Review

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The Role of Epitope/Antigen Spreading in CAR T-cell Immunotherapy

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Immunotherapy using chimeric antigen receptor (CAR)-engineered T-cells has proven transformative in the management of selected haematological malignancies. However, it remains much less effective against solid tumours. In part, this is due to heterogeneity of antigen expression and the profoundly immunosuppressive nature of the tumour microenvironment. Epitope spreading entails the diversification of the immune response, allowing primed T-cells to target additional epitopes within the same antigen (intra-molecular) or a distinct antigen (inter-molecular; also known as antigen spreading). The occurrence of epitope spreading has been described in some immune competent mouse models and clinical studies of CAR T-cell immunotherapy. Interferon γ and granulocyte macrophage colony-stimulating factor have been implicated in this process through the recruitment and activation of antigen-presenting cells and T-cells. However, epitope spreading is not universally observed following CAR T-cell immunotherapy, prompting efforts to boost this. Accordingly, CAR T-cells have been engineered to produce a range of immunostimulatory factors. Moreover, alternative host cells including natural killer (NK) T-cells and macrophages have been proposed to more efficiently support epitope spreading. Additionally, a range of combination treatment strategies have been evaluated. Thus, CAR T-cells have been co-administered with vaccines, radiotherapy, stimulator of interferon genes (STING) agonists, tumour-tropic bacteria, oncolytic viruses and lipid nanoparticles. Effective harnessing of epitope spreading offers the potential to overcome key limitations of CAR T-cell therapy for solid tumours, nullifying the impact of tumour antigen loss and heterogeneity. However, it may trigger clinically significant autoimmunity in some patients.

Keywords: CAR; epitope spreading; antigen spreading

Introduction

Immunotherapy using chimeric antigen receptor (CAR)-engineered T-cells has greatly facilitated the effective management of selected haematological cancers. However, efficacy against solid tumours lags far behind. A key challenge is heterogeneity of target antigen expression within solid tumours. Accordingly, mono- or oligo-specific CAR T-cells cannot engage the entire and varied landscape of malignant cells within the tumour microenvironment (TME). One strategy that may help to address this entails the harnessing of endogenous immune effector mechanisms, tantamount to the induction of "therapeutic autoimmunity".

Autoimmune responses typically begin with the recognition of immunodominant epitopes within autoantigens. Thereafter, responses frequently diversify to include additional cryptic and non-cross reactive epitopes—a process known as antigen or epitope spreading [1]. Epitope spreading requires the uptake, processing and presentation of antigens by professional antigen-presenting cells (APCs), a process that requires tumour cell death. Cross-presentation is believed to be of particular importance whereby exogenous antigens are ingested by APCs, degraded and then loaded onto major histocompatibility complex (MHC) class I molecules [2]. Epitope spreading encompasses both intra-molecular and inter-molecular (also known as antigen spreading) forms (Fig. 1). In the former, APCs are believed to uptake antigen and present cryptic epitopes from the same molecule to T-cells, a concept first demonstrated in the autoimmune disease model, experimental autoimmune encephalitis [1]. In that setting, initial T-cell responses were focused on a single immunodominant epitope, followed thereafter by reactivity against several additional peptide epitopes within the same molecule. By contrast, inter-molecular spreading is believed to depend upon T-cell responses to a dominant epitope within a multiprotein complex that enables the subsequent priming of responses against discrete protein antigens. This process was elegantly demonstrated for both T-cell and antibody responses

