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Anti-tumour Treatment

Clinical application of cytokine-induced killer (CIK) cell therapy in colorectal cancer: Current strategies and future challenges

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ABSTRACT

Colorectal cancer (CRC) remains a significant global health burden and is the second leading cause of cancer-related death. Cytokine induced killer (CIK) cell therapy is an immunotherapy which has the potential to meet this need. Clinical trials of CIK cell therapy for the management of CRC have reported improved clinical outcomes. However, production and delivery protocols varied significantly, and many studies were reported only in Chinese language journals. Here we present the most comprehensive review of the clinical CIK cell therapy trials for CRC management to date. We accessed both English and Chinese language clinical studies, and summarise how CIK cell therapy has been implemented, from manufacturing to patient delivery. We discuss current challenges that impede wider adoption of CIK cell therapy in CRC management.

Background

With 1.9 million diagnoses each year, colorectal cancer (CRC) is the third most common cancer and the second leading cause of cancer-related death worldwide [1]. Patients with locally advanced CRC, including regional lymph node metastasis, have a 5-year overall survival (OS) rate of 75 %, which decreases to 15 % with distant metastases [2]. This emphasises the unmet need for more effective therapies for CRC. Immunotherapy, now considered the fourth pillar of cancer treatment, can complement surgery, radiotherapy and chemotherapy to substantially improve patient outcomes [3]. Immunotherapy can be broadly described as therapeutic strategies that direct an immune response against cancers. There has been interest in immunotherapy for decades, but it has been re-invigorated by the success of immune checkpoint inhibitors. Today there are thousands of immunotherapies being developed, making it one of the fastest-growing fields in oncology [4]. Nevertheless, immunotherapies can still be broadly classified into three main types: immune checkpoint inhibitors (ICI), cancer vaccines, and adoptive cell therapies (Fig. 1) [5].

ICIs have had the most impact in the immuno-oncology field to date. They inhibit negative regulators of the immune response which are commonly upregulated by cancers. Currently, monoclonal antibodies targeting cytotoxic T lymphocyte antigen 4 (CTLA-4) and programmed cell-death protein 1 (PD-1) related proteins are clinically available and have had success in treating liquid and some solid cancers [6–8]. In CRC, targeting PD-1/PD-L1 has produced significant survival benefits, to the point of being curative for some CRC patients. However, this benefit is limited to CRCs with high microsatellite instability/mismatch repair deficiency which represents a small cohort in both early-stage (~15 %) [9] and late-stage metastatic CRC (3–5 %) [10].

Cancer vaccines prime the immune response to attack cancer cells. There are many types of cancer vaccination strategies in development [11]. However, there are currently only two US Food and Drug Administration (FDA) approved prophylactic vaccines to prevent cancers caused by viruses (hepatitis B virus and human papillomavirus) and one FDA-approved dendritic cell (DC) vaccine (Sipuleucel-T) to treat prostate cancers [12]. Bacillus Calmette-Guérin (BCG) vaccines, originally used to prevent tuberculosis, have also been approved by the FDA for the treatment of bladder cancers [13]. Cancer vaccines are in development for CRC [14].

In adoptive cell therapy *ex vivo* expanded lymphocytes, primarily T or NK cells, are infused into the patient to treat the cancer. These lymphocytes can come from the same patient (autologous) or from MHC-matched (allogenic) donors. Further, T cells can be genetically engineered to express T cell receptors (TCR) that recognise tumour antigens

