Immunogenicity of CAR T cells in cancer therapy

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Abstract | Patient-derived T cells genetically reprogrammed to express CD19-specific chimeric antigen receptors (CARs) have shown remarkable clinical responses and are commercially available for the treatment of patients with certain advanced-stage B cell malignancies. Nonetheless, several trials have revealed pre-existing and/or treatment-induced immune responses to the mouse-derived single-chain variable fragments included in these constructs. These responses might have contributed to both treatment failure and the limited success of redosing strategies observed in some patients. Data from early phase clinical trials suggest that CAR T cells are also associated with immunogenicity-related events in patients with solid tumours. Generally, the clinical implications of anti-CAR immune responses are poorly understood and highly variable between different CAR constructs and malignancies. These observations highlight an urgent need to uncover the mechanisms of immunogenicity in patients receiving CAR T cells and develop validated assays to enable clinical detection. In this Review, we describe the current clinical evidence of anti-CAR immune responses and discuss how new CAR T cell technologies might impact the risk of immunogenicity. We then suggest ways to reduce the risks of anti-CAR immune responses to CAR T cell products that are advancing towards the clinic. Finally, we summarize measures that investigators could consider in order to systematically monitor and better comprehend the possible effects of immunogenicity during trials involving CAR T cells as well as in routine clinical practice.

Chimeric antigen receptors (CAR) are fusion proteins that redirect T cell specificity towards surface molecules expressed on tumour cells independently of the conventional T cell receptor (TCR)–major histocompatibility complex (MHC) interactions. CARs are introduced into T cells through gene transfer[1,2]. The antigen-recognition domain most often consists of a mouse-derived monoclonal antibody as a continuous peptide single-chain variable fragment (scFv) steered through an extracellular spacer domain that provides flexibility. ScFvs engage with their target epitopes and confer activation signals through modular intracellular signalling domains. Currently, CAR T cells are generated ex vivo from peripheral blood-derived T cells, which are typically transduced with replication-deficient vectors that integrate the CAR expression cassette into the T cell genome. These CAR T cells are subsequently expanded to large numbers in culture. After infusion into patients, these cells can recognize and eliminate tumour cells expressing the target antigen.

Autologous (patient-derived) and, occasionally, allogeneic (donor-derived) CAR T cells have successfully progressed from preclinical to clinical development and are currently used to treat patients with cancer[3–5]. The most successful CAR T cell products to date, of which three have received FDA approval (tisagenlecleucel, axicabtagene ciloleucel and brexucabtagene autoleucel), target the B cell lineage antigen CD19. CD19-targeted CAR T cells have become an important treatment option for patients with acute B cell lymphoblastic leukaemia (B-ALL) or certain aggressive B cell non-Hodgkin lymphomas (NHLs), inducing complete remissions in a subset of heavily pre-treated patients with extensive disease. These CAR T cells are also associated with cytokine-release syndrome, immune effector cell-associated neurotoxicity syndrome and other immune-mediated adverse events[6]. To date, the success of CAR T cells in patients with haematological malignancies has not been replicated in patients with solid tumours owing to several barriers, which have

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Key points

- Pre-existing and/or treatment-induced immunity to chimeric antigen receptor (CAR) constructs containing mouse-derived single-chain variable fragments are associated with treatment failure in certain patients and might limit the success of redosing strategies.

- The possible effects of immunogenicity on CAR T cell persistence and function are currently poorly understood.

- Novel technologies designed to enhance CAR T cell performance and/or the application of allogeneic CAR T cells might further amplify the likelihood of anti-CAR immune responses, thus necessitating strategies to overcome such risks.

- Various monitoring, mitigation and management approaches can be used to reduce the risk of anti-CAR immunity, although validated assays enabling adequate assessments of anti-CAR immune responses remain an unmet need.

- We advocate for the inclusion of CAR-associated immunogenicity analysis in both preclinical and clinical investigations of CAR T cell therapy.

been reviewed in detail elsewhere[7,8]. Therefore, current efforts aim to increase the potency of CAR T cells and/or to combine them with other treatments designed to overcome these barriers.

CAR T cells have the potential to induce both humoral and cellular anti-CAR immune responses to non-self components of the CAR construct or to residual proteins originating from the gene-transfer vectors, which are inherently immunogenic (FIG. 1). This reaction might, in turn, contribute to limiting the efficacy and thus inhibiting the success of subsequently administered CAR T cells[9–11]. Furthermore, the scFvs of many CARs currently in clinical development are derived from mouse or other non-human monoclonal antibodies

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(Supplementary Table 1). Pre-existing antibodies that broadly recognize the scFvs of mouse immunoglobulins, called human anti-mouse antibodies (HAMAs), have been detected in subsets of patients[12,13]. Human or humanized scFvs can also contain non-self sequences because the variable binding fragments are generated through multiple gene recombination events and somatic hypermutation. Antibodies directed towards a specific antibody sequence (such as those contained in a human scFv) are known as anti-idiotype antibodies[14,15]. Moreover, the expression of proteins encoded by several human genes in a single peptide CAR chain creates fusion sequences at junctions that do not normally exist in humans. Thus far, no conclusive evidence exists that such anti-CAR immune responses contribute to other reported adverse events such as cytokine-release syndrome and immune effector cell-associated neurotoxicity syndrome; therefore, such toxicities are not the focus of this Review.

Cellular immune responses to genetically modified cytotoxic T cells have been documented in patients with cancer and in those with HIV, sometimes in association with immune rejection of the adoptively transferred T cells[9–11]. Particularly, T cells with specificity for the CAR transgene or residual proteins originating from gene-transfer vectors have been shown to deplete and inactivate the infused CAR T cells[10]. Similarly, the presence of humoral immunity and the development of antibodies to the CAR construct might interfere with CAR T cell activity by neutralizing the antigen-binding fragment and/or promoting early CAR T cell apoptosis[16]. A small risk of severe systemic anaphylaxis also exists owing to IgE-mediated HAMA-triggered mast cell degranulation after CAR T cell application in patients who are pre-sensitized to CAR T cells with mouse-derived scFvs and/or mouse-derived monoclonal antibodies[17,18] (FIG. 1). Nevertheless, the effects of CAR-specific immunogenicity on the clinical outcomes after CAR T cell infusion remain both poorly understood and poorly investigated. Therefore, as new CAR constructs are developed to improve efficacy and persistence, understanding the origins and mechanisms of CAR T cell immunogenicity remains crucial, particularly in patients with solid tumours. Herein, we review published clinical data on immune responses to CAR T cells in patients with haematological or solid malignancies and discuss the strategies to investigate, mitigate and manage the risk of immunogenicity at different stages of CAR T cell product development.

Clinical evidence of anti-CAR immunity

Antibodies to CD19-directed CARs do not appear to impair initial clinical responses. Pre-existing humoral immunity to CD19-specific CAR T cells has been observed with both tisagenlecleucel and axicabtagene ciloleucel[19–22]. The vast majority of patients with B-ALL (84.6%; $n = 88$)[22] who received tisagenlecleucel had pre-existing anti-CAR antibodies, with a similarly high percentage (91.4%) reported in patients with refractory diffuse large B cell lymphoma[19]. Treatment-emergent immunogenicity, defined as an increase in anti-CAR antibodies following infusion, has been reported in

