rally in patients with primary malignant pleural mesothelioma or secondary metastatic disease (29, 49). In line with the effort to develop local delivery of CAR T cells is the parallel development of implantable biomaterials to manipulate cell and tissue properties and to enhance persistence of locally delivered cells (Fig. 1B). Encapsulating or embedding CAR T cells in implantable biopolymer scaffolds resulted in a better persistence and antitumor effects in mouse models of pancreatic cancer and melanoma (50). Furthermore, biopolymer scaffolds can not only accommodate cells but also be loaded with costimulatory molecules, cytokines, and small molecules that can further enhance the antitumor activity of CAR T cells or target the TME (50).

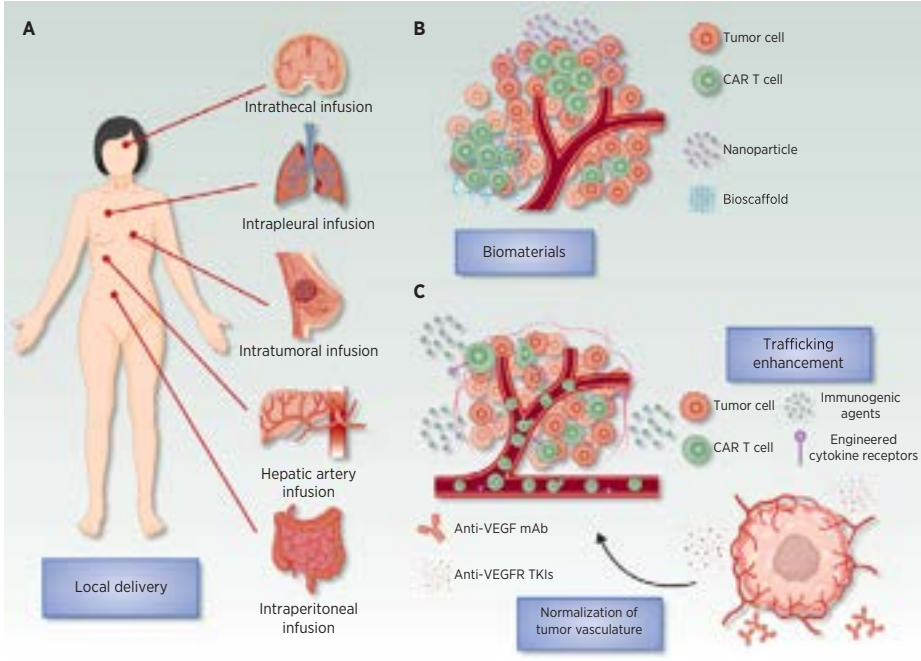

Figure 1.
Strategies to overcome physical barriers in solid tumors. Local T-cell delivery can maximize the accumulation of CAR T cells at the tumor site and may also improve the safety profile (**A**). Biomaterials can be used to enhance the persistence and functionality of locally delivered CAR T cells (**B**). CAR T cells can be combined with antiangiogenic drugs in the effort of normalizing the intratumoral blood flow or with immunomodulating agents or engineered with cytokine receptors to enhance the trafficking (**C**).

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Overcome the subverted tumor vasculature

The first obstacle for CAR T cells in reaching the tumor bed upon intravenous infusion is represented by the subverted tumor blood and lymphatic vasculature. To overcome the aberrant tumor vasculature, CAR T cells can be combined with antiangiogenic drugs in the effort of normalizing the intratumoral blood flow (Fig. 1C). Administration of the anti-VEGF agent bevacizumab boosted the tumor infiltration and antitumor activity of CAR T cells targeting the GD2 antigen in an orthotopic xenograft model of neuroblastoma (51). Similarly, sub-pharmacologic concentrations of the tyrosine-kinase inhibitor with anti-VEGFR activity sorafenib enhanced the infiltration and antitu-mor activity of CAR T cells targeting GPC3 in a mouse model of hepatocellular carcinoma (52). In contrast, pharmacologic concentra-tions of sorafenib showed inhibitory effects on CAR T-cell prolifer-ation and cytotoxic activity suggesting that combinatorial strategies involving CAR T cells and kinase inhibitors with broad targeted specificity should be carefully investigated preclinically to avoid any deleterious effects on CAR T cells (53). T cells can also be directly engineered to target the tumor vasculature via targeting of the VEGF receptors (54). A phase I/II clinical study (NCT01218867) targeting VEGFR2 via CAR T cells in patients with metastatic solid tumors has been conducted at NCI. Treatment was well tolerated and even if no significant antitumor responses were observed, the study paves the possibility to develop a dual targeting strategy attacking both tumor vasculature and tumor cells.