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Abbreviations

ADCC	Antibody-dependent cell-mediated cytotoxic
AMPK	AMP-activated protein kinase
BiTE	Bi-specific T-cell engager
CAR	Chimeric antigen receptor
CTLA-4	Cytotoxic T lymphocyte antigen 4
CIK	Cytokine-induced killer
CTLs	Cytotoxic T lymphocyte
CRC	Colorectal cancer
c-Met	Mesenchymal-epithelial transition factor
DC	Dendritic cell
DC-CIK	Dendritic cell-cytokine induced killer
DNAM-1	DNAX Accessory Molecule-1 receptor
DFS	Disease-free survival
EGF	Epidermal growth factor receptor
FCγ	Fragment crystallizable gamma
FAS-L	FAS ligand
FBS	Fetal Bovine Serum
FOLFOX	Folinic acid, fluorouracil and oxaliplatin
GMP	Good manufacturing practice
HIF-1α	Hypoxia-inducible factor-1α
HR	Hazard ratio
ICI	Immune checkpoint inhibitor
IFN-γ	Interferon gamma
IFN-12	Interferon-12
IL-1	Interleukin-1
IL-2	Interleukin-2
IL-6	Interleukin-6
IL-15	Interleukin-15
ICAM-1	Intercellular Adhesion Molecule 1
KRAS	Kirsten rat sarcoma
LFA-1,2,3	Lymphocyte function-associated antigen-1,2,3
MHC	Major Histocompatibility Complex
MMR	Mismatched repair
MSI	Microsatellite instability
NK	Natural killer
NKCE	Natural killer cell engager
NKG2D	Natural killer group 2 member D receptor
Nkp30	Natural killer protein 30
OS	Overall survival
ORR	Overall response rate
PBMC	Peripheral blood mononuclear cell
PD-1	Programmed cell death protein 1
PD-L1	Programmed cell death protein–ligand 1
PDTO	Patient-derived tumour organoid
RCT	Randomised controlled trial
RFA	Radiofrequency ablation
scRNA-seq	Single-cell RNA sequencing
TACE	Transarterial chemoembolization
TCR	T cell receptor
TIL	Tumour-infiltrating lymphocyte
TRAIL	TNF-related apoptosis-inducing ligand
TNF-α	Tumour necrosis factor alpha
VEGFR	Vascular endothelial growth factor receptor
Wnt	Wingless-related integration site
WT1	Wilmas' tumour 1
Xelox	Oxaliplatin and capecitabine

through MHC molecules, while T and NK cells can also be genetically engineered to express chimeric antigen receptors (CAR) to induce MHC-independent cancer-killing. Both these techniques rely on the identification of tumour specific antigens. Currently, there are several candidates for CAR-T cell therapy in CRC being investigated [15]. Adoptive cell therapies that do not require genetic engineering include tumour infiltrating lymphocytes (TIL) therapy and cytokine-induced killer (CIK) cell therapy. TIL therapy relies on the expansion of tumour-derived lymphocytes, which enriches for cancer-specific T cells. TIL therapy has been shown to produce positive outcomes in a KRAS-positive CRC patients [16]. At the time of writing, there were six ongoing clinical trials for CRC TIL therapy [17].

CIK cells are produced by stimulating and expanding peripheral blood mononuclear cells (PBMCs) to produce a mixture of T cells with both TCR and NK-mediated cancer recognition [18]. CIK cell therapy has been used for solid and liquid tumours. CIK cell therapy is the only form of adoptive cell therapy which has been trialled extensively for CRC management. Our systematic review of 70 clinical studies involving 6,743 CRC patients shows that CIK cell therapy can significantly improve patient outcomes [19]. These findings warrant wider adoption of CIK cell therapy for CRC. However, protocols for CIK cell therapy production and delivery are heterogeneous and highly dependent on the treatment centre. This lack of standardised implementation protocols could deter inexperienced centres from adopting this technology.

In this review, we summarise the clinical implementation of CIK cell therapy for CRC treatment. We cover the production methods, quality checks, and infusion protocols reported in the 70 studies (Table 1) involving 3,203 patients who received CIK cell therapy as part of the intervention arm, that met our inclusion criteria. Additionally, we discuss potential research gaps that, if addressed, could enhance the broader adoption of CIK cell therapy.

Cytokine-induced killer (CIK) cell therapy

CIK cell therapy was first described by Schmidt-Wolf *et al.,* in 1991 [20]. Since its inception, CIK cell culture has followed a general protocol where peripheral blood mononuclear cells (PBMCs) are cultured in the presence of interferon (IFN)-γ, an anti-CD3 antibody, interleukin (IL)-1 and IL-2 [21].