5% and 36.7% of patients with diffuse large B cell lymphoma and B-ALL, respectively[19,21]. Far fewer patients receiving axicabtagene ciloleucel have been reported to have pre-existing anti-CAR antibodies (3%; $n = 94$)[20], while no pre-existing anti-CAR antibodies have been confirmed in patients receiving brexucabtagene autoleucel using a combination of two different assays[23]. All three of these products contain the same mouse-derived scFv (FMC63); therefore, the differences in the reported percentages of patients with pre-existing antibodies
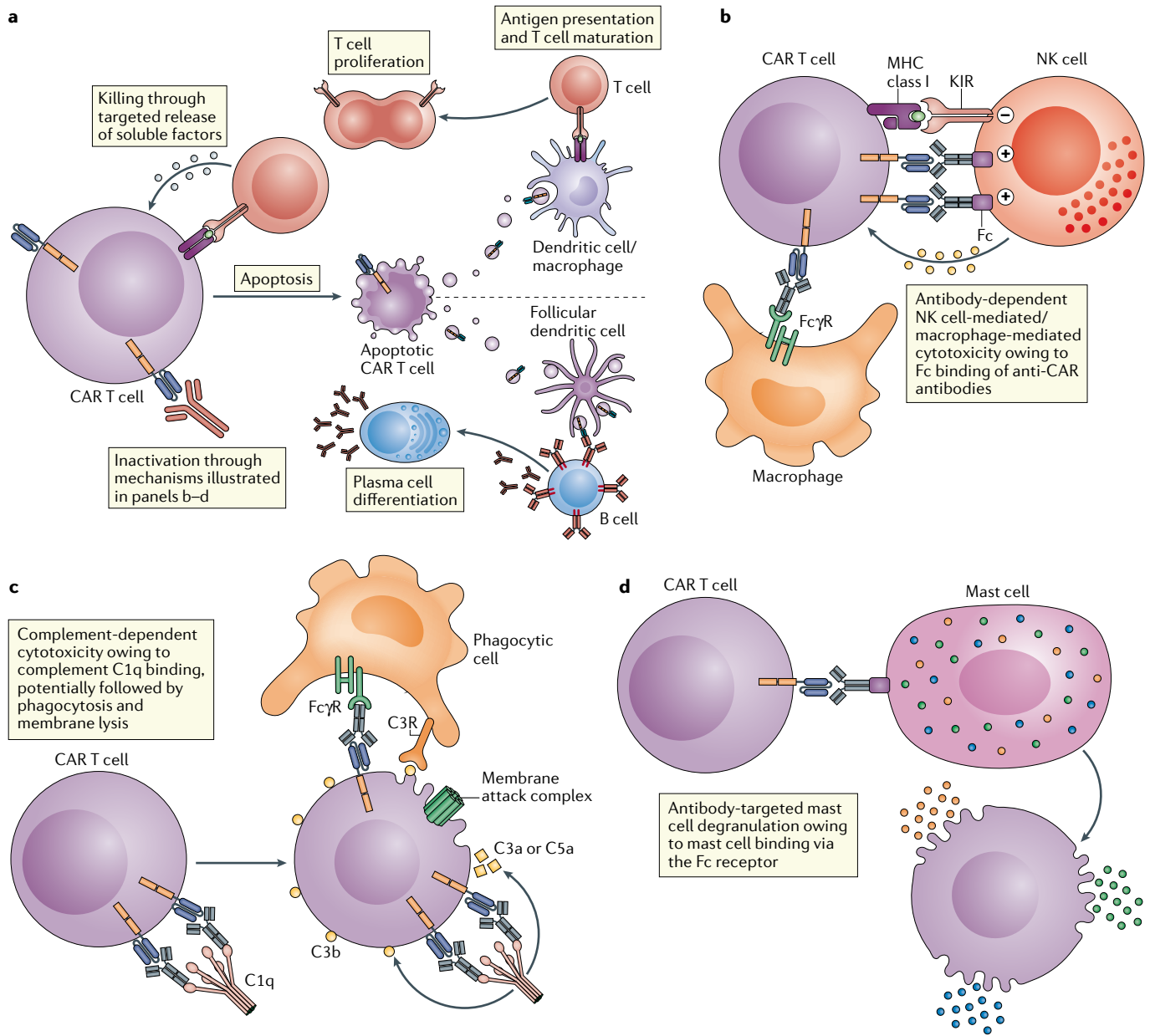
Fig. 1 | **Mechanisms of action of anti-CAR immune responses.** Acquired anti-chimeric antigen receptor (CAR) immune responses can be humoral or cellular. Cellular immunity probably arises from the processing and cross-presentation of foreign (mouse, viral or non-self human) sequences to the CAR molecule in the context of a major histocompatibility complex (MHC). CAR peptides from apoptotic or necrotic CAR T cells can be displayed by antigen-presenting cells and used to prime T cell responses in secondary lymphoid organs (panel **a**). CAR-specific CD8+ T cells or cytotoxic CD4+ T cells[153] can eliminate CAR T cells that present fragments of the CAR molecule via MHC-mediated recognition. Humoral immunity can be primed through CAR proteins in apoptotic bodies presented by follicular dendritic cells to B cells. Supported by anti-CAR T cells, CAR-specific B cells can expand and then undergo class switching and plasma cell differentiation, producing different classes of immunoglobulins with distinct functions. Anti-CAR antibodies can potentially induce the death of CAR T cells via several mechanisms, including antibody-dependent cellular cytotoxicity, whereby interactions between CAR-bound antibodies and the Fc receptor (FcR) domains of innate immune cells, such as natural killer (NK) cells or macrophages, leads to cytotoxicity via the release of perforin and/or granzymes, or phagocytosis (panel **b**) and complement-dependent cytotoxicity owing to CAR-bound antibodies that activate the complement cascade, leading to the formation of membrane attack complexes and cell lysis (panel **c**). Following CAR engagement, anti-CAR IgE could also bind to the FcR of mast cells, thus promoting degranulation and CAR T cell death (panel **d**). Excessive release of multiple vasoactive mediators in mast cell granula could lead to systemic anaphylaxis, which was lethal in one patient[17]. KIR, killer immunoglobulin-like receptor.

are noteworthy and could reflect the different assays used for detection. Apparently, the presence of pre-existing antibodies or their increase after the infusion of these products was not associated with worse clinical responses. This lack of clinical consequences has also been observed in other studies involving CAR T cells with mouse scFvs targeting CD19 or CD20 (REFS[24,25]).

Post-infusion cellular responses to CD19-specific CARs could result in treatment failure. In contrast to pre-existing antibodies, the presence of CAR-specific cytolytic T cells after infusion has been associated with treatment failure in some but not all clinical trials[9,10,26–28]. The first report of cellular immunity possibly negating therapeutic efficacy involved two patients with follicular lymphoma receiving multiple dose-escalating infusions of first-generation autologous CD19-directed CAR T cells. These CAR T cells were produced through an inefficient manufacturing process using T cell clones with integrated plasmid vectors that required extensive ex vivo culture[9]. The cells were detectable in both patients at 24 hours after the first infusion (10^8 cells/m²), but they failed to persist for even 1 week. Despite re-infusion at higher doses and the use of IL-2 support, the CAR T cells did not survive in vivo. This failure might in part reflect the manufacturing approach but could have been exacerbated by a T cell-mediated anti-CAR response that existed prior to the first infusion that then expanded after administration. This response was directed towards either the CAR itself or the co-delivered hygromycin phosphotransferase-HSV1-thymidine kinase fusion (HyTK) suicide gene[9]. Similarly, T cell-mediated anti-CAR responses have been detected in subsequent trials involving second-generation CD19-directed CAR T cells using mouse-based scFvs and, to a lesser extent, with the use of fully human CAR constructs[27,29]. Notably, intensified lymphodepletion with cyclophosphamide and fludarabine has been identified as a factor that might reduce the extent of anti-CAR cellular immunity[27,28]. Conditioning regimens containing both of these agents are currently considered the standard of care approach prior to initial administration of CD19-targeted CAR T cells[30–32].

The success of CD19-specific CAR T cell re-infusion might be limited by cellular immunity. Despite the high complete response rates to the first infusion of CD19-directed CAR T cells after lymphodepletion in patients with several haematological malignancies, disease recurrence remains an issue, with approximately 30–50% of patients having disease relapse within 12 months[33]. Antigen escape through loss of CD19 expression has been seen in 7–25% of patients, depending on the trial[34]. For those with CD19-positive disease relapse, a repeat infusion of the original CD19-targeted CAR T cell product seems appealing. However, clinical responses to second or subsequent infusions have generally been suboptimal, with complete remissions typically seen in <25% of patients[35]. Furthermore, populations of cytotoxic T cells with specificity towards the CAR have been shown to expand after initial infusion in a subset of patients despite intensified lymphodepletion[27].