Enhance trafficking of CAR T cells at the tumor site

CAR T cells show homing to lymphoid tissues upon intravenous infusion in patients with B-cell malignancies, while trafficking outside the lymphatic system and into solid tumors remains suboptimal. Several strategies are currently under investigation in the effort to induce an effective trafficking of CAR T cells at the tumor site (Fig. 1C). Classical cytotoxic drugs can have different immunomod-ulatory effects, and the choice of the right combination of cytotoxic drugs to be used within the lymphodepletion regimen prior to CAR T-cell administration may promote CAR T-cell trafficking (55). Doxo-rubicin and IL12 showed synergistic effects in promoting tumor homing of TILs in a xenograft mouse model of melanoma (56).

Combination of doxorubicin and IL12 induces the release of the chemokines CXCL9 and CXCL10 binding to the receptor CXCR3, which is highly expressed in T cells, and promoting their migration to the tumor site. As alternative to chemotherapy, other biological agents can be used to alter the TME to support a more favorable milieu to T-cell trafficking and persistence.

Oncolytic viruses selectively infect, lyse, and replicate in malignant cells, while leaving nonmalignant cells unaffected (57). These viruses thus represent an appealing platform to promote CAR T-cell migra-tion and survival within the TME (58). Similarly, physical modifica-tions of the tumor architecture that can be caused by local mild hyperthermia can promote the recruitment and effector function of CAR T cells (59). It is also possible to engineer CAR T cells to express the receptors of chemokines that are highly expressed by tumor cells. For example, some tumor cells produce the chemokine CCL2, but its receptor CCR2 is expressed at low lev-

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els on ex vivo expanded T cells. Promoting the expression of CCR2b in CAR T cells have been demonstrated to increase the trafficking of CAR T cells to the tumor (60). Finally, after ex vivo manipulation, CAR T cells also show reduced ability of degrading the extracellular matrix due to the lack of expression of heparanase, an enzyme that degrades the heparan sulfate proteoglycans. CAR T cells engineered to re-express heparanase effectively degrade the extracellular matrix, infiltrate the tumor, and exert more profound antitumor activity (61).

The field of CAR engineering has been so far dominated by the expression of CARs in activated T lymphocytes, but other cell subsets can be redirected against tumor cells via CAR gene transfer. In particular for the clinical setting of solid tumors, natural killer (NK) cells and classical natural killer T (NKT) cells, also known as invariant NKTs, possess unique properties such as enhanced trafficking at the tumor site and CD1d restricted cytotoxic activity for NKT cells and innate cytotoxicity activity against tumor cells for NK cells. Both NK cells and NKTs can be genetically manipulated to express CARs and acquire antigen specificity, while maintaining their innate proper-ties (62, 63). Phase I clinical studies with CAR-engineered NKTs or NK cells are currently ongoing (NCT03294954, NCT03579927, NCT03056339).

Coping with the Tumor Microenvironment

The TME is a complex network that comprehends the extracel-lular matrix and several nonmalignant cells such as fibroblasts, macrophages, and myeloid-derived suppressor cells (MDSC) that contribute to tumor progression and immune evasion (Fig. 2). The expression of inhibitory molecules, also known as inhibitory immune checkpoints, by tumor and stromal cells is one of the most important mechanisms impairing T-cell effector function. The neu-tralization of inhibitory immune checkpoints can be carried out by either combining CAR T cells with the systemic administration of immune checkpoint inhibitors or knocking down inhibitor receptors such as PD1 in CAR T cells (64, 65). Both strategies are currently under clinical investigation, and at the moment it is difficult to anticipate if selective blockade of inhibitory immune checkpoints in CAR T cells achieved by knocking down of PD1 is advantageous as compared with a more generalized blockade effect achieved with the systemic administration of immune checkpoint inhibitors. An alter-native strategy to counteract the PD1/PD-L1 axis is to revert the inhibitory signal of PD1 in T cells coupling PD1 extracellular domain with the signaling domain of costimulatory molecules such as CD28 (66). Phase I clinical trials with CRISPR-Cas9–mediated PD1 gene-knockout in anti-mesothelin CAR T cells or with a combination of anti-mesothelin CAR T cells and pembrolizumab are actually ongoing (NCT03545815, NCT02414269), as well as phase I studies with CAR T cells administered in combination with nivolumab alone or with nivolumab and ipilimumab in patients with glioblastoma (NCT04003649, NCT03726515).