Anti-cancer mechanisms

The biology of CIK cells has been extensively reviewed [22–24]. Briefly, CIK cells are characterised by the presence of T cells (CD3+ CD56−), NK-like T cells (CD3+ CD56+), and a small population of NK cells (CD3− CD56+). While most of the CD3+ T cells are reported to be CD8+ cytotoxic T lymphocytes (CTLs), CD4+ regulatory and helper T cell subsets are also present [25–27]. The anti-cancer activity of CIK cell therapy is primarily mediated by NK-like T cells. These cells are predominantly CD8+, with a small percentage being CD4+. Cancer recognition is mediated by three proposed pathways. First, CIK cells possess polyclonal TCRs that allow them to recognise tumours in an MHC-restricted fashion. Second, CIK cells also express the NK receptors NKG2D, DNAM-1 and Nkp30 for MHC-unrestricted tumour recognition [28,29], with NKG2D being the most critical [30]. NKG2D and TCR activation is complemented by lymphocyte-function-associated antigen (LFA)-1 expression which enhances the binding of CIK cells to cancer cells expressing LFA-1 ligands ICAM-1, -2 and -3 [31,32]. Finally, some CIK cells are reported to express the NK cell-associated FC γ receptor CD16 which allows for antibody-dependent cell-mediated cytotoxicity (ADCC) (Fig. 2) [33,34].

Following cancer cell recognition, CIK cells trigger cellular cytotoxicity by releasing cytolytic granules containing granzymes and perforin, or by signalling through death ligands like FAS-L and TNF-related apoptosis-inducing ligand (TRAIL) [35]. Additionally, CIK cells can

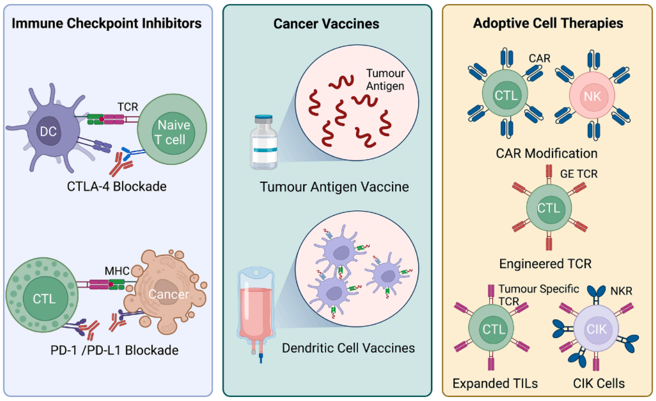

Fig. 1. Common forms of immunotherapies. Currently available immunotherapies can be broadly classified into 3 groups, immune checkpoint inhibitors (ICI), cancer vaccines, and adoptive cell therapies. ICIs are monoclonal antibodies against CTLA-4, which promotes T cell activation, and PD-1/PD-L1, which promotes T cell mediated cancer killing. Cancer vaccines deliver tumour antigens as vaccine formulations similar to pathogen-based vaccines, or by infusing tumour antigen loaded dendritic cells (DC). Finally, adoptive cell therapies are the infusions of *in vitro* expanded T and NK cells into cancer patients. These cells may be expanded tumour infiltrating lymphocytes (TILs) or cytokine induced killer (CIK) cells, or cells modified by the addition of chimeric antigen receptors (CAR) or genetically engineered (GE) expression of tumour antigen specific T cell receptors (TCRs). CIK cells naturally express both TCRs and natural killer cell receptors (NKRs).

release pro-inflammatory cytokines such as tumour necrosis factor (TNF)-α, and IFN-γ, amplifying the anti-tumour responses (Fig. 2) [36]. In CRC, CIK cells have been observed to dampen AMP-activated protein kinase (AMPK), Notch/Wnt and hypoxia-inducible factor-1α (HIF-1α) signalling pathways. However, the precise mechanisms remain unknown [37].

Production for clinical use

To generate CIK cells, patient-derived PBMCs are isolated by density-gradient centrifugation, and then immediately cultured with IFN-γ to promote a Type 1 T cell phenotype [21]. After 24 h, anti-CD3 and IL-2 are introduced to provide T cells with mitogenic signals and to promote survival and expansion, respectively [38]. Fresh IL-2 supplemented media is required every 3–4 days to maintain cell expansion. IL-1, while used in the original description of CIK cell culture [20], was not used in all centres reporting CRC clinical studies (Table. 2). The CIK cell therapy product is ready for patient infusion after 14–21 days of culture [21]. This general method can generate bulk CIK cell cultures containing >90 % CD3+ T cells, with the expansion of CD3+ CD56+ NK-like T cells ranging from 8 to 65 % [37,39,40]. Even though the anti-cancer activity is reported to be mediated by the NK-like T cells, further enrichment of this population does not appear to increase the anti-cancer effects compared to bulk cultures [41]. It is known that the NK-like T cells arise from the CD3+ CD56- population in uncultured PBMCs [40]. It is hypothesised that bulk culture transfers have precursors that sustain the CD56+NK-like T cell population *in vivo*. To date, all CRC clinical trials of CIK cell therapy have used bulk CIK cell cultures.