The detection of such cells has been associated with poor expansion of the CAR T cell product after redosing[27]. To date, little is known about which specific epitopes within the CAR construct are recognized by anti-CAR cytotoxic T cells. The few studies available mostly found T cells with specificity to the mouse scFv FMC63 used in several CD19-specific CARs[27,29] as well as, to a lesser extent, to other parts of the transgene (such as the signal peptide linker or hinge domains in 3 of 19 patients receiving CD19-specific CAR T cells)[29] (Supplementary Table 1).

Immunogenicity in haematological indications beyond CD19 CAR T cells. Another promising CAR T cell candidate currently in clinical development targets plasma cell malignancies. Specifically, CAR T cells redirected to recognize B cell maturation antigen (BCMA) have induced potent antitumour responses in patients with advanced-stage multiple myeloma in seven large clinical cohorts (as of July 2020), although rates of complete remission are thus far lower and responses are less durable than with CD19-targeted CAR T cells in patients with B-ALL or NHL[36–38]. All trials used lymphodepletion in the majority of patients (with cyclophosphamide or fludarabine and cyclophosphamide), and only one study provided data on CAR immunogenicity[36–42]. In a recent approach described by Xu et al.[40], a bispecific CAR targeting two distinct BCMA epitopes using two camel-derived antigen-binding domains combined within a single CAR construct induced the highest yet reported complete remission rate of 76% (13/17) in patients with multiple myeloma. Anti-CAR antibodies were detected in all patients with disease relapse after an initial complete remission and correlated with a decrease in circulating CAR T cell numbers. Thus, humoral immunity could be associated with CAR T cell inactivation and treatment failure in patients receiving products that do not deplete endogenous B cells. Indeed, plasma cell depletion by BCMA-directed CAR T cells might be insufficient to blunt the early B cell responses that drive humoral immunity towards CARs (FIG. 2).

Several novel CAR T cell approaches are in development for other indications such as T cell malignancies (NCT03590574)[43]. Introducing CAR constructs with specificity for pan T cell-lineage antigens has thus far proved challenging owing to CAR T cell fratricide, a phenomenon involving self-killing among CAR T cells[44,45]. This process could both limit effective manufacturing of the product and reduce persistence of the cells in vivo. An exception to these limitations is provided by the development of anti-CD5 CAR T cells[46], in which a subpopulation of CD5-specific CAR T cells downregulates endogenous CD5 expression, thus avoiding fratricide and enabling expansion for clinical application. Early reports from an ongoing phase I trial suggest efficacy of such CD5-targeted CAR T cells in patients with T cell acute lymphoblastic leukaemia or T cell lymphoma, without T cell aplasia[47]. The continued depletion of endogenous T cells could theoretically impede adaptive immune responses towards tumours and pathogens but might also limit the extent of anti-CAR immunity, thus improving CAR T cell persistence.
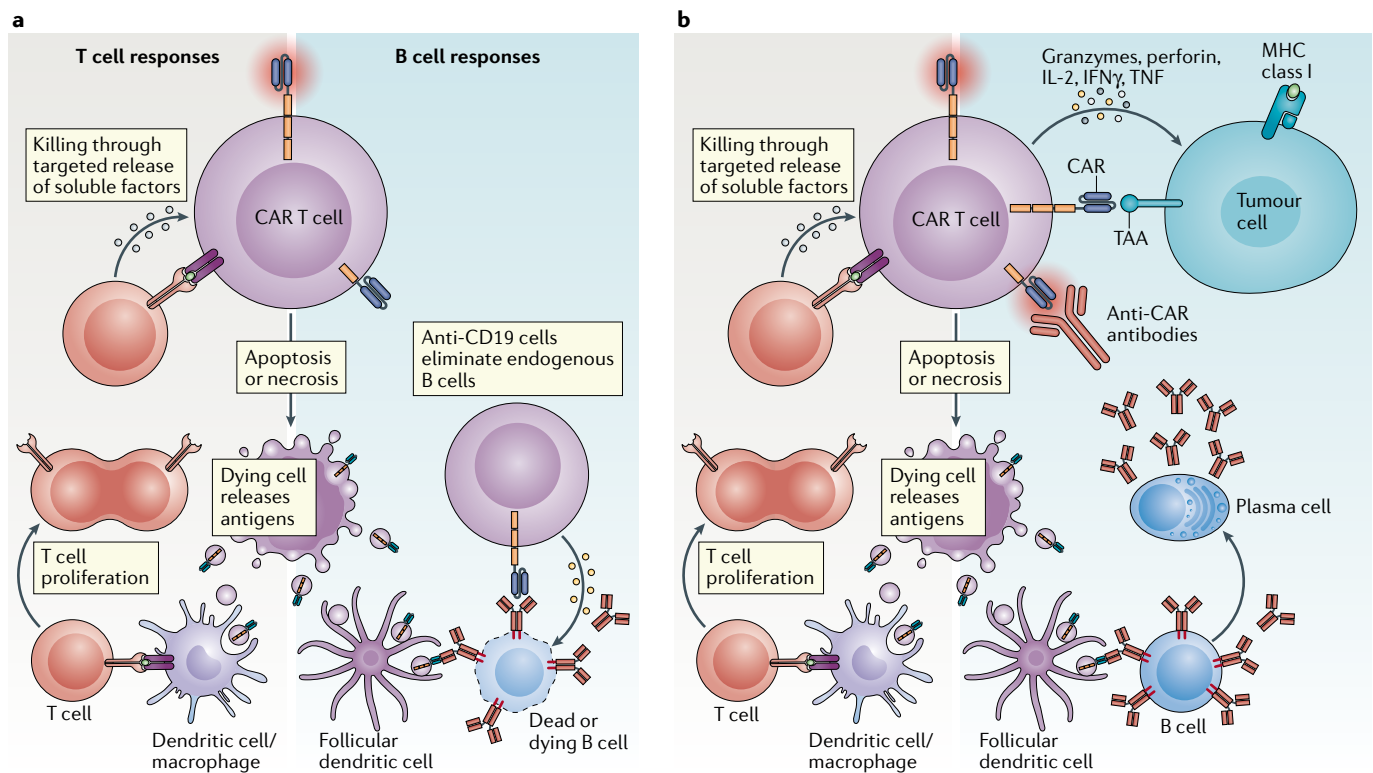
Fig. 2 | **Differences in anti-CAR immune responses following the targeting of B cell versus solid-tumour antigens.** **a** | Chimeric antigen receptor (CAR) T cells targeting B cell malignancies induce aplasia of non-malignant B cells, thus reducing the potential for anti-CAR humoral immunity. **b** | CAR T cells directed to solid-tumour antigens (targets not expressed on B cells) might have an increased risk of inducing both cellular and humoral anti-CAR immunity. MHC, major histocompatibility complex; TAA, tumour-associated antigen.

Early trials of CAR T cell therapy in solid tumours revealed anti-CAR immunity. Eliciting clinical responses with CAR T cells in patients with advanced-stage solid tumours has also proved challenging, in part owing to poor T cell infiltration of the tumours and/or limited CAR T cell persistence[7,8], which have been linked, in some cases, to anti-CAR immune responses[48,49] (FIG. 2). For example, Kershaw et al.[16] conducted a phase I trial to evaluate the safety of adoptive immunotherapy with autologous first-generation CAR T cells targeting the α-folate receptor (FRα) in patients with metastatic ovarian cancer. These investigators observed an antibody-mediated immune response to the CAR T cells that inhibited both IFNγ release (an indicator of anti-FRα activity) and cytotoxic activity against the FRα-positive tumour cells. This response substantially reduced the efficacy of the CAR T cells and might have led to their rapid clearance, as observed in the trial[16].