Physiologically, the interaction between the receptor Fas (CD95) on T cells and its ligand FasL (CD95L) represents an immune homeostasis mechanism rapidly leading to T-cell apoptotic death. FasL can be overexpressed in the TME as an immune-escape mechanism and the disruption of the Fas/FasL pathway by engi-

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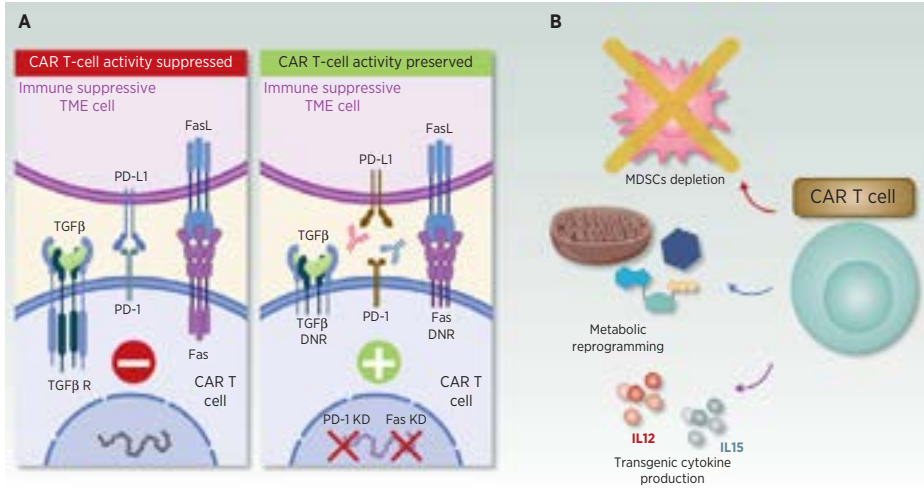

Figure 2.
Strategies to counteract the immune suppressive tumor microenvironment. Neutralization of inhibitory mechanisms can be carried out by either knocking down specific receptors in CAR T cells such as PD1 or Fas, by engineering CAR T cells to express dominant negative receptors (DNR) and by combining CAR T cells with the systemic administration or transgenic production of immune checkpoint inhibitors (**A**). Other strategy to overcome the immune suppressive tumor microenvironment consist in MDSCs depletion, metabolic reprogramming of CAR T cells, and transgenic cytokine production (**B**).

neering CAR T cells to express a dominant negative receptor, as well as by Fas knockdown, showed promising results in preclinical models (67, 68).

One of the main soluble inhibitory factors within the TME is the TGFb, produced by cancer cells as well as by regulatory T cells (Treg), fibroblasts, macrophages, and platelets that inhibits T-cell prolifera-tion and effector function. TGFb signaling in T cells can be blocked by engineering T cells to express a dominant-negative TGFb receptor and this strategy has been already safely tested in a clinical study using virus specific T cells (69). A similar approach can be applied to CAR T cells and is actually under clinical investigations in patients with metastatic prostate cancer using anti-PSMA CAR T cells (NCT03089203).

The TME is also characterized by nutrient deprivation and the presence of catabolites that impair persistence and effector function of T cells. Metabolic reprogramming is emerging as a strategy to enhance the functionality of CAR T cells within the metabolically disrupted TME of solid tumors, and genetic manipulations of glucose or acetate metabolisms in adoptively transferred T cells showed promising results in preclinical models (70, 71).

Among the immunosuppressive catabolites that are enriched in the TME, adenosine has been well characterized. Adenosine inhibits effector functions by engaging the cell-surface receptor A2AR in T cells. A2AR blockade showed the potential to profoundly increase CAR T-cell efficacy in mouse models of HER2þ solid tumors, partic-ularly when combined with PD-1 blockade (72).

Cellular components of the TME such as MDSCs are considered major players in hampering immune responses. Depleting MDSCs with a nanoformulation of auroyl-modified gemcitabine improved CAR T-cell efficacy in preclinical models of solid tumors (73). MDSCs can also be effectively targeted by blocking GM-

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CSF, and the com-bination of anti-GM-CSF agents with anti-CEA CAR T cells was effective in a mouse model of colorectal cancer with liver metasta-ses (74). Engineering T cells to release cytokines that reshape the TME, such as IL12 may reduce the inhibitory effects of M2-type tumor-associated macrophages and Tregs. Unfortunately, clinical experience with TILs engineered to express IL12 in response to NFAT in melanoma patients did not prevent the occurrence of the toxic effects caused by IL12 in human subjects (75). However, a clinical trial with anti-MUC16 CAR T cells engineered to secrete IL-12 is currently ongoing in patients with MUC16-positive solid tumors (NCT02498912).