Current clinical applications of CIK cell therapy in CRC

CIK cell therapy combined with dendritic cell vaccines

Dendritic cells (DCs) play a critical role as antigen-presenting cells, capturing and processing antigens for presentation to T cells. The adoptive transfer of DCs, loaded with tumour-associated antigens, for cancer therapy (DC vaccines) has been frequently investigated in combination with CIK cell therapy (DC-CIK therapy). The interactions between CIK cells and DCs have been shown to result in changes in the expression of immunostimulatory surface molecules in both populations, with significantly greater IL-12 release by DCs, and enhanced CIK cell cytotoxicity against cancer cell lines with resistant phenotypes [42].

In a meta-analysis of 12 randomised controlled trials (RCTs) incorporating 826 patients with solid cancers, DC-CIK therapy together with chemotherapy demonstrated significantly improved OS, disease-free survival (DFS) and overall response rate (ORR) over chemotherapy alone [43]. However, further meta-analysis comparing outcomes from 10 CIK cell monotherapy against 16 DC-CIK therapy trials suggested that DC-CIK therapy did not provide additional benefit to CIK cell therapy combined with chemotherapy [19]. In the English language literature reporting DC-CIK therapy for CRC, tumour antigens loaded to DCs included a synthetic WT1 peptide [44] and a lysed SW480 CRC cell line [45], while another failed to report if DCs were loaded with tumour antigen [46]. DC vaccines are an emerging immunotherapy with even more diversity in production protocols than for CIK cells [47]. We hypothesise that the lack of observed benefit may be due to protocol diversity, including the type of tumour antigens loaded. Given that more resources are required to generate paired DC vaccines for CIK cell therapy, a clinical trial comparing DC-CIK over CIK cell therapy is required to determine its benefit in CRC treatment.

Table 1
CIK cell therapy type and adjunct anti-cancer therapy.

Adjunct therapy types	CIK therapy type	
	CIK (25studies)	DC-CIK (45studies)
Alone	–	1
Chemotherapy	21	37[a]
Concurrent chemoradiotherapy	–	1
Chemotherapy & radiotherapy	2	1
Chemotherapy & radiofrequency ablation (RFA)	–	2
RFA	1	–
Radiotherapy and microwave hyperthermia	1	–
Transarterial chemoembolization (TACE)	–	1
Chemotherapy or no therapy	–	1
Unspecified[b]		1

[a] Includes 1 study where DC-CIK therapy plus chemotherapy was administered with/without radiotherapy.
[b] Adjunct anti-cancer therapy was reported to be "routine treatment".

CIK cell therapy combined with chemotherapy

The clinical use of ICI in CRC is currently limited to patients with microsatellite instability (MSI)-high and DNA mismatch repair (MMR)-deficient disease [48]. Chemotherapy remains one of the most common treatments for CRC, and combined treatment of chemotherapy with ICIs is a well-accepted approach in other solid cancers, such as low PD-L1 expressing non-small cell lung cancer, where the efficacy of ICI monotherapy is marginal [49,50]. Therefore, combining CIK cell therapy with chemotherapy, as reported in most CRC CIK cell therapy trials (Table 1), appears prudent. We reported that 66 of 70 studies combined CIK therapy with chemotherapy, FOLFOX or XELOX being the most common regimes [46,51–105]. Combination treatment with chemotherapy has an advantage over CIK monotherapy as it likely achieves more rapid cytoreduction. The resulting tumour cell death may further increase tumour antigen-presentation to immune cells, enhancing CIK cell therapy benefit. In a single study comparing DC-CIK therapy to no active treatment in Stage III-IV CRCs there was a comparable ORR of 1.8 % and 0 % in the respective treatment arms [106]. It appears that adjunct anti-cancer treatments such as chemotherapy augment the clinical benefit of CIK cells in CRC patients.