Hege et al.[48] reported outcomes from the first two clinical trials (conducted in the 1990s) evaluating CAR T cells in patients with solid tumours, with a focus on persistence and immunogenicity. These first-generation CAR T cell products contained a humanized antibody (huCC49) directed towards a tumour-associated glycoprotein (TAG-72) and elicited an anti-idiotype immune response via the formation of anti-CAR antibodies to the mouse-derived TAG-72-binding domain of huCC49. This immune response led to the elimination of the infused TAG-72 CAR T cells in <14 weeks, despite

repeated high-dose infusions of CAR T cells (1×10^{10}) in the majority of patients[48]. A preceding study using the fully mouse-derived variant of CC49 revealed that the antibody itself might have antigen-binding epitopes with particularly high immunogenic potential, resulting in HAMA responses in the majority of patients (54%)[13]. Follow-up investigations led to next-generation huCC49 humanized constructs bearing only the specificity-determining residues of the mouse TAG-72-binding epitopes grafted onto human antibodies[50]. These early trials demonstrate the importance of minimizing the inclusion of mouse components when designing CAR T cell products for solid tumours.

Profiling the immunogenicity of next-generation CARs in solid tumours. Early studies involving first-generation CAR constructs helped to elucidate the likelihood of immunogenic responses in patients with solid tumours[16,48,49]. However, the limited efficacy of these CAR T cells prompted a shift towards the use of second-generation CARs after improved antitumour responses were observed in preclinical models and in patients with haematological malignancies[51–55]. For example, Ahmed et al.[56,57] used autologous T cells expressing a second-generation CAR based on the mouse HER2-specific monoclonal antibody FRP5. In one trial, FRP5-scFv HER2-exodomain-specific CAR T cells were administered to 19 patients with advanced-stage HER2-positive sarcomas. These investigators

observed low but detectable levels of HER2-specific CAR transgenes in peripheral blood for up to 2 years after repeat infusions[56]. Similar CAR T cell kinetics were observed in another clinical study conducted by the same group involving patients with progressive glioblastoma[57]. Despite these as well as other trials showing unsatisfactory clinical responses[56–59], the role of cellular and humoral anti-CAR responses was not intensely investigated as a factor that may have contributed to treatment failure.

The aforementioned studies, which involved patients with solid tumours, did not include lymphodepletion prior to CAR T cell infusion. In contrast, in a case report published in July 2020, three cycles of lymphodepletion followed by infusions of second-generation CAR T cells targeting HER2 enabled the peripheral expansion and bioavailability of the infused cells[60]. This approach resulted in a complete response in a child with metastatic rhabdomyosarcoma, which was then consolidated with repeat CAR T cell infusions without further lymphodepletion but with an anti-PD-1 antibody. Longitudinal immune monitoring in this child revealed T cell-repertoire remodelling with immunodominant clones and serum autoantibodies reactive to oncogenic pathway proteins. Despite an evident strong endogenous immune response, the patient remained HAMA-negative even after receiving a total of 15 infusions of a CAR T cell product containing a mouse scFv. Drawing definitive conclusions based on observations from a single patient is difficult; therefore, further investigations are warranted to better elucidate the anti-CAR immune responses and their potential

interplay with lymphodepletion, particularly where redosing is required. Ultimately, mitigating immunogenicity using intensified lymphodepletion might contribute to improving CAR T cell engraftment in patients with solid tumours[61–63].

Reducing inherent CAR immunogenicity

Several components of CAR constructs have the potential to trigger anti-CAR immune responses in patients. Various approaches can be used to render these components less immunogenic.

Humanizing tumour-reactive scFvs. Several investigators are developing and testing humanized scFvs to circumvent the anti-CAR responses associated with mouse-derived scFvs. Humanized constructs are likely to be less immunogenic and might also be preferable for salvage therapy in patients with disease relapse after initial mouse-derived CAR T cell infusion (FIG. 3) or as an option for CAR T cell-naive patients[29,50,64–66]. The first treatment of patients with B cell NHL using a fully human CD19-directed CAR induced fewer CAR-specific T cell responses compared with a similar cohort who received CAR T cells containing a mouse-derived scFv[29]. Another strategy is to substitute traditional scFvs with immunoglobulin heavy-chainonly recognition domains that lack light chains and potentially immunogenic linker sequences (such as heavy-to-light chains or other associated junctions)[67]. These heavy-chain CAR constructs have shown robust target affinity and efficacy in preclinical models[68–70]. The first clinical proof-of-efficacy of a BCMA-specific
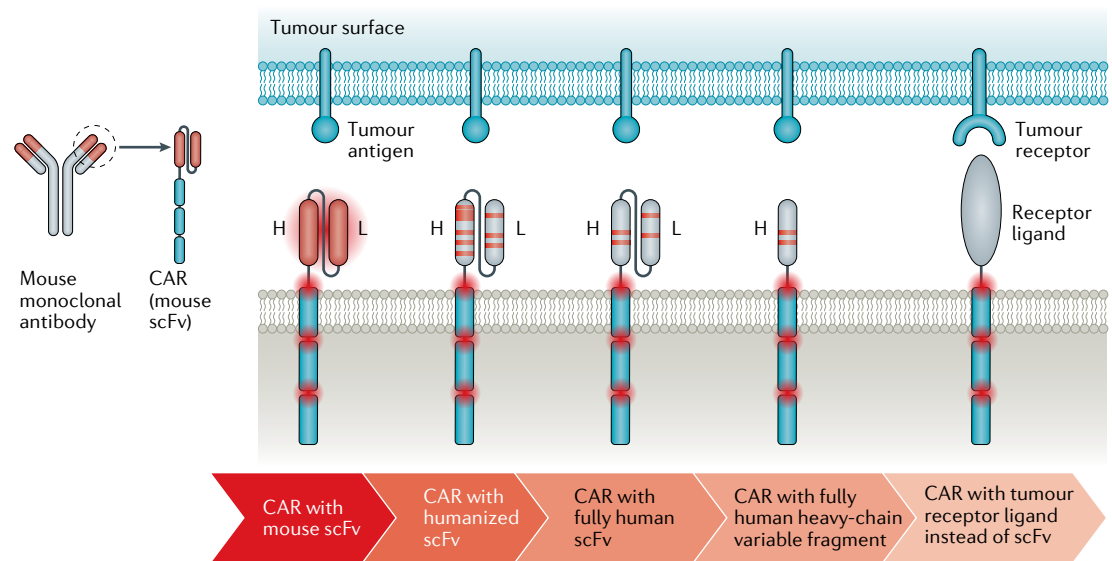
Fig. 3 | **Engineering CAR T cells to reduce their inherent immunogenicity.** Improving chimeric antigen receptor (CAR) design in order to limit inherent immunogenicity involves selecting tumour-binding moieties with the least immunogenic potential. Here, we show methods of replacing elements of single-chain variable fragments (scFvs) derived from mouse antibodies with human sequences or replacing the entire mouse-derived scFv with human-derived heavy-chain variable fragments or fully human scFvs. CARs with heavy-chain-only variable fragment tumour-recognition domains have the additional advantage of not requiring linkers, which are another possible source of immunogenicity. As an alternative to scFvs, the ligand of a tumour-associated receptor (or vice versa) can be attached to the constant regions of the CAR. Such constructs exploiting interactions between endogenous receptors and their ligands might enable tumour targeting with minimal risk of immunogenicity.

fully human heavy-chain-only construct was reported in November 2019 (REF.[71]). Theoretically, constructs derived solely from human protein components could still initiate immune responses involving anti-idiotype antibodies in patients[48,72]. Therefore, head-to-head clinical trials directly comparing the immunogenicity of mouse-derived and humanized scFvs are warranted.