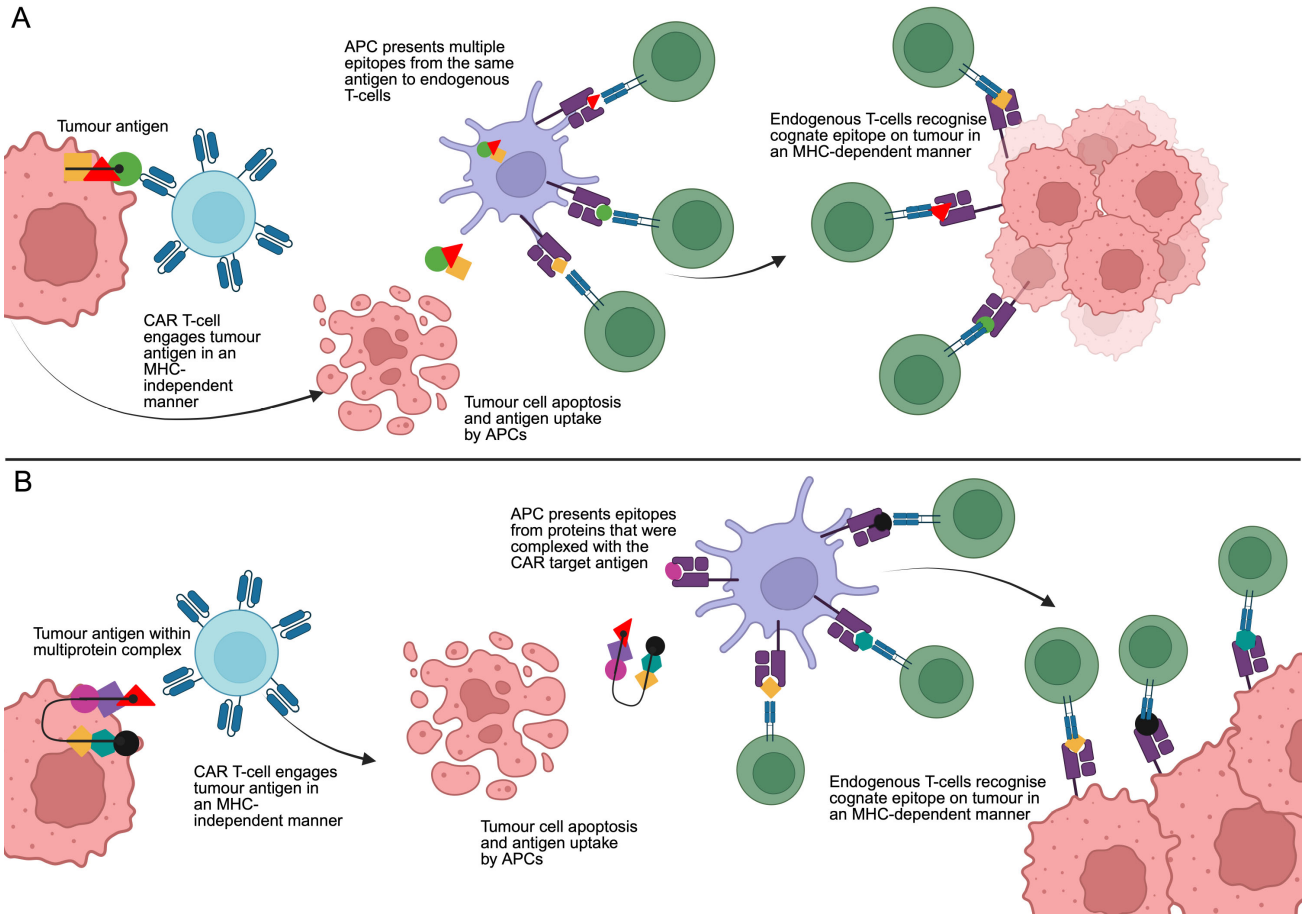

Fig. 1. Putative mechanisms of chimeric antigen receptor (CAR) T-cell induced epitope spreading. (A) Intra-molecular epitope spreading. A CAR T-cell is shown engaging a native cell surface tumour antigen containing three epitopes, denoted by a green circle, red triangle and yellow square. The CAR engages the green circular epitope triggering T-cell activation, tumour cell apoptosis and uptake of released tumour antigen by professional antigen-presenting cells (APCs). In some circumstances, this facilitates APC presentation and/or cross-presentation of other epitopes (e.g., red triangle/yellow square) in a major histocompatibility complex (MHC)-dependent manner to endogenous T-cells that express cognate T-cell receptor. If the tumour also displays these MHC-dependent epitopes, it may be recognised by these endogenous T-cells. If the APC is a B-cell, antibody may also be produced against these additional tumour-associated epitopes. (B) Inter-molecular epitope spreading (antigen spreading) differs in that the CAR tumour antigen forms part of a protein complex, meaning that epitopes derived from different molecules within the complex may similarly be presented by APCs. In turn, this may lead to MHC-dependent tumour recognition by endogenous T-cells.

(via western blotting and immunoprecipitation) against individual components of the small nuclear ribonucleoprotein complex [3].

A key question is which APC types drive epitope spreading. Batf3-dependent dendritic cells (DC) which efficiently cross-present exogenous tumour antigen onto MHC class I have been strongly implicated in this process in mouse models [4,5]. However, important roles have also been assigned to additional DC subtypes, macrophages and B-cells, which may also produce tumour-specific antibodies [6–9].

Epitope spreading has been identified in many processes including autoimmune disease [10], allograft rejection, allergy immunotherapy [11], tumour vaccination [2,12] and immune checkpoint therapy [13]. It represents one form of "bystander effect" which bolster the evolving immune response [14]. Additional distinct bystander mechanisms triggered by CAR T-cells include the induction of Fas-mediated apoptosis [15], activation of cluster of differentiation (CD)8^+ T-cells that express natural killer (NK) cell markers [16] and remote T-cell release of interferon (IFN)-γ within the TME [17].

Epitope Spreading in Pre-clinical Studies of CAR T-cell Immunotherapy

Several pre-clinical studies have provided evidence that epitope spreading can accompany CAR T-cell immunotherapy. Zhang *et al.* [18] targeted natural killer group 2 D (NKG2D) ligands using a CAR in which

full length NKG2D was fused to the CD3ζ endodomain (chNKG2D). They argued that this was accompanied by epitope spreading in several pre-clinical tumour models. Rauscher murine virus A (RMA) lymphoma cells are insensitive to chNKG2D T-cells since they do not naturally express NKG2D ligands. However, tumour cells acquired sensitivity to CAR-mediated destruction when engineered to express the mouse NKG2D ligand, retinoic acid inducible in embryonal carcinoma cells (Rae)-1 [18]. *In vitro* cytotoxicity of chNKG2D CAR T-cells against Rae-1$^+$ RMA tumour cells was dependent on perforin and Fas ligand, but not granulocyte macrophage colony-stimulating factor (GM-CSF) or IFN-γ [19]. Strikingly, however, *in vivo* efficacy was not diminished if CAR T-cells lacked either perforin or Fas ligand - but was abrogated or impaired if CAR T-cells were deficient in IFN-γ or GM-CSF respectively. Importantly, mice that were rendered free of Rae-1$^+$ RMA tumours by chNKG2D CAR T-cells also rejected subsequent re-challenge with parental RMA tumour cells, even though these tumour cells lacked the CAR target antigen. Conceivably this could have been due to an alternative bystander mechanism such as Fas-mediated killing (despite the lack of role of this pathway in primary tumour control), or a distant effect of remodelling of the primary TME. However, lymph node and spleen cells collected from surviving mice contained CAR negative CD4$^+$ and CD8$^+$ T-cells that produced IFN-γ when cultured with parental RMA tumour cells, but not an irrelevant control tumour cell line. Collectively, these findings are supportive of the induction of an antigen-specific memory response against tumour cells that lacked the CAR target—i.e., epitope spreading. Evidence would have been strengthened with additional studies using MHC blocking antibodies, enzyme linked immunosorbent (ELIS)pot analysis and immune deficient/knockout host mice.