It is not clear if treatment with chemotherapy alters the CIK cells

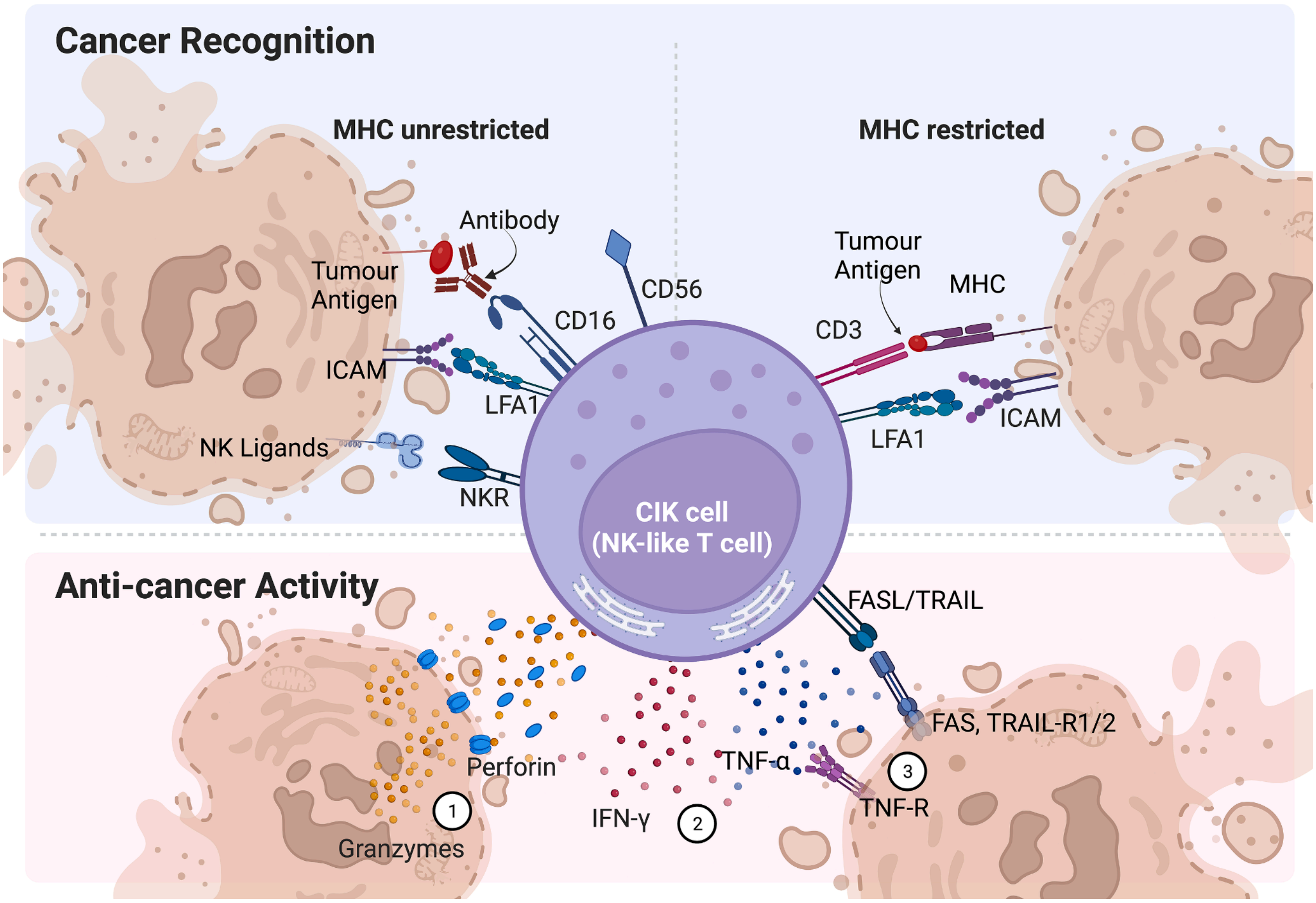

Fig. 2. Cancer recognition and anti-cancer activity of CIK cells. CIK cells, primarily the NK-like T cell subset identified by CD56 and CD3 expression, recognise tumours using both MHC restricted and unrestricted pathways. MHC restricted pathways rely on TCR-MHC engagement. MHC unrestricted recognition relies on Natural Killer cell Receptors (NKRs), NKG2D, DNAM-1 and NKp30. Engagement between cells is supported by LFA-1/ICAM binding. Finally, some CIK cells are reported to express CD16 which allows for Antibody-Dependent Cell-Cytotoxicity. Upon cancer recognition, CIK cells mediate anti-cancer activity by (1) release of cytolytic granules containing granzymes and perforin to induce cell death (2), release of proinflammatory cytokines IFN- γ and TNF-α to induce an anti-cancer microenvironment, and (3) use of the Death ligand/Receptor pathway using TNF, FASL and TRAIL to induce cell death.

produced from a patient. Pan et al. retrospectively compared adjuvant chemotherapy with or without CIK therapy in Stage II-IV patients who had complete resection of CRC [80]. The authors performed flow cytometric phenotyping of CIK cells generated from blood taken at each cycle of treatment for the first 4 cycles. The results showed that the proportion of the CD3+ CD8+ cells remained unchanged, while by the fourth treatment cycle, CD3+ CD4+ cells were reduced with an increase of CD3-CD56+ and CD3+ CD56+ cells. The analysis on patients with unresectable metastatic CRC demonstrated similar results and together hint that collection of PBMC from patients on chemotherapy may not compromise CIK cell production [79].

Evaluation of new cancer therapeutics in patients after complete surgical resection is more difficult than in patients with advanced disease, where treatment efficacy can be measured quickly using imaging or tumour markers. Risk-benefit assessment of cancer therapeutics is also more complex in the adjuvant setting as risks including toxicities of drugs are likely, and drug administration can only impair and not improve the quality of life in the absence of cancer and cancer-related symptoms. While our systematic review indicated no difference in the survival benefit of CIK therapy when it was administered concurrently or sequentially with chemotherapy, the majority of metastatic CRC patients require ongoing administration of chemotherapy, frequently until disease progression or