Exchanging scFvs with alternative tumour-specific domains. Aside from scFv-based CARs, tumour cells can also be targeted via endogenous tumour-specific receptor–ligand interactions. The scFv portion of the CAR can be replaced either by the extracellular domain of a receptor or by a ligand that binds to receptors overexpressed by tumours. Moieties currently in preclinical and early clinical evaluation include chimeric NKG2D receptors[73–75], membraned-tethered IL-13-cytokine CARs targeting IL-13Rα2-expressing tumour cells[76–78], integrin $\alpha_v\beta_6$-binding peptides[79,80] and heregulin-ζ chimaeras[81]. Such tumour-specific receptor–ligand interactions are expected to be less immunogenic than traditional scFvs because they have human peptide sequences and are therefore likely to be recognized as self-proteins. Furthermore, universal CAR designs with extracellular domains that can be loaded with tumour-targeting antibodies in vivo might enable renewed redirection of CAR T cell specificity in the event of antigen escape[82,83]. However, no immunogenicity data are available thus far regarding the use of such universal receptor strategies.

Mutating CAR spacers. Optimal engagement of the scFv with the target epitope is paramount for effective CAR T cell therapy. In particular, proximal epitopes located close to the tumour cell membrane might require CAR T cells with longer and more flexible hinge domains. Many groups have used the constant regions of various IgG heavy chains as hinges[84,85]. In their original form, the CH2 and CH3 domains within these IgG-derived spacers could be targeted by Fc-receptors on innate immune cells, such as macrophages, granulocytes and natural killer (NK) cells, and might pose an immunogenicity risk (FIG. 1). Indeed, the presence of an Fc-binding spacer containing the CH2 domain might have contributed to the poor persistence of CD19-targeted CAR T cells reported by Jensen et al. as well as in other clinical studies[9,25,86]. This phenomenon can be avoided by mutating the Fc receptor-binding epitopes within the spacer, thereby preventing innate immune cell activation[87,88].

Immunogenicity of novel CAR technologies
Cytokine self-support.
Cytokine support technologies designed to enhance the antitumour efficacy of CAR T cells, particularly with the goal of overcoming cytokine starvation in tumours with immunosuppressive microenvironments, are an area of extensive preclinical and clinical investigation[7,89]. The systemic administration of cytokines, such as IL-15, increases the risk of immunogenic responses in patients and can cause toxicities such as fever, rigors, hypotension, thrombocytopenia and lymphocytopenia, among others[90]. Therefore, other approaches using engineered CAR T cells to locally

release soluble cytokines have been used, demonstrating potent activity in preclinical models[91–93]. Bystander T cell activation is a theoretical risk inherent to such paracrine cytokine support strategies[94,95]. Early clinical results with this approach are encouraging[96], although whether paracrine cytokine support might amplify any anti-CAR responses remains to be elucidated. Alternatively, a constitutively active cytokine receptor or a cytokine receptor signalling domain within the CAR molecule can provide immunomodulatory cytokine signalling solely to the CAR T cells without affecting bystander lymphocytes[97,98] and this strategy is currently being investigated in clinical trials (NCT03635632 and NCT04099797).

Suicide genes and elimination markers. Despite their effectiveness in patients with certain haematological malignancies, CAR T cells also pose safety risks due to on-target, off-tumour toxicities and/or the unpredictable adverse effects of add-on technologies. Suicide genes and elimination markers have been investigated as strategies that can be co-introduced alongside the CAR transgene to enable the targeted depletion of gene-modified cell products upon the emergence of severe adverse events[11,99,100]. However, such approaches are not without a risk of immunological complications. For example, Berger et al.[99] identified multiple immunogenic epitopes in T cells expressing herpes simplex virus thymidine kinase (HSV-TK) as an inducible suicide gene, resulting in rapidly evolving anti-CAR T cell responses mediated by CD4^{+} T cells and CD8^{+} T cells. Thus, the HSV-TK suicide gene might also have contributed to the rejection of CD19-directed CAR T cells observed in the trial conducted by Jensen et al.[9], as mentioned earlier.

Safety systems adapted from proteins of human origin (such as surface-bound elimination markers, including truncated EGFRs or the iCaspase 9 suicide system) avoid introducing xenogeneic components that might increase the risk of immunogenicity[100–102]. To implement safety genes in CAR T cell products, many researchers choose to co-express the CAR construct and a suicide switch on a single polycistronic construct separated by an internal ribosome entry site or a viral 2A sequence that contains self-cleaving peptides. Virus-derived 2A sequences have not been shown to be immunogenic in ex vivo studies[103], although only prospective clinical trials can definitively address the issue of their immunogenicity in patients. Notably, research published in 2000 indicates that internal ribosome entry site insertion might reduce the expression level of downstream transgenes such as the suicide switch[104]. More recently, gene editing has been used to create transgene-free suicide switches that avoid the risk of transgene-related immunogenicity, although this approach currently requires further investigation[105].

Gene editing with transiently delivered nucleases. Programmable nucleases provide a method of enhancing CAR T cell functionality via gene editing. This approach has been used to disrupt the expression of endogenous immune-checkpoint proteins and thus boost the performance of CAR T cells[106–108] and, potentially, to enable their co-administration with therapeutic monoclonal

antibodies[109]. Furthermore, gene editing might limit the extent of fratricide owing to reduced cell-surface antigen expression, enabling clinical investigations of CAR T cells for common T cell antigens beyond CD5 (such as CD3 and CD7)[44,45]. Importantly, gene editing could also be used to evade T cell-mediated anti-CAR immunity by eliminating cell-surface MHC expression as has been demonstrated in vitro and in vivo using allogeneic CAR T cells[106,110,111] (FIG. 4).