Similar findings were reported using an intraperitoneal ID8 mouse ovarian tumour model, in which Rae-1 is naturally expressed [20–24]. Tumour-bearing mice achieved long-term disease-free status following repeated intraperitoneal chNKG2D T-cell dosing, although CAR T-cell persistence was shorter than 7 days. Therapy resulted in perforin-dependent killing of tumour-associated regulatory T-cells (Treg; which express NKG2D ligands) and conversion of the TME from immunosuppressive to immunostimulatory. Once again, disease control absolutely required CAR T-cell-derived IFN-γ and was reduced if they lacked GM-CSF or perforin. Importantly, host anti-tumour immunity was also mobilised, indicated by intratumoural (i.t.) influx of activated NK cells, DCs, nitric oxide-producing macrophages CD4$^+$ T-cells and CD8$^+$ T-cells. These changes were all dependent on CAR T-cell derived IFN-γ and GM-CSF and host IFN-γ receptor expression, all of which conspired to activate myeloid APC function and release of the T-cell chemokines, cysteine-x-cysteine ligand (CXCL)9 and CXCL10. Increased production of IFN-γ and other pro-inflammatory cytokines by endogenous CD8$^+$ T-cells, NK cells and, to a lesser extent, CD4$^+$ T-cells was demonstrable in the spleen and tumour over several weeks. Once again, this host immune response was dependent on the triumvirate of CAR T-cell derived IFN-γ, GM-CSF and perforin. Cross-presentation of tumour antigens was indicated by the ability of splenic and lymph node APCs to stimulate tumour antigen-specific T-cells. Both endogenous CD4$^+$ and CD8$^+$ T-cells contributed to tumour elimination and memory responses. Accordingly, tumour-free mice rejected re-challenge with inoculated derived (ID)8 tumour cells and splenic CD8$^+$ and CD4$^+$ T-cells from cured mice produced IFN-γ and tumour necrosis factor (TNF)-α when exposed to ID8, but not RMA control cells. The occurrence of epitope spreading also explains how chNKG2D CAR T-cells controlled ID8 (and RMA) tumours in which some tumour cells lacked Rae1 expression [25]. Remarkably, tumour control was still evident if as few as 7% of ID8 tumour cells expressed Rae1. Host immune cell requirements for efficacy included the production of IFN-γ, the presence of T-cells/ B-cells, peritoneal macrophages and a partial dependence on perforin, but not host GM-CSF.

Similar effects were later shown by the same group in a myeloma model, 5T33MM [26]. This was indicated by enhanced activation of endogenous T-cells and NK cells within the bone marrow and spleen. Mice also manifested increased serum IFN-γ (at a time when CAR T-cells had largely disappeared) and developed splenic CD4$^+$ and CD8$^+$ T-cells that produced IFN-γ when cultured with 5T33MM, but not irrelevant tumour cells. As a result, mice rejected re-challenge with 5T33MM cells but not an irrelevant tumour cell line.

Epitope spreading has also been reported following treatment with other CAR T-cell types, particularly in brain tumour models. Sampson *et al.* [27] showed that gliomas could be eradicated with high doses of CAR T-cells specific for the epidermal growth factor variant III (EGFRVIII) splice variant in a manner that required lymphodepletion using total body irradiation. Notably, cured mice were protected from re-challenge with tumour cells that lacked EGFRVIII. This was accompanied by an expansion of EGFRVIII-specific CAR T-cells, even though tumours were confirmed to lack this target. While mechanisms were not further explored, this speculatively raises the possibility that the CAR T-cells themselves contributed to EGFRVIII-independent recognition of tumour cells.

Subsequently, it was shown using two orthotopic glioma models that interleukin 13 receptor (IL-13R)α2-targeted CAR T-cell mediated cure was followed by protection against re-challenge with tumour cells that lacked IL-13Rα2 [28]. Similarly, established tumours in which 50% of cells lacked CAR target antigen could be eradicated via these mechanisms. This protective response was dependent on the initial treatment of 7 days (but not 4 days) tumours

in which host T-cell and myeloid cell influx was more established. The infiltrating leukocyte compartment was dramatically re-organised by CAR T-cell activation. This was indicated by evidence of activation and type 1 polarity of T-cell subsets and upregulation of APC-associated and IFN-γ stimulated gene sets in myeloid cells. Moreover, IFN-γ deficient CAR T-cells demonstrated poor efficacy in these models while anti-tumour activity was also blunted if IFN-γ producing CAR T-cells were transferred into mice lacking IFN-γR. Overall, these studies indicate a bidirectional positive feedback mechanism between CAR T-cells and host immune cells that is dependent on IFN-γ.

One intriguing CAR design that fostered epitope spreading in pre-clinical studies was described by Cieniewicz *et al*. [29]. They fused the T-cell/ transmembrane, immunoglobulin and mucin domain (TIM)-4 ectodomain to a trimodular endodomain comprising CD28 + CD3ζ + Toll/Interleukin-1 receptor (TIR)-1 domain of Toll-like receptor (TLR)2 [29]. TIM-4 binds to phosphatidylserine (PS), which is commonly over-expressed on transformed cells. As a result, T-cells which engaged PS were activated and displayed phagocytic and antigen cross-presenting function *in vitro*. Moreover, if TIM-4 CAR T-cells were co-cultured with a human papilloma virus $(HPV)^+$ squamous cell carcinoma cell line, they could subsequently present antigen and stimulate the human leukocyte antigen (HLA) class I-dependent activation of T-cells that expressed an E7 HPV epitope-specific T-cell receptor (TCR).