intolerable toxicities, to achieve better disease control. For these reasons, it may be reasonable for clinical research around establishment of CIK therapy in CRC management to focus on advanced staged patients with CIK therapy being infused in a concurrent manner with chemotherapy. This approach potentially allows limiting the exposure to chemotherapy and hence minimising the cumulative chemotherapy-related toxicities. Many chemo-immunotherapy protocols now exist for various solid cancers where induction treatment consists of chemotherapy combined with ICIs, with administration of ICI alone continuing in the maintenance phase [107,108].

Production and patient delivery of CIK cells in CRC

CIK cell culture

The CIK cell culture conditions utilised are summarised in Table 2. Overall, there was considerable variation between the studies in the CIK cell culture methods. RPMI-1640 was used in 20 studies [100,106,109–114,51–54,60,64,65,85,89,92,94,96], followed by GT-T551 medium in nine studies [103,104,44,46,66,88,90,97,98] and AIM V medium in eight studies [61,64,72,76,95,101,105,111]. Other less commonly used media included X-VIVO 15, TexMACS GMP, SuperCulture L500, GT-T503 and KBM 551 media [115,116,73,79–81,87,91,97]. In four studies, two different media were used at different timepoints of CIK cell culture [64,66,97,111]. The use of serum to enhance CIK cell culture was generally poorly described in the studies examined. In 26 studies [102,117–121,55–59,62,63,67–71,75,77,78,82,84,86,93,99], no details were provided for either the media or serum used, and in 37 studies the information was only available for the media [100,101,103–106,110,111,113,114,122,44,46,51–54,61,64,65,72–74,

Table 2
Summary of CIK culture methods.

	Concurrent (45studies)	Sequential (18studies)	Others (7studies)[a]
Culture media			
AIM-V	3	2	1
GT-T551	7	–	–
KBM 551	1	–	–
L500	1	–	–
RPMI 1640	9	6	3
"Serum-free media"	1	–	1
TexMACS GMP	–	1	–
X-VIVO 15	2	1	–
AIM V and RPMI 1640	2	–	–
GT-T551 and X-VIVO	1	–	–
GT-T503 & GT-T551	1	–	–
Not reported	17	8	2
Serum			
Human serum incl. self-serum or plasma	4	–	1
FBS	1	1	–
Not reported[b]	40	17	6
CIK culture duration			
7–13 days	17	6	2
14–21 days	17	8	2
7–21days[c]	4	1	1
>21 days	–	1	–
Not reported	7	2	2
Supplements			
IFN-γ + IL-2 + anti-CD3	16	6	2
IFN-γ + IL-2 + anti-CD3 + IL-1α	7	6	3
IFN-γ + IL-2 + anti-CD3 + IL-1	1	–	–
IFN-γ + IL-2 + anti-CD3 + IL-15	1	–	–
IL-2 + anti-CD3 + IL-1α	1	–	–
TNF-γ + IL-2 + anti-CD3	1	–	–
IFN-γ + IL-2	1	–	–
IL-2	2	–	–
Not reported	15	6	2