Aside from unintended off-target gene editing events, the induction of host immune responses combined with pre-existing humoral and cellular immunity has been identified as a risk factor associated with the CRISPR–Cas9 genome editing system[112–117] that could lead to the T cell-mediated elimination of Cas9-expressing cells[117,118]. Nevertheless, Stadtmauer et al.[108] demonstrated that the temporary delivery of CRISPR–Cas9 components as ribonucleoprotein complexes did not affect the persistence of autologous TCR-redirected T cells in three patients. The rapid degradation of the immunogenic protein as well as dilution during ex vivo expansion probably explain these results. Thus, transient delivery, as opposed to constitutive overexpression, could be the preferred method of CAR T cell gene editing in cells with intact antigen processing and warrants further investigation.
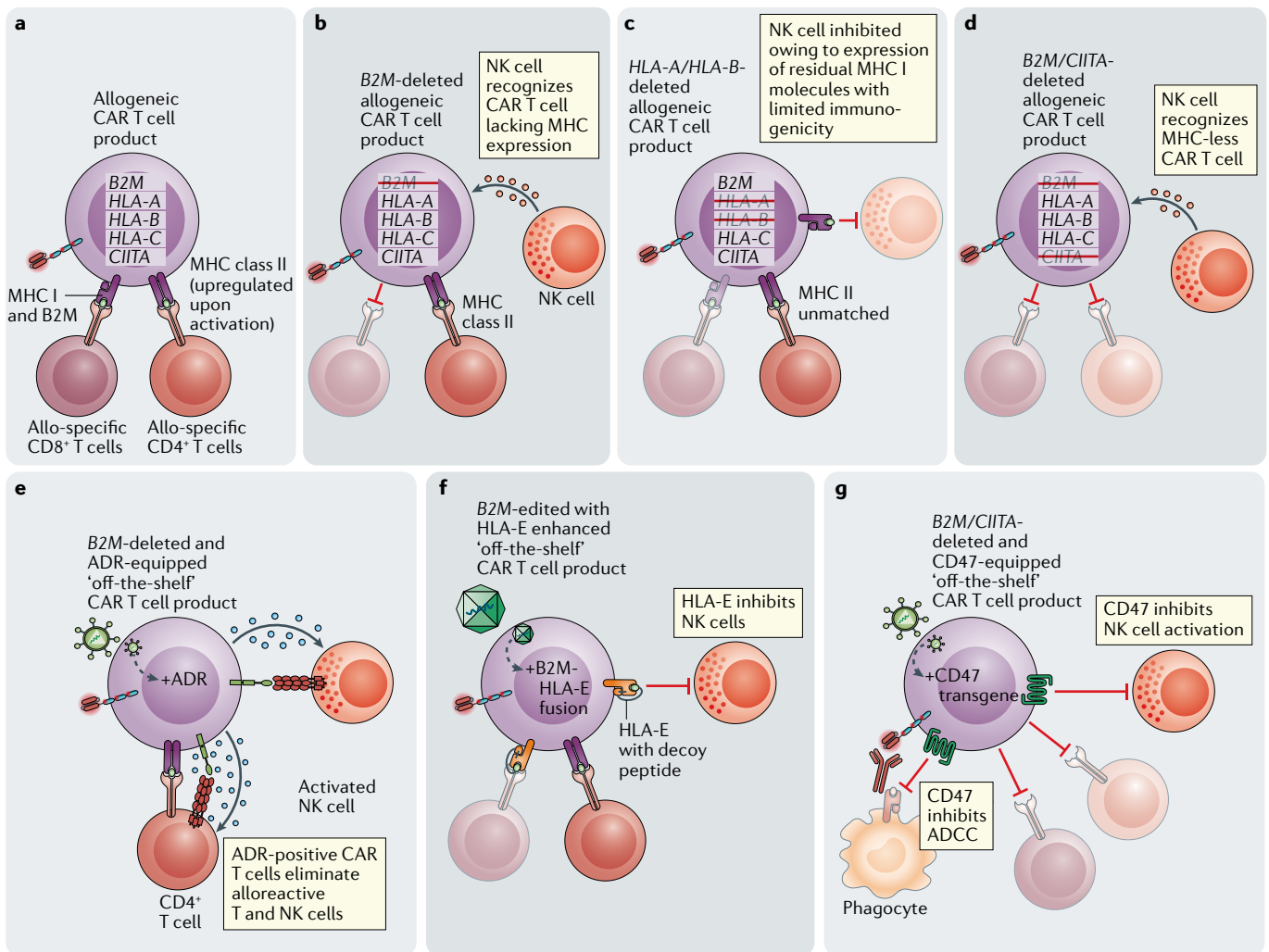
Fig. 4 | **Strategies to avoid immune elimination of allogeneic CAR T cells.** **a** | The presence of incompatible major histocompatibility complexes (MHCs) can boost anti-chimeric antigen receptor (CAR) responses to allogeneic CAR T cells. Resting T cells express MHC class I (MHC I), while T cells can also upregulate MHC class II (MHC II) after activation. **b** | MHC acts as a potent inhibitory receptor via the killer immunoglobulin-like receptor (KIR) on natural killer (NK) cells. Eliminating MHC I from the surface of resting CAR T cells can prevent alloimmune reactions while also increasing the risk of NK cell-mediated cytotoxicity. **c** | Multiplex gene editing can be used to eliminate CAR T cell-surface MHC expression while also sparing the less-diverse human leukocyte antigen (HLA)-C subtype, which might minimize the risk of rejection by NK cells. Careful MHC matching of donor-derived products should also be performed. **d** | Disrupting the functional expression of *CIITA*, which encodes the master transcriptional regulator of MHC II, or of the common constant chain CD74 could be used to remove MHC II from the cell surface without provoking alloimmune responses to activated CAR T cells. **e** | Alloimmune defence receptors (ADRs) targeting the activation marker 4-1BB on allospecific T cells might improve the persistence of allogeneic CAR T cells. ADRs introduced into *B2M*-negative and MHC I-deficient CAR T cells are likely to actively eliminate the risk of NK cell lysis. **f** | Site-specific insertion of universal MHC I constructs (such as those based on a decoy peptide-loaded monomorphic HLA-E) into the *B2M* gene prevent NK cell targeting of allogeneic-induced pluripotent stem-derived cells. **g** | Alternatively, the overexpression of CD47 prevents effective alloreactive T cell/NK cell activation and antibody-dependent cellular cytotoxicity (ADCC) by acting as a 'do not eat me' signal.

Immunity and allogeneic CAR T cells

Off-the-shelf allogeneic CAR T cell products created using cells from healthy donors are an attractive alternative to the autologous approach and could potentially broaden the clinical applicability of these cells and ensure timely availability[119]. However, non-self MHCs expressed on these CAR T cells could elicit host-derived immune responses that lead to immune-mediated elimination of the product. Allogeneic CAR T cells could be targeted by the patient's alloreactive T cells with specificity to foreign major MHC antigens[120,121]. Even with partially human leukocyte antigen (HLA)-matched donors, minor antigen differences owing to polymorphisms could create additional immunogenic epitopes[122]. Indeed, data published in December 2020 (REF.[123]) indicate that preconditioning with chemotherapy plus the anti-CD52 antibody alemtuzumab, which depletes both B cells and T cells, is required for the short-term antitumour activity of allogeneic CD19-specific TCR-edited T cells in both adult and paediatric patients with treatment-refractory B-ALL. Importantly, alemtuzumab would also diminish CD52-expressing CAR T cells after infusion; therefore, *CD52* was deleted in the CAR T cells used in this trial. Despite the addition of alemtuzumab to lymphodepletion, the allogeneic CAR T cells displayed limited persistence after endogenous T cell reconstitution[123]. These results highlight the need for strategies to overcome the barriers to allo-reactive immunity created by even minor HLA mismatches.

Several approaches have been used to generate CAR T cells with reduced expression of MHC antigens, thus enabling the treatment of patients regardless of HLA disparities (FIG. 4). The elimination of MHC class I (MHC I) by direct editing of the HLA locus or by disruption of the gene encoding β2-microglobulin (*B2M*), which is required for MHC I expression, have the potential to prevent allo-rejection by CD8+ T cells[106,110]. However, because T cell activation causes the upregulation of MHC II, allospecific CD4+ T cell-mediated CAR T cell rejection could also occur. The elimination of MHC II expression via the deletion of *CIITA*, the master transcription factor for MHC II genes, could mitigate this rejection[111].

These approaches offer some promise against T cell-mediated rejection; however, paradoxically, the loss of HLA molecules renders allogeneic CAR T cells vulnerable to NK cell killing. Therefore, NK cell inhibitory transgenes have been developed to promote the persistence of genetically modified cells in immunocompetent hosts (FIG. 4). For example, Gornalusse et al.[124] developed cells that are resistant to rejection by both CD8+ T cells and NK cells by expressing the non-polymorphic HLA-E molecule fused to B2M and a decoy antigen in human pluripotent stem cells. Other strategies, for example, involving multiplex gene editing, might be used to eliminate highly polymorphic *HLA-A* and *HLA-B* alleles while sparing the less diverse *HLA-C*, thus retaining an important endogenous NK cell-inhibitory receptor[125]. Alternatively, the overexpression of CD47 can inhibit alloreactive T cells and NK cell activation as well as providing a 'do not eat me' signal to phagocytes that prevents antibody-dependent cellular cytotoxicity[126,127]. In July 2020, Mo et al.[128] published a report describing an 'alloimmune defence receptor' that enables CAR T cell-mediated killing of alloreactive T cells and NK cells through recognition of the activation marker 4-1BB in preclinical models without the need for genetic modifications of the HLA system. Intriguingly, the expression of alloimmune defence receptors by engineered immune cells compromised neither allogeneic CAR T cell expansion nor the antitumour immune response in mouse models[128].

Extensive lymphodepletion can also reduce the risk of immediate allorejection[129,130]. The downside of this approach is the potential of graft-versus-host disease (GvHD) caused by persistent allogeneic CAR T cells or the proliferation of endogenous non-CAR donor T cells retaining alloreactive TCR expression. To mitigate the risk of GvHD, investigators have transferred CAR transgenes into virus-specific T cells, thus generating safer products because the TCRs of such cells are less likely to be alloreactive[57,131–134]. Alternatively, CARs can be introduced into non-conventional T cells with restricted TCR repertoires (such as γδT cells, mucosa-associated invariant T cells, NK T cells and invariant NK T cells), as these all pose a lesser risk of GvHD[135–137]. As a further alternative, the TCRs of allogeneic CAR T cells could be disrupted using gene editing[109].