Cytokines such as IL15 that do not have direct effects on the TME may however play a critical role in sustaining expansion, survival, and function of CAR T cells and partially compensate the inhibitory effects of the TME (76). IL15 expressing CAR T cells are currently under investigation in patients with liver cancer targeting glypican-3 (NCT04093648) and in patients with neuroblastoma targeting GD2 (NCT03721068).

Conclusion

Targeting solid tumors with CAR T cells in the clinical setting remains challenging. Since its first conception in late eighties, the strategy of using chimeric molecules as functional receptors with antibody-type specificity to arm T cells against solid tumors has clearly evolved. Nowadays CAR T cell-based therapy encompasses sophisti-cated genetic engineering techniques and combinatorial approaches to counteract biological barriers, tumor heterogeneity and immunosup-pressive properties of the TME. Nevertheless, further hypothesis-driven research is needed to speed up the preclinical development and fill the gap in the clinic between CAR T cell-based treatment in hematological malignancies and solid tumors. Moreover, new strat-egies are rapidly emerging for the redirection of T cells against solid tumors with the premise of a better safety profile, as in the case of CD3-bispecific engagers (BiTE) that allow cytotoxic killing of tumor cells by patients T cells without ex vivo manipulation (77). Optimal strategies to pursue among TILs, CAR T cells, and BiTEs are likely to be tailored to different clinical settings and tumor types.

As outlined in this review article, only translational, cooperative, and interdisciplinary efforts including clinical oncologists, immunol-ogists, and biomedical engineers would succeed in decoding and effectively implement strategies to address the complex pathophysi-ology of solid tumors and immune surveillance.

Disclosure of Potential Conflicts of Interest

G. Dotti and B. Savoldo hold patents in the field of T-cell engineering. B. Savoldo is a paid consultant for Tessa Therapeutics, and reports immediate family members who have received commercial research grants from Bluebird Bio, Cell Medica, and Bellicum Pharmaceuticals. G. Dotti is a paid consultant for MolMed s.p.a, Bellicum Pharmaceuticals, and Tessa Therapeutics, and reports receiving commercial research grants from Cell Medica. No potential conflicts of interest were disclosed by the other authors.

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New developments in adoptive cancer immunotherapy

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In recent years, various therapeutic strategies have been developed and evaluated in clinical trials aimed at preserving the beneficial effects of donor lymphocytes, such as GVL and immune reconstitution, while minimizing the price of GVHD.

Most of these approaches involve the infusion of cells, collected and in some cases selected, expanded and / or genetically modified, under conditions of Good Manufacturing Practice (GMP). Among these, there are the selective infusion of specific donor lymphocytes for viral antigens or for minor histocompatibility antigens expressed by leukemic cells. An alternative is represented by the infusion of polyclonal populations of lymphocytes depleted of alloreactive specificity. Recently, the role of lymphocytes with regulatory activity, potentially capable of modulating alloreactivity, has also begun to be studied. Alternatively, it is possible to introduce a suicide gene into donor polyclonal lymphocytes, to confer selective sensitivity to a drug. With this strategy it is possible to infuse patients with a wide lymphocyte repertoire, being able to selectively eliminate the cells in case of GVHD. Finally, the role of immunomodulating mesenchymal cells infused to prevent or treat GVHD is being evaluated.

Gene therapy, or the transfer of one or more genes into human cells for therapeutic purposes, is a relatively new procedure, tested in clinical trials since the 1990s. Today there are more than 1300 clinical studies, mainly phase I-II, active in about 30 countries on neoplastic, genetic, infectious and degenerative diseases. The manipulation of cells affects somatic cells in humans and causes genotypic and phenotypic modifications of the cell itself and of the tissues generated by them. For obvious ethical and social reasons, manipulation is limited to somatic cells and does not involve germ cells. The possible applications of these principles are congenital and neoplastic diseases and congenital and acquired immunodeficiencies.

The milestones for gene therapy are as follows:

- isolation of the gene to be transferred;
- introduction of the gene into vectors;
- the transfer of the gene of interest into somatic cells can be performed directly in vivo, through the infusion of the vector to the patient, or, more frequently, in vitro, through the exposure of the patient's cells (or healthy cells from a donor, in some cases) to the transfer carrier. In vitro manipulation requires GMP working conditions and numerous quality control tests before infusing the ge-

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netically modified cells into the patient. In some cases, once the gene transfer has taken place, the genetically modified cells are selected, in order to enrich the cell population infused to the patient, in corrected cells.