[a] Includes 1 study where CIK was infused both concurrently and sequentially with chemotherapy, and another study where timing of CIK therapy infusion in relation to chemotherapy was not described.

[b] Includes studies where only media details were given without any reference to serum. Twenty-four, nine and four studies met this description from concurrent, sequential and others categories, respectively.

[c] Includes studies where CIK culture duration could not be categorised for 7–13 days or 14–21 days as the reported range of culture duration incorporated both categories.

76,79–81,88–92,94–98], making it unclear if serum supplementation was employed or not. Commercial human serum supplementation was used in five studies [60,66,83,87,109] and fetal bovine serum (FBS) was used in two [85,112]. The utilisation of FBS in CIK cell cultures can expose recipients to xenoimmunization and the possibility of zoonotic disease transmission [123]. FBS use does not comply with good manufacturing practice (GMP) guidelines and future CIK cell therapies should utilise GMP-grade serum-free media [124,125].

IFN-γ, IL-2 and anti-CD3 were used to supplement CIK cell cultures in 42 studies [105,106,109–114,122,44,46,51–54,60,61,64–68,72–74,76,79–81,83,85,88–92,94,95,97,99–101]. While the concentration of IFN-γ was generally 1000 U/mL, that of IL-2 and anti-CD3 ranged from 300 to 1000 U/mL and 10 to 350 ng/mL, respectively. IL-1 or IL-1α were used in conjunction with IFN-γ, IL-2 and anti-CD3 in 17 studies with their addition to CIK cell cultures occurring after the first 24 h of incubation [101,106,109,110,113,114,52,53,61,64,66,73,79,80,83,89,92]. IL-1 has been shown to increase cytotoxicity of CIK cells when combined with IFN-γ, IL-2 and anti-CD3 [20]. The most commonly utilised IL-1α concentration was 100 U/mL. IL-15 was added to the CIK cell culture as an additional supplement to IFN-γ, IL-2 and anti-CD3 in one study [85]. In 23 studies, the details of cytokines used to supplement CIK cell culture were not provided [102,103,117–121,55–59,62,63,69–71,75,77,78,82,84,93].

The most commonly used incubation period for CIK cell cultures was 14–21 days (27studies) [100,101,105,109,111,112,121,46,51,52,56,57,60–62,65–67,73,76,77,79–81,83,85,95], followed by 7–13 days in 25 studies [102,106,113,114,122,44,53,54,58,68,69,71,72,74,78,84,86,87,90,92–94,96,99]. The longest incubation period reported was 28 days in one study [89].

Quality assurance

Laboratory assessment of cells prior to delivery to patients is an important quality assurance step in the manufacture of autologous or allogenic T cell products. It typically involves flow cytometry to ensure an adequate number of CIK cells, sterility testing, and testing for cell cytotoxicity [124]. Evaluation of these parameters varied across the 70 studies (Sup. Table 1). Seven studies performed phenotyping on cultured CIK cells prior to infusion [106,112,44,54,67,79,80], 27 studies on peripheral blood after at least one infusion of CIK cell therapy [103,110,113,114,51–53,56,57,60,61,65,72,74,78,82,83,85–89,91,94,97–99] and seven studies on both cultured CIK cells and peripheral blood [46,68,76,95,103,105,111]. Commonly studied surface markers were CD3+, CD4+, CD8+ and CD56+, while some studies additionally analysed CIK cells or peripheral blood for surface markers of NK cells (CD16+), B cells (CD19+) or IL-2 receptor expression (CD25+). Testing for cytokines IFN-γ, IL-2, IL-6, IL-10, IL-12 and/or TNF-α was undertaken on peripheral blood in six studies [112,65,74,87,88,91]. Cytotoxicity of cultured CIK cells was checked in six studies using cell lines or autologous tumour cells as targets [60,67,97,101,106,109]. The observed low rate of phenotyping of the infused CIK cells is a concern as it could help in understanding differences in efficacy of CIK cells [126].