Anti-CAR immune responses in the clinic

Longitudinal investigations of anti-CAR immune responses would be particularly important in understanding how immunogenicity can lead to treatment failure. Current professional society guidelines for the use of CD19-directed CAR T cells do not include recommendations regarding the evaluation or clinical management of suspected anti-CAR immunity[32,138–140]. In this section, we outline the need to develop validated assays to adequately monitor the immunogenicity of CAR T cell candidates and enable investigators to assess the clinical relevance of these responses and, if appropriate, implement suitable evidence-based mitigation and management strategies (FIG. 5).

Monitoring anti-CAR immunity in clinical trials and clinical practice. The observation of lethal anaphylaxis attributed to HAMA after infusion of mesothelin-specific CAR T cells[17] might have prompted regulatory agencies to recommend addressing the risk of immunogenicity in phase I safety trials if an immunological reaction is suspected (FIG. 5). Several assays have been designed in various clinical trials to test for humoral or cellular immune responses to CAR T cells (BOX 1). Beyond HAMA, however, these assays are rarely certified according to Clinical Laboratory Improvement Amendments and are currently not widely available. At the same time, HAMA is, by definition, only of value for the detection of antibodies to mouse-derived scFvs. Particularly subsequent infusions of mouse-derived CAR T cells could be preceded by HAMA evaluation, which might help to elucidate possible mechanisms of primary resistance and/or limited responses to second or subsequent infusions[141]. As novel constructs are humanized, HAMA assessments might become less
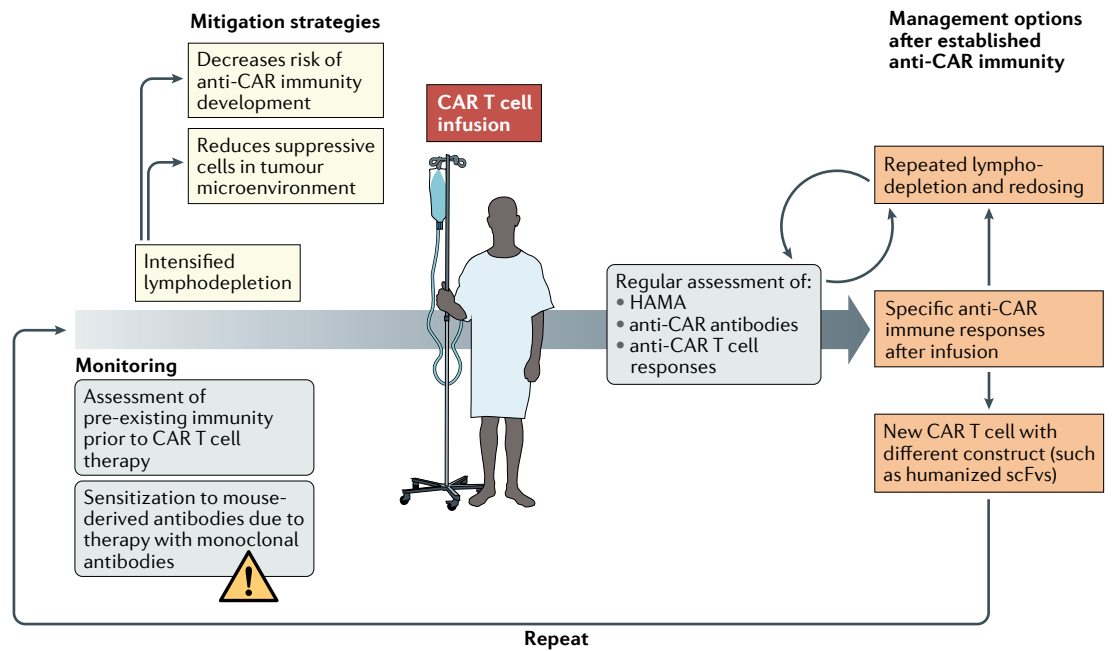
Fig. 5 | **Monitoring, mitigation and management of anti-CAR immunity in the clinic.** Mitigation of anti-CAR immunity through lymphodepletion regimens can reduce the numbers of circulating lymphoid cells as well as of antigen-presenting cells prior to CAR T cell infusion. Furthermore, cytoreductive treatments might reduce the numbers of certain immuno-suppressive cells in the tumour microenvironment, leading to tumour cell death and creating a pro-inflammatory milieu that promotes the antitumour efficacy of CAR T cells[154]. The monitoring of patients receiving CAR T cells should begin as soon as a patient is considered for therapy. Conventional cancer treatment regimens, including monoclonal antibodies of mouse origin[155], can induce human anti-mouse antibodies (HAMAs) in patients[12,13]. Therefore, careful evaluation of pre-existing immunity is important for the avoidance of anaphylaxis after CAR T cell infusion[17]. Subsequently, monitoring in clinical trials should include surveillance for possible HAMAs and specific anti-CAR immune responses, including antibody and T cell analysis. The management of low-level anti-CAR immunity can be achieved using repeat lymphodepletion to reduce anti-CAR immunity prior to redosing with the same CAR T cell product. Humanized CAR T cell constructs could be considered in patients who progressed or relapsed after a previous infusion of CAR T cells containing mouse single-chain variable fragments (scFvs) or who required subsequent infusions to prevent disease progression. However, this approach could be associated with additional costs and/or treatment delays.

relevant and developing new assays, both for humanized constructs and potentially for those derived from non-mouse, non-human sources, will be a growing need. Until such companion assays are available, investigators could consider cryopreserving serum samples and peripheral blood mononuclear cells collected at multiple time points for later analysis.

Very few clinical studies involving patients with solid tumours have investigated anti-CAR antibody responses beyond HAMAs, despite reports indicating potent immune responses to non-human CAR sequences and mutant oncogenic proteins[49,60]. Given that CAR T cell expansion and persistence remain suboptimal in trials for solid tumours, the role of immune responses to the CAR constructs used is becoming more relevant and should be revisited. Thus, establishing validated assays for the characterization of humoral and cellular immune responses to CAR constructs and the associated trans-genes is of paramount importance. Assays designed to detect anti-CAR antibodies can be cell-based or enzyme-linked immunosorbent assay (ELISA)-based and should ideally be able to measure the immunogenicity of all CAR components expressed on the T cell surface and predict their likely effects on the cell product (such as cytotoxic potential)[142] (BOX 1). For cellular immune responses,

assays that enable mapping of the most immunogenic epitopes within the CAR constructs are most likely to inform better CAR design and the development of novel technologies that improve efficacy. Overall, these efforts could also benefit from the implementation of common terminologies and the lessons learned from the assessment and reporting of immunogenicity for biologics (such as therapeutic proteins)[143–146].

Mitigating immunogenicity with intensified lympho-depletion. Lymphodepleting conditioning regimens administered prior to CAR T cell therapy are expected to affect not only the lymphoid compartment (including B cells and T cells) but also myeloid cells. The transient removal of professional antigen-presenting cells could reduce the likelihood of antigen cross-presentation when CAR T cells are expanding in the circulation and within lymphoid tissues. Cyclophosphamide-based chemo-therapy is also known to deplete regulatory T cells[147], which can otherwise limit both the antitumour efficacy and persistence of CAR T cells. Most investigators routinely incorporate fludarabine/cyclophosphamide-based lymphodepletion to improve treatment outcomes and overcome the possibility of immunological rejection with initial CAR T cell infusions[27,28,32]. Intensifying this

regimen is also being considered for subsequent infusions. Interestingly, Gauthier et al.[35] demonstrated that intensified lymphodepletion (with fludarabine plus cyclophosphamide versus cyclophosphamide alone) prior to the first CAR infusion is associated with improved responses to a second, higher dose of CD19-targeted CAR T cells in a subset of patients.