Despite these examples, CAR T-cell induction of epitope spreading has not been a universal finding in pre-clinical models. Klampatsa *et al*. [30] showed that mesothelin-specific CAR T-cells could eradicate tumours only if all cells were target antigen positive. It was concluded that "bystander effects", which could include epitope spreading—were negligible in this model. To rectify this, co-treatment with anti-programmed death (PD)1, anti-cytotoxic T-lymphocyte antigen (CTLA)4, CD40 agonist antibody or indoleamine dioxygenase inhibitor were all evaluated, but none enabled the eradication of antigen heterogeneous tumours. By contrast, a non-lymphodepleting dose of cyclophosphamide enabled CAR T-cells to achieve cure, in a manner that was dependent on endogenous $CD8^+$ T-cells, but not basic leucine zipper ATF-like transcription factor (Batf)3-dependent DCs. In keeping with this, epitope spreading to enable the recognition of the model tumour antigen, ovalbumin (OVA), was demonstrated by tetramer staining. A mathematical model based on this paper and other pre-clinical animal models concluded that when tumours were heterogeneous for target antigen expression (e.g., $<$70% positive), therapeutic success was correlated with maximum cytotoxic activity of bystander cells within the TME, but was not influenced by CAR T-cell dose beyond a certain threshold number, or CAR T-cell number within the TME [31]. The relative importance of epitope spreading versus other bystander mechanisms warrants further consideration in this context. From a clinical perspective, such modelling predicts that CAR T-cell dose may be a poor predictor of outcome in antigen heterogenous solid tumours, unless tumour-infiltrating cytotoxic T-cell density is considered. However, species-dependent differences in tumour kinetics, immune exclusion and T-cell exhaustion between the mouse and human setting may further limit predictive value.

Barriers to Epitope Spreading

The inconsistent nature of CAR T-cell induced epitope spreading is likely contributed to by multiple factors. These include tumour-associated factors such as the production of immunosuppressive cytokines (e.g., transforming growth factor b), small molecules (e.g., adenosine) and infiltrating stromal cell types (e.g., cancer-associated fibroblasts, mesenchymal stromal cells, regulatory T-cells, myeloid-derived suppressor cells and tumour-associated macrophages) [32, 33]. Moreover, lymphodepletion is generally used to condition patients for CAR T-cell immunotherapy in order to facilitate expansion of the cells *in vivo* [34]. However, these agents are preferentially cytotoxic to the very cells required to mediate epitope spreading.

Engineering Strategies Designed to Potentiate CAR T-cell Mediated Epitope Spreading

Given the foregoing considerations, several efforts have been made to amplify this process using additional genetic engineering strategies, as summarised in Fig. 2A.

Interleukin 12

A number of cytokines have been implicated in the induction of epitope spreading, including the potent pro-inflammatory cytokine, IL-12. Although lymphodepletion is typically required for efficacy, this requirement was obviated for IL-12-armoured CD19 CAR T-cells when tested in an immunocompetent mouse lymphoma model [35]. Activity was absolutely dependent on IFN-γ production (a key downstream mediator of IL-12) by the CAR T-cells. These findings were confirmed in a different lymphoma model in which splenocytes from cured mice lacked CAR T-cells, but nonetheless contained tumour-reactive T-cells, as demonstrated by IFN-γ ELISpot [36]. Transfer of splenocytes from these mice into lymphoma-bearing third party mice resulted in disease eradication in the majority of cases. These findings strongly implicate epitope spreading in this protective response.

An important concern with IL-12 armouring is risk of severe and sometimes lethal toxicity, as was seen when this cytokine was administered systemically to cancer patients [37]. Indeed, a clinical trial in which *ex vivo* expanded tumour-infiltrating lymphocytes were engineered to