There are numerous hematological and non-isolated congenital disease genes cloned thanks to molecular PCR techniques. The elements of greatest difficulty today are represented by the functional transfer of genes with complex regulation, such as the globin gene, or of large dimensions, such as the dystrophin gene.

For insertion it is necessary to have vectors, capable of binding the gene (transgene) and transferring it within the cell. The vectors are of two kinds, viral and non-viral. The first are represented by retroviruses, lentiviruses, adenoviruses and adeno-associated viruses (AAV, Adeno-Associated Virus). Since these are pathogenic viruses, the original genome of the viral vectors must be modified and made unable to replicate within the target cell, damaging it. The structural genes are replaced by the genes to be transferred and those that allow their integration and transcription are left intact. The result, in the case of retroviruses and lentiviruses, is a defective virus, capable of integrating and expressing its genome in that of the host cell, but unable to replicate itself and give rise to new viral particles because it is deprived of the necessary genetic information. Non-viral vectors are mainly represented by liposomal particles or directly by DNA or RNA introduced by electroporation.

Clinical applications are summarized in Table 62.8. In some pathologies, such as immunodeficiencies due to adenosine deaminase deficiency or γ-chain receptor deficiency, the clinical application of gene therapy has already shown complete and

Table 1 - Gene Therapy clinical applications

Clinical applications	Diseases	Clinical aims
Congenital disorders	Immunedeficiencies Metabolic disorders Tesaurismosis Amaurosi Leber Epidermolisis bollosa	Enzimatic levels activity for disease correction
Hematology	Hemoglobinopahies Haemophilia Fanconi anemia	Transfusion independence
Allogeneic HSCT	GVHD	GvHD control by suicide-gene activation
Oncology	Soldi tumors Leukemia Lymphoma Multiple Myeloma	Anti-tumor response
Infectious diseases	HIV EBV and others pathogens	Anti-viral response
Neurology	Inflammatory and degenerative disorders	Disease progression control
Cardiology	Ischaemic and inflammatory myocardial disease	Disease progression control

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lasting clinical responses. In the case of immunodeficiency due to γ-chain receptor deficiency, however, undesirable effects were also observed. It has been seen that, if the integration of a retroviral vector containing the γ-chain gene (which codes for a receptor for growth factors) occurs near the oncogene LMO2 in hematopoietic stem cells, it can transform the cells and give to leukemic transformation pictures. We are therefore working on the development of new, safer vectors for this type of pathology.

Therapeutic protocols are underway for some congenital haematological diseases, such as chronic granulomatous disease, Fanconi's anemia, Wiskott-Aldrich syndrome, haemophilia, transfusion-dependent β-thalassemia.

Among the applications in acquired pathologies, possible objectives are:

– activation of tumor suppressor genes (ie insertion of the p53 gene);
– increase in tumor immunogenicity through the insertion of genes that encode tumor specific antigens (TSA), identified by cytotoxic lymphocytes infiltrating the tumor or by genes that induce tumor cells to produce cytokines that amplify the antitumor immune response, such as IL-2, TNF-α and IFN-γ;
– gene modification of T lymphocytes with the insertion of a T cell receptor (TCR) or a chimeric antigen (CAR) that confers specific cytotoxicity for a tumor antigen.

New approaches to immunotherapy

The last few years have been characterized by the introduction of new personalized cancer therapies that act directly on the patient's immune system to make it able to recognize and destroy cells.

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Diffuse large B-cell lymphoma and Primary mediastinal B-cell lymphoma

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15

CAR T cells: beyond tumour targeting...

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Paediatric haematology and oncology are a prime example of the success of translational interdisciplinary research that has dramatically improved the outcome for children with cancer. Today the 5-year survival rates of these children have risen to ~80%, in most high-income countries, and only ~30% in lower-to-middle-income countries (1-3). This progress reflects the optimized use of conventional therapies through better risk stratification and the introduction of innovative immunotherapeutic approaches. Although selected patient groups require less invasive procedures and abbreviated courses of chemotherapy, most paediatric patients need intensive systemic and multimodal interventions that may cause unavoidable long-term toxicity and an increased secondary malignancy rate (4). Despite considerable progress in the first-line therapy, relapses of paediatric cancers still carry a dismal prognosis. Therefore, the development of new, more efficient and less toxic therapies is an urgent clinical need. Besides novel molecular medicines, immunotherapeutic approaches represent a highly innovative and promising field in cancer treatment. The repertoire of immunotherapeutic drugs is broad and comprises different monoclonal antibodies to target immune or cancer cells, therapeutic vaccines to induce antitumor immune responses, oncolytic viruses, artificial proteins crosslinking tumour and immune cells, or most recently genetically engineered immune cells that engage in an enforced interaction with cancer cells. Importantly, resistance to conventional therapies does not appear to confer resistance to immune-based therapies (5). To combine the beneficial effects of both humoral and cell-mediated components of the antitumour response, T lymphocytes can be genetically modified to express:

a) chimeric proteins known as chimeric antigen receptors (CARs) that combine the antigen-binding specificity of a monoclonal antibody with the effector endodomain of the CD3/T cell receptor (TCR) complex (ζ chain) (6) or
b) HLA-bound tumour peptides (so-called TCR transgenic T cells). Both modes of gene transfer lead to T cells with the capability of an enforced immune-tumour cell contact and superior tumour cell killing capacities.

Dramatic anti-tumour activity is observed in patients treated with CD19. CAR-T cells for B-cell malignancies, such as acute lymphoblastic leukaemia and non-Hodgkin lymphomas, which recently has been translated into commercially available drugs (Kymriah - Novartis, Axicabtagene Ciloleucel - Kite Pharma, Brexucabta-

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gene Autoleucel - Kite Pharma, Lisocabtagene Maraleucel - Juno Therapeutics and Idecabtagene Vicleucel - Bluebird Bio/BMS). However, challenges to achieve similar effective responses in patients with relapsed solid tumours are still considerable (7, 8). These challenges are due to several factors that are currently under intense investigation:

1. Tumour heterogenicity and different tumour-antigen expression (9, 10);
2. composition and characteristic of tumour microenvironment (TME) (11-13);
3. development of immune-escape mechanisms during treatment (14, 15);
4. invasion, persistence and fitness of the CAR T cells (16, 17);
5. loss of metabolic plasticity of CAR T cells (18, 19);
6. CAR-T cell design (20, 21).

T cells are currently the major cellular platform for CAR. However, since normally they are harvested from the patient's blood (autologous use) their fitness is compromised from the intensive and prolong treatments. Besides T cells, a second group of immune cells, the Natural Killer (NK) cells are currently under investigation as a second cell type with the potential to be armed by a CAR receptor. These cells are, in fact, a crucial component of the innate immune system and play an important role in the host response against viral infections and cancers (22). They have the capacity to work as "serial killers", eliminating multiple targets without requiring antigen recognition and are efficient producers of soluble factors important for regulating both innate and adaptive immune responses (23). During cancer progression, malignant cells often activate several escape mechanisms including reduction, or even loss of the expression of HLA-class I molecules on their surface, thus evading T cell recognition and killing, while still allowing activation of NK-mediated killing. The implementation with CAR represents a promising approach to further enhance NK cell function against tumours and prolong their persistence in vivo. Moreover, since NK cells do not cause graft-versus-host disease, they represent an ideal off-the-shelf and ready-to-use product for the treatment of solid as well as hematologic malignancies, which potentially eliminates the challenges of patient-specific products that plague current CAR-T cell therapies (24).

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T cell subset selection for CAR engineering

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Immunotherapies based on the adoptive transfer of T cells engineered with chimeric antigen receptors (CAR) are revolutionizing medical oncology. While these treatment modalities have demonstrated remarkable activities against hematologic malignancies, their efficacy against solid tumors remains limited. Most of CAR T cells in the market and under clinical evaluation are manufactured from unselected T cell populations isolated from patients' or donors' peripheral blood, resulting in phenotypically and functionally heterogeneous cell products. It is now well established that the differentiation state of transferred T cells is a critical parameter influencing treatment efficacy. Specifically, CAR products enriched in early memory T cells endowed with stem cell-like attributes have been associated with successful antitumor responses across several studies. Thus far, the clinical exploitation of stem-like T cells has been hindered by their relative paucity in the circulation and the lack - until recently - of robust, clinical-grade manufacturing protocols capable of generating and maintaining this cell type *in vitro*. These strategies rely on programming and redirecting stem-like T cells from enriched naïve precursors through pharmacologic or genetic induction of stem cell pathways. Reprogramming strategies to de-differentiate short-lived effector subsets into stem-like T cells are also under development. Conferring stemness to antitumor CAR T cells might unleash the full potential of cellular therapies.

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