Managing relapsed disease with humanized CARs and other constructs. To avoid premature CAR T cell inactivation resulting from immune responses to the mouse-derived scFvs, alternative CAR T cell products using humanized scFvs can be considered. Early studies involving humanized CD19-targeted CAR T cell constructs reported varied clinical response rates, including in patients who progressed or relapsed after a previous infusion of CAR T cells containing mouse scFvs. For example, Maude et al.[64,148] infused 16 patients with B-ALL who either did not respond to or had disease progression on CTL019 CAR T cells (which include the mouse-derived FMC63 scFv) with an alternative CD19-specific CAR T cell product (CTL119, containing a humanized form of FMC63). In this phase I study, 9 of 16 (56%) patients had complete remissions

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Box 1 | Assays designed to monitor anti-CAR immunity

Cellular immunity

Cytotoxicity assays
Cytotoxicity assays measure the killing of chimeric antigen receptor (CAR)-transduced target cells (including T cells and autologous lymphoid cell lines) by ex vivo expanded T cells and/or mononuclear cells from peripheral blood samples via stimulation with irradiated CAR-expressing targets. Such assays typically use ^{51}Cr labelling for quantification of target cell death after co-culture for 4–6 hours[10,24,27,99].

Degranulation assays
Degranulation assays measure CD107a (also known as Lamp1) surface expression, which indicates recent secretion of cytolytic vesicles, on ex vivo expanded patient-derived T cells from peripheral blood samples co-cultured with irradiated CAR-expressing T cells using flow cytometry[10,156].

In vitro proliferation assays
In vitro proliferation assays measure the ex vivo proliferation of patient-derived T cells after stimulation with irradiated CAR-expressing target cells or CAR–peptide mixtures typically through ^{3}H-thymidine incorporation or flow cytometric assessment (dilution of a proliferation dye such as carboxyfluorescein diacetate succinimidyl ester or similar)[26].

Enzyme-Linked Immunosorbent Spot (ELISpot) assays
ELISpot assays detect IFNγ released by ex vivo expanded patient-derived CAR-specific T cells. These cells are typically derived from peripheral blood mononuclear cells followed by 2-week expansion in the presence of irradiated CAR-expressing T cells and IL-2. After co-culture, these cells are exposed to either irradiated CAR-expressing target cells or CAR-derived peptide pools. This approach has the advantage of enabling the identification of the distinct protein fragments recognized by anti-CAR T cells[28,29,103].

Intracellular cytokine staining and flow cytometry
Intracellular cytokine staining and flow cytometry involve the detection of IFNγ or other cytokine-producing T cells among peripheral blood mononuclear cells after stimulation with mouse-derived CAR sequence-spanning peptides (or potentially non-mouse CAR peptides). This approach uses intracellular staining with fluorochrome-labelled antibodies typically including but not limited to IFNγ, TNF, and/or IL-2 and/or extracellular staining of activation markers such as CD137 and CD40 ligand followed by flow cytometry for quantification purposes[19,21].

T cell receptor (TCR) repertoire analysis
TCR repertoire analysis involves either next-generation sequencing or TCR Vβ spectratyping using flow cytometry. This approach enables the identification of expanded T cell clones in vivo based on individual TCR complementarity defining regions or TCR Vβ usage. Such analysis can be used to detect infused clones if TCR repertoire analysis is conducted before infusion[9].

Humoral anti-CAR immunity

Enzyme-linked immunosorbent assays (ELISAs)
ELISAs are standardized assays that enable the semi-quantitative analysis of human anti-mouse antibodies (HAMAs)[17]. To this end, patient serum is added to mouse immunoglobulin-coated wells. After washing, human immunoglobulins bound to the mouse immunoglobulins are visualized using secondary antibody staining with an anti-human IgG typically linked to a reporter enzyme (such as horseradish peroxidase). Subsequently, enzyme-mediated luminescence or colour changes in a solution are monitored for quantification. Standards run in the same assay enable the estimation of HAMA levels.

Cell-based flow cytometry
Cell-based flow cytometry involves the incubation of CAR-expressing target cells (such as Jurkat T cells or Chinese hamster ovary (CHO) cells) with patient serum samples, followed by washing and secondary staining for anti-human IgG. Additional steps are required to remove the HAMA signal and thus identify specific anti-CAR/idiotype antibodies unrelated to HAMA[9,40,86,142].

ELISA anti-drug antibody bridging assays
ELISA anti-drug antibody bridging assays use two soluble CAR proteins, one that binds to the vessel's surface while the second is linked to a reporter such as an alkaline phosphatase or horseradish peroxidase. This complex only becomes fixed during the wash stages in the presence of an anti-CAR antibody. Additional steps are needed to remove the HAMA signal. This approach does not enable the identification of specific conformational CAR epitopes, which would require anchoring of the entire CAR to a cell membrane[10,17,31,157].

at 1 month. However, in the same trial, 22/22 patients with CAR T cell-naive disease were in complete remission at this time point after receiving the CTL119 CAR T cell product[64]. Whether the relatively low rate of complete remissions in patients who received reinfusions reflects an immune response to epitopes shared between mouse and humanized CAR transgenes or is due to other mechanisms of resistance remains unknown[29,64–66,149].

Targeting an alternative antigen is another approach that might circumvent an anti-CAR T cell response if attributed to specific epitopes within a given scFv or another specific part of the CAR construct. Among these strategies, CD22-directed CAR T cells are currently the most established alternative for B cell malignancies. Thus far, CAR T cells targeting CD22 containing a fully human scFv have demonstrated a high degree of efficacy in patients with disease relapse on anti-CD19 CAR T cells with mouse scFvs and are equally effective regardless of CD19 expression[150–152].

Conclusions

CAR T cells are currently an important therapy for patients with certain relapsed and/or refractory haematological malignancies. Despite robust reported levels of efficacy, anti-CAR immune responses are associated with treatment failure, although direct causation has, in most studies, not been established. Nevertheless, the depletion of endogenous B cells by either direct CAR targeting or lymphodepletion can reduce the risk of immune responses to CAR T cells. However, CAR T cells directed to non-B cell malignancies, such as solid tumours, could induce both T cell and B cell responses, thus increasing the risk of immunogenicity (FIG. 2). This issue adds to the hurdles of designing effective CAR immunotherapies for solid tumours, where — among other challenges — a balance between the induction of potent endogenous immunity to cancer cells and responses against transgenic CAR T cells is required.

Developing technologies that improve the safety and/or functionality of CAR T cells, such as suicide genes or cytokine self-support, might also introduce new immunogenic risks. Moreover, the emergence and development of allogeneic donor-derived CAR T cells has drawn attention to the issue of immunogenicity. Indeed, the potential success of 'universal' off-the-shelf CAR T cells merits the inclusion of strategies designed to avoid immune recognition, protect against allospecific immunity and/or reduce the risk of immunogenic responses. CAR constructs with humanized scFvs and/or other less immunogenic components might also reduce the risk of anti-CAR immunity. In the clinic, intensified lymphodepletion remains vital to mitigate or modulate anti-CAR immune responses and promote better CAR T cell persistence.

Efforts to decrease the incidence and/or severity of anti-CAR immune responses should be accompanied by appropriate monitoring of immunogenicity in patients receiving CAR T cells in clinical trials. As we report, anti-CAR immunity has been investigated in fewer than a third of CAR T cell trials with available data (Supplementary Table 1). Additionally, many of the assays used to date (BOX 1) lack both standardization and validation, thus limiting the strength of correlations and performance comparisons. We strongly advocate for the inclusion of CAR-associated immunogenicity analysis in both preclinical and clinical investigations to enable both immunogenic responses and their implications to be studied in detail. The more widespread use of CAR T cells supports the need to monitor anti-CAR immunity and demands vigilance regarding possible CAR-related immunogenicity in order to ensure the safety and efficacy of CAR T cells in patients with cancer. This knowledge will increase our understanding of the clinical relevance of immunogenicity and inform the development of future CAR T cell strategies.

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Review criteria

We conducted a literature screen and identified 120 clinical trial publications describing the use of chimeric antigen receptor (CAR) T cells, out of which 34 reported on the investigation of immunogenicity using laboratory assays (Box 1). A complete list of these trials, including information on the assays used to characterize immunogenicity, CAR design, trial outcomes and preconditioning regimens, is available in Supplementary Table 1. Data cut-off July 15, 2020.

Supplementary information

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