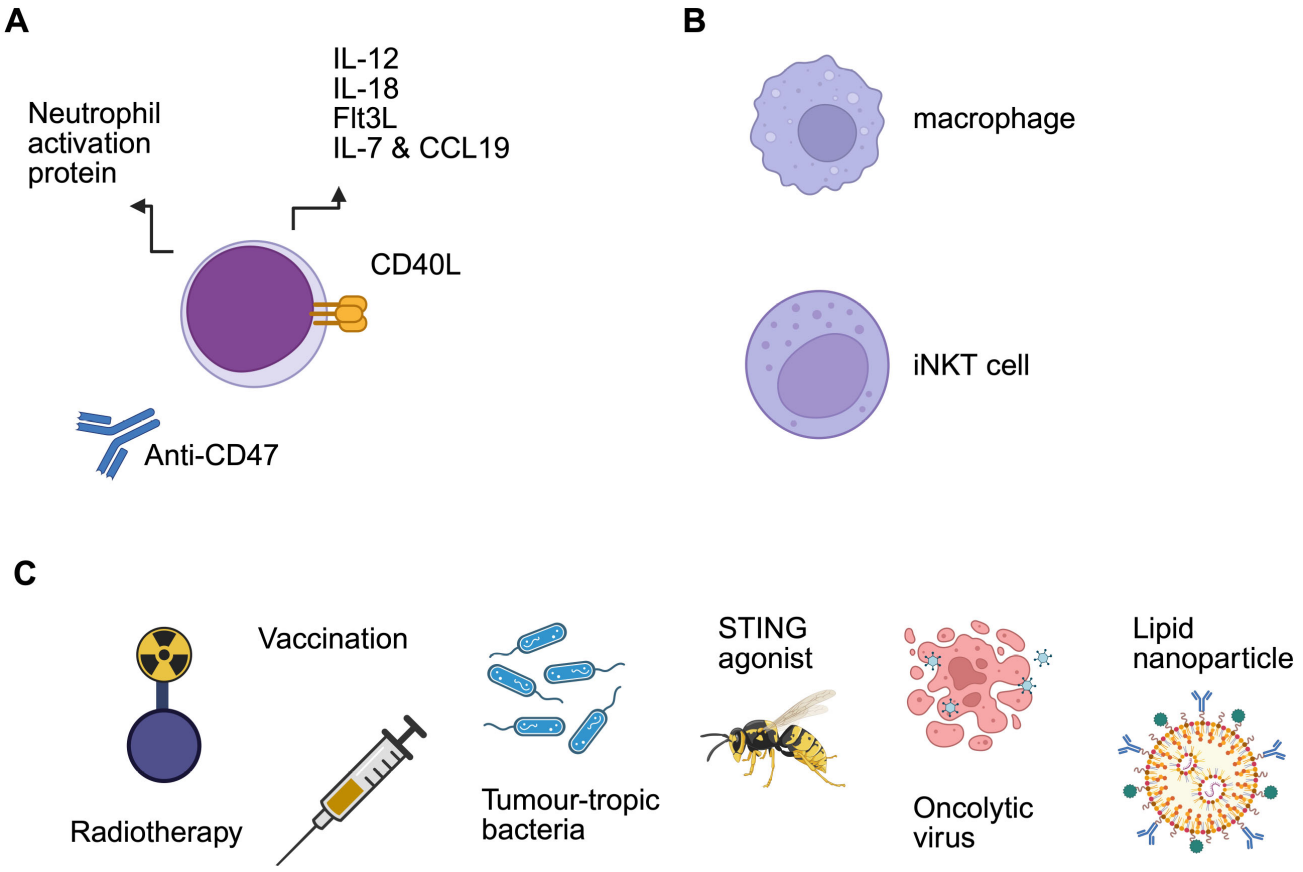

Fig. 2. Strategies to boost the impact of epitope spreading in CAR T-cell immunotherapy. Three broad strategies are currently being employed. (A) CAR T-cells may be genetically armoured to produce factors which are known to stimulate the growth and activation of APCs and to promote epitope spreading. (B) Some evidence indicates that alternative host cells for CAR engineering offer even greater potential to foster epitope spreading when compared to T-cells. (C) Combination approaches that involve the co-administration of CAR T-cells with a second therapeutic modality can stimulate this process under some circumstances.

produce IL-12 under the control of a nuclear factor of activated T-cells (NFAT) promoter was also marred by unacceptable toxicity [38]. To mitigate this risk, tumour-specific T-cells were engineered by mRNA electroporation to transiently produce IL-12 [39]. When delivered using the i.t. route, this intervention safely potentiated the efficacy of tumour-specific T-cells, with induction of abscopal tumour regression that was mediated by epitope spreading.

An alternative system to safely combine systemically administered CAR T-cells with IL-12 involves the use of tumour-targeted derivatives of this cytokine. One such strategy entails the fusion of a collagen-binding domain (CBD) derived from the von Willebrand factor A3 domain to each of the p35 and p40 subunits of IL-12 [40]. Owing to the disordered vasculature of solid tumours, collagen exposure is enhanced in solid tumours, leading to increased CBD IL-12 delivery and TME remodelling. Similar effects would be anticipated with other TME stroma-targeted delivery systems, such as antibodies targeted to fibroblast activation protein for example [41].

CD40 Ligand

One potent trigger for IL-12 release by DCs is the stimulation of CD40 on these cells by T-cell-associated CD40 ligand (CD40L) [42]. To harness this, CD19-specific CAR T-cells have also been engineered to constitutively express CD40L. Once again, efficacy of these armoured CAR T-cells was demonstrated against CD19-expressing lymphoma in the absence of lymphodepletion [43]. CD40L armoured CAR T-cells were shown to activate DCs in spleen and lymph nodes, but not the tumour. Using congenic CAR and non-CAR T-cells, it was further shown by IFN-γ ELISpot that endogenous MHC class I-restricted T-cells had been activated to undergo epitope spreading. As a result, mice that exhibited tumour control with CD40L-armoured CAR T-cells were protected against delayed rechallenge with CD19 negative tumour cells.

Interleukin 18

Given issues with safety surrounding IL-12, the functionally related cytokine, IL-18 has also been used to armour CAR T-cells. Interleukin 18 is produced as an inac-

tive precursor (pro-IL18) that acquires biological activity when the N-terminal pro-peptide is cleaved by caspase 1, an enzyme that is not highly active in T-cells. Consequently, the approach generally taken has been to fuse biologically active (i.e., cleaved) IL-18 downstream of a leader peptide. This directs the cytokine into the secretory pathway and, since the leader peptide is cleaved, IL-18 is released as a constitutively active cytokine. CAR T-cells engineered to secrete IL-18 have been shown to stimulate NK cells, NKT-cells, M1 polarised macrophages, DCs and endogenous memory CD8 T-cells within the TME, inducing epitope spreading involving other MHC class I-restricted cells (as determined by ELISpot) and protection against the effects of antigen loss [44]. Depletion of macrophages using liposomal clodronate compromised efficacy, emphasising the role of these APCs in efficacy. Recently, the first clinical trial of IL-18 armoured CAR T-cells was reported [45]. In contrast to clinical experience with IL-12 expressing T-cells, safety was acceptable with grade 3 cytokine release syndrome in only 14% of participants. Objective responses were seen in an impressive 81% of subjects, all of whom had relapsed following prior CAR T-cell immunotherapy. Analysis of translational samples collected in that study will provide an opportunity to evaluate whether IL-18 armouring is accompanied by epitope spreading in human cancer subjects.

Flt3 Ligand

CAR-T cells have been armoured to secrete the DC growth factor, Fms-like tyrosine kinase (Flt)3 ligand [46]. This led to expansion of intra-tumoural CD103$^+$ type 1 DCs and substantially increased T-cell activation when combined with the immune adjuvants, poly (I:C) and anti-4-1BB antibody. Combination therapy led to induction of epitope spreading against the model tumour antigen, OVA. Anti-tumour efficacy was also increased, despite preconditioning of tumour-bearing mice with a low dose of total body irradiation. Moreover, tumour re-challenge with cells that lacked either the CAR target (Human epidermal growth factor receptor (HER)2) or OVA resulted in delayed tumour growth.

Interleukin 7 and CCL19

Alternatively, CAR T-cells have been engineered to co-release IL-7 and CCL19 (7x19 CAR-T cells), leading to enhanced infiltration into solid tumours and boosted efficacy [47]. More recently, this strategy has been shown to increase cross-presenting DCs and induce epitope spreading in black 16 frequency 10 (B16F10) and methylcholanthrene induced (MC)38 tumour models [48].

Anti-CD47

A further engineering strategy targets the CD47 protein which imparts a “don’t eat me” signal, thereby compromising the potential uptake of tumour antigen by phagocytic APCs. Secretion of an anti-CD47 nanobody by PD-L1 specific CAR T-cells enabled epitope spreading in the B16F10 melanoma model, indicated by ELISpot analysis and occurrence of melanin loss in one tumour [49].

Neutrophil Activating Protein

Bystander T-cell responses against solid tumours have also been achieved when CAR T-cells were engineered to express a pro-inflammatory neutrophil-activating protein (NAP) from *Helicobacter pylori* [50]. This resulted in attraction and maturation of DCs within an immunologically hot TME, leading to enhanced tumour control and protection of cured mice from antigen negative tumour rechallenge. Splenocytes from cured mice produced IFN-γ when exposed to CAR antigen negative parental tumour. The occurrence of epitope spreading was further confirmed using OVA as a model antigen.

Use of Distinct CAR Cell Hosts to Trigger Epitope Spreading

Most CAR-based studies primarily employ conventional ab T-cells. However, a number of recent studies suggest that other cell types may also support the induction of epitope spreading (Fig. 2B).

CAR NKT-cells

CAR NKT-cells have been reported to outperform CAR T-cells when assessed for anti-tumour activity and the induction of epitope spreading in mouse models [51]. Potential mechanisms include DC activation within the TME and selective depletion of CD1d high M2 polarised tumour-associated macrophages (TAM)s by these cells. CD1d is the restricting element for the invariant NK TCR and was implicated in both mechanisms.

CAR Macrophages

Pre-clinical studies have demonstrated that CAR macrophages also induce epitope spreading [52]. Intratumoural delivery of HER2-specific CAR macrophages elicited a complete response in over a third of mice with HER2$^+$ CT26 tumours. All mice were protected from subsequent re-challenge with HER2 negative parental tumour cells, in contrast to naive mice. Depletion of T-cells resulted in loss of protection against re-challenge. CD8$^+$ T-cells reactive with the immunodominant antigenic peptide restricted by H-2^{ld} (AH1) peptide were induced, consistent with epitope spreading. Imaging studies indicated that CAR macrophages had resculpted the TME, indicated by influx and activation of T-cells, NK cells and pro-inflammatory macrophages. Efficacy was further enhanced by PD-1 blockade.

In a separate study, macrophages that expressed a fragment crystallizable (Fc) receptor γ-based CAR were armoured to secrete anti-CD47 nanobody-IgG2a Fc fusion

that had been silenced for Fc receptor binding and glycosylation [53]. This resulted in enhanced tumour cell phagocytosis and repolarisation of the TME in favour of immunostimulatory myeloid cells and T-cells with lowered immunosuppressive properties. Cross-presentation of tumour antigen on macrophage-associated MHC class I to $CD8^+$ T-cells was also evident. As a result, anti-tumour efficacy was potentiated and was accompanied by resistance to tumour rechallenge with tumour cells that lacked the CAR target antigen.

Combination Approaches to Foster CAR T-cell Mediated Epitope Spreading

Several combination approaches have been used to enable epitope spreading following CAR T-cell administration (Fig. 2C).

Vaccination

Vaccine administration has frequently been combined with CAR T-cell immunotherapy. Subcutaneous injection of amphiphile CAR-T cell ligands results in their delivery to and presentation on the surface of APC in the draining lymph nodes, providing a potent stimulus to circulating CAR T-cells [54,55]. Importantly, subsequent re-challenge with parental tumour cells that lacked the CAR T-cell ligand was rejected. Although CAR T-cell treatment was administered following lymphodepletion, epitope spreading was confirmed by IFN-γ ELISpot analysis of splenocytes when stimulated with parental tumour cells [54]. Efficacy of the vaccine was further boosted if combined with an adjuvant such as stimulator of interferon genes (STING) or Toll-like receptor (TLR) agonist. This combination resulted in priming of endogenous tumour reactive $CD4^+$ and $CD8^+$ T-cells and enhanced control of tumours that exhibited heterogeneous expression of the CAR target antigen [55]. Vaccine boosted CAR T-cells also exhibited enhanced metabolic fitness [55]. Blockade of either IFN-γ or IL-12 completely abrogated both efficacy and epitope spreading, once again implicating this type 1 cytokine axis in therapeutic impact. Interferon-γ originated both from CAR engineered (autocrine loop) and endogenous T-cells [55]. Indeed, the role of vaccination could be replaced in large part by the engineering of CAR T-cells to co-express NFAT-inducible IFN-γ, while addition of the vaccination protocol further boosted epitope spreading [55].

A novel strategy used to combine vaccination with T-cell engineering was described by Slaney *et al*. [56] who developed a transgenic mouse strain in which T-cells co-expressed both a HER2-specific CAR and gp100-specific TCR. Following lymphodepletion by total body irradiation, T-cells were infused into HER2 tumour-bearing mice alone or in combination with IL-2 and a vaccinia virus that encoded the gp100 antigen. The combination therapy controlled a range of tumours including orthotopic breast tumours and large liver tumours. Durable responses required Cysteine-x-cysteine motif receptor (CXCR)3 and exogenous IL2 but were independent of IFN-γ. Mice were resistant to tumour rechallenge, in keeping with induction of epitope spreading.

A recently described vaccination technology that has been used in combination with HER2-specific CAR T-cells is a self-assembling peptide hydrogel that had been loaded with HER2 peptides. Following CAR T-cell delivery within the hydrogel, endogenous humoral and cellular immune responses were also triggered in humanised mouse models of breast cancer [57].

Radiotherapy

Low dose fractionated radiotherapy has also been shown to induce abscopal systemic anti-tumour responses. When tested in an immune competent mouse lymphoma model, the combination of radiotherapy with CD19 CAR T-cell immunotherapy resulted in enhanced control of irradiated tumours in addition to shrinkage of non-irradiated tumours [58]. Mechanistically, this was accompanied by activation of activation of damage-associated molecular pattern (DAMP) pathways and the cGAS (cyclic guanosine adenosine synthetase)/STING pathway, chemokine release, infiltration of DCs within irradiated tumours and epitope spreading, indicated by tetramer staining studies. As a result, effector T-cells within non-irradiated lesions were also enhanced. Intriguingly, administration of the same dose of radiation as a single larger fraction promoted more traditional effects including mitotic catastrophe, apoptosis and senescence, rather than the immunomodulatory effects seen with dose fractionation.

Tumour Tropic Bacteria

A number of non-pathogenic bacteria colonise hypoxic solid tumours, including the facultative anaerobe, *Escherichia coli* K12 DH5α. To exploit this, DH5α bacteria were engineered to express IL-18 (resistant to IL-18 binding protein) in the outer membrane. When injected i.t., $CD8^+$ T-cell and NK cell responses were induced in tumours, accompanied by abscopal and recall tumour protection. Intratumoural delivery of these modified bacteria also enhanced the i.t. recruitment of mesothelin-specific specific CAR NK cells into a mesothelioma xenograft [59].

STING Agonist

The STING pathway represents a potent immunomodulatory system that may drive endogenous anti-tumour immune responses. Pre-clinical studies have investigated the combined use of the STING agonist, 2′3′-cyclic guanosine monophosphate-adenosine monophosphate (cGAMP) in combination with CAR T-cells [60]. Mice with prostate-specific membrane antigen- or gp75-expressing black (B)16 melanoma tumours were treated intravenously with antigen-specific CAR T-cells alone or followed by

i.t. cGAMP, making further comparison with cGAMP alone, or combined with irrelevant antigen control CAR T-cells. While STING agonist plus control CAR T-cells did elicit a small increase in tumour-reactive T-cells in both treated and untreated tumours, the antigen-specific CAR T-cell/cGAMP combination achieved best efficacy. This included abscopal shrinkage of tumours implanted in the opposite flank. Using CD45 congenic mice, investigators showed that the combination treatment increased influx of both CAR-expressing and endogenous T-cells within the tumour. Epitope spreading was indicated by the occurrence of vitiligo, circulating and i.t. TCR clonotypic alterations and amplification of T-cells with reactivity for endogenous tumour antigens (identified by tetramer staining) in blood, tumours and both draining and contralateral lymph nodes. Mechanistically, this was dependent on host STING signalling, Batf3-dependent DCs, perforin release and STING signalling in CAR T-cells.

Passive Tumour Sensitisation to CAR T-cells

A number of approaches have been undertaken to deliver CAR T-cell target antigens to tumour cells, with the added benefit of mobilisation of endogenous immune defences. An oncolytic orthopoxvirus was used to express CD19 in solid tumour cells, thereby sensitising them to CD19-specific CAR T-cell immunotherapy [61]. Using an immune competent MC38 colonic adenocarcinoma model, mice which were cured using this combination therapy were protected from subsequent challenge with parental MC38 cells, consistent with epitope spreading.

A more recent study describes the lipid nanoparticle-mediated delivery of synthetic antigens that are orthogonal to the human proteome, in order to mitigate risk of on-target off-tumour toxicity [62]. Investigators used a camelid single-domain (VHH) antibody as the synthetic antigen, thereby sensitising tumours to subsequent infusion of anti-VHH CAR T-cells. This approach also elicited epitope spreading, control of heterogeneous tumours and protection against tumour rechallenge.

Clinical Evidence of CAR T-cell Associated Epitope Spreading

A number of clinical studies have indicated that epitope spreading may occur in CAR T-cell treated patients although it should be acknowledged that evidence in support of this phenomenon is weak. This was first reported in two non-lymphodepleted subjects treated repeatedly with mRNA electroporated mesothelin-specific CAR T-cells (NCT01355965) [63]. Evidence of humoral epitope spreading was demonstrated in both cases (one of whom achieve a partial response), indicated by the appearance of novel anti-self antibodies. Antibodies detected included those targeted against mesothelin itself and other molecules implicated in neoplastic transformation (e.g., septin 6, p21-activated kinase) [63].

A related phenomenon was seen in a second mesothelin-specific CAR T-cell clinical trial, again involving the repeated administration of mRNA transfected cells to six subjects with pancreatic cancer (NCT01897415). Although stable disease was the best response achieved, a number of antibody reactivities directed against selected immune-related molecules appeared *de novo* or were up-regulated after treatment [8,64]. Antibody targets included molecules implicated in immunomodulation (e.g., PD-L1 [64]) and disease pathogenesis (e.g., epidermal growth factor receptor and HER 2) [8].

A child with metastatic rhabdomyosarcoma who achieved complete remission after multiple infusions of HER2-specific CAR T-cells also exhibited evidence of humoral epitope spreading. This was indicated by an increase in autoantibodies directed against several targets, including some that were implicated in tumour invasiveness and metastasis [65]. These alterations were accompanied by remodelling of the TCR repertoire, in keeping with the known T-cell dependence of IgG antibody responses.

Clinical evidence of epitope spreading was also observed in two subjects with mantle cell and follicular lymphoma respectively. Both achieved remissions that lasted over 7 years post CD20-specific CAR T-cell immunotherapy (NCT00621452), despite lack of robust CAR T-cell expansion or B-cell aplasia [66]. Both subjects only received cyclophosphamide but not fludarabine conditioning, which is known to be intensely lymphocytotoxic and thus may conceivably hinder epitope spreading. One subject had also received radiotherapy. The occurrence of epitope spreading was supported by positive ELISpot response (IFN-γ) to autologous tumour and anti-tumour antibody formation. Clonal TCR sequences were identified in one subject that were absent in the CAR product but increased in circulation and tumour close to the time of remission onset. These cells were also noted in close proximity to cross-presenting DCs in tumour biopsy material.

A recent analysis of data collected in the ZUMA-1 registrational clinical trial of axicabtagene ciloleucel (NCT02348216) is also consistent with the occurrence of epitope spreading in some patients. Long-term remission was associated with elevated native cytotoxic and proinflammatory effector T-cells and post-infusion expansion of effector memory T-cell clonotypes of ill-defined (perhaps anti-tumour) but nonviral specificity [67].

A patient with multifocal glioblastoma achieved a complete response of their disease following multiple locoregional infusions of IL-13Rα2-specific CAR T-cells (NCT02208362). This response was all the more impressive given that the tumour did not homogeneously express the CAR target antigen [68]. Endogenous CAR negative T-cells isolated from this subject demonstrated enhanced tumour-specific proliferation and IFN-γ production when

stimulated with an irradiated autologous tumour cell line that lacked IL-13Ra2 [28], once again pointing towards the occurrence of epitope spreading.

Despite these findings, epitope spreading has not been uniformly observed in clinical studies of CAR T-cell immunotherapy. In one study of lymphoma patients following CD19 CAR T-cell immunotherapy, no differences were observed in the frequency of precursor T-cells responding to a range of established tumour antigens [69]. Notably, patients were conditioned with fludarabine and cyclophosphamide which may have compromised the possible emergence of endogenous anti-tumour immunity. Similar negative findings were reported in non-lymphodepleted subjects with head and neck cancer treated with i.t. panErbB-specific CAR T-cells. No increase in ELISpot reactivity against MAGE-A3 or A4 tumour antigens was noted in any subject [70]. In this case, SPECT CT imaging demonstrated that T-cells did not penetrate beyond the site of injection, which may have hindered uptake of antigen by APCs.

Conclusions

Epitope spreading enables immune responses to achieve broadened specificity, thereby adapting to the heterogeneous expression of target antigens by tumours and facilitating the development of memory immune responses. Emerging clinical evidence suggests that the occurrence of endogenous immune anti-tumour responses, very likely involving epitope spreading, is a key determinant of clinical outcome following CAR T-cell immunotherapy. Interferon-γ has been consistently implicated as a key driver of this process in a number of preclinical studies. This cytokine is known to upregulate MHC expression [71,72], promote myeloid cell activation [73] and transiently induce immunoproteasome formation, which alters the immunopeptidome and potentially unmasks cryptic epitopes [74]. Moreover, IFN-γ can diffuse widely throughout the TME to mediate effects at a considerable distance from the CAR T-cell of origin [75]. Consequently, strategies that boost IFN-γ release such as armouring with IL-12, IL-18 or CD40 ligand have also been investigated in an attempt to potentiate this process.

A number of caveats should be noted in respect of the role of IFN-γ in epitope spreading. First, IFN-γ is a pleiotropic cytokine that exerts double-edged effects with respect to cancer immunity and has been implicated in the triggering of some immune escape mechanisms. These include the upregulation of PD-L1 and indoleamine dioxygenase, both by malignant and associated stromal cells in the TME [73]. Nonetheless, tumours may acquire mutations in genes that contribute to downstream signalling (e.g., Janus-associated kinase (JAK)1 or JAK2), highlighting that the IFN-γ pathway commonly remains the subject of negative selective pressure, even when tumours enter the escape phase of immune editing [73]. Second, IFN-γ exerts anti-tumour activity via alternative mechanisms to the promotion of antigen presentation and epitope spreading [76]. These include induction of tumour cell cytostasis, senescence and programmed cell death, in addition to inhibitory actions on tumour angiogenesis. To disentangle direct and indirect effects of IFN-γ on tumour growth, studies using compartment-specific IFN-γ receptor 1 knockout models would be useful.

A further important consideration is the fact that clinical evidence in support of CAR T-cell induced epitope spreading is considerably weaker than that seen using in bred mouse models. However, extrapolation of pre-clinical mouse data to humans is problematic. Murine APC subsets differ from human subsets, potentially over-estimating the contribution of Batf3-dependent DCs [77]. In addition, transplantable mouse tumours (e.g., ID8 ovarian carcinoma [78]) often carry a far simpler mutational landscape than human solid tumours.

Combination strategies also offer an interesting opportunity to harness epitope spreading for clinical benefit. However, i.t. delivery (e.g., STING agonists, vaccines) requires an accessible tumour lesion, is invasive and subject to difficulties in achieving widespread drug distribution within the tumour, while avoiding systemic leakage and off-target effects. Radiation may also elicit off-target toxicity in normal tissues in the field, although this can be mitigated using more focused delivery systems such as intensity-modulated radiotherapy. While vaccines combined with CAR T-cells may induce epitope spreading without prior lymphodepletion in some mouse models, clinical extrapolation is challenged by species-specific differences and the fact that lymphodepleted specific pathogen-free mice have a much smaller “immunological space” than human patients, which may inflate vaccine efficacy [79].

While T-cells are traditional hosts for CAR T-cell immunotherapy, alternatives such as iNKT cells and macrophages have been reported to more efficiently induce epitope spreading. Nonetheless, these host cells may impose additional challenges for clinical deployment, especially in respect of scalable clinical manufacture.

There is a clear need to better understand the mechanisms whereby CAR T-cells can trigger epitope spreading, how this may be influenced by intensity of lymphodepletion and which armouring strategies achieve an optimal balance between induction/ amplification of this process while minimising tumour immune counterattack mechanisms. Effective harnessing of epitope spreading offers great potential as a bridge to improved efficacy of CAR T-cell products against solid tumours. On a cautionary note, induction of B-cell and T-cell-mediated epitope spreading may elicit clinically significant autoimmunity, akin to toxicity commonly observed in patients treated with immune checkpoint blockade. Treading the fine line between tumour control and autoimmune disease may be the price to be paid for success.

Availability of Data and Materials

Not applicable.

Author Contributions

JM and KM contributed to the conception. Both authors were involved in drafting and critical revision and have read and approved the final manuscript. Both authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

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Conflict of Interest

J. Maher is Chief Scientific Officer, paid consultant and shareholder in Leucid Bio. Figs. 1,2 were created using BioRender (https://www.biorender.com/). The authors have no financial or personal relationship with BioRender, and the use of this tool does not imply any endorsement.

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