Testing for contamination with pathogens or endotoxins was performed in 12 studies [101,105,112,44,46,68,74,75,79,80,84,95]. Although unreported sterility testing cannot be excluded, low rates of sterility testing is worrisome as the contamination of CIK cells would place recipients at risk. For CAR-T cell therapy, sterility testing is recommended for each of cultured batch prior to patient infusion [15,125].

Timing of blood collection

The timing of blood collection varied greatly between the studies (Table 3). There was a correlation observed between PBMC collection timing and whether CIK therapy was being administered concurrently or sequentially with chemotherapy. Of the 45 studies where CIK therapy was administered concurrently with chemotherapy, in 30 PBMC were collected before chemotherapy [100–103,111,44,46,51–53,56,57,60,63,65,73–75,77–79,83–86,88,90,93,97,98] and in one study after [64]. Timing of PBMC collection was not described in the remaining 14 studies [55,59,66,76,87,91,96,99,104,114,69–72]. Among the 18 studies with sequential administration of CIK therapy with chemotherapy, three studies reported PBMC collection before [61,81,94] and three after [80,95,112] chemotherapy administration. For studies where PBMC were collected before chemotherapy administration, it happened either on the day of chemotherapy or 1–3 days before the chemotherapy commencement in 25 studies [100–103,111,46,51,52,54,56,60,63,65,73–75,77,83–86,90,93,97]. The longest gap between PBMC collection and chemotherapy infusion was 14 days [61,79]. The immunomodulatory effects of chemotherapy and their potential to affect PBMC profiles have been raised previously [127,128], however, the exact extent and the nature of their impacts on the subsequent CIK cell composition and anti-cancer activity is currently unknown.

CIK cell therapy delivery

The timing of CIK cell patient infusion was variable (Table 3). For the concurrent studies, CIK cell infusion occurred early in the chemotherapy cycle in three studies [46,52,97], mid cycle in seven studies [57,63,70,77,78,99,100] and late cycle in 13 studies

Table 3
CIK therapy infusion protocols.

	Concurrent (45studies)	Sequential (18studies)	Others (7studies) [a]
PBMC collection timing			
0–3 days before chemotherapy	25	–	–
7–14 days before chemotherapy	2	1	–
Before chemotherapy, details unknown	3	2	–
After chemo, details unknown	1	3	–
Not reported	14	12	5
7 days before RFA or TACE	–	–	2
CIK therapy Infusion timing[b]			
Early cycle	3	–	–
Mid cycle	7	–	–
Late cycle	13	–	–
Not reported	22	4	5
≤2 weeks after chemotherapy	–	6	–
>2 weeks after chemotherapy	–	8	–
7 days after RFA	–	–	1
Between 2 cycles of TACE	–	–	1
CIK therapy cycle number			
1	1	4	2
2	8	1	–
3	8	4	–
4	1	–	–
5	1	1	–
6	2	1	–
12	2	–	–
Not reported	17	2	3
Variable (1–160)	5	5	2
Number of CIK therapy infusions per cycle			
1	8	4	–
2	8	–	–
3	11	4	–
4	1	1	1
5	–	1	2
8	–	1	–
Variable (3–8)	1	1	–
Not reported	16	6	5
Infused CIK/DC-CIK cell number per cycle			
$<5 \times 10^9$ cells	6[c]	3[d]	2
5×10^9–2×10^{10} cells	7	8	2
$\geq 2 \times 10^{10}$ cells	1	1	1
Not reported	31	6	2

[a] Includes 1 study where CIK was infused in both concurrent and sequential manners with chemotherapy and another study where timing of CIK therapy infusion in relation to chemotherapy was not described.
[b] Timing of CIK therapy infusion was described in reference to chemotherapy cycles.
[c] Includes 2 study where 1.5–6.3 × 10^9 cells and 1.5–6.2 × 10^9 cells were infused, respectively.
[d] Includes 1 study where 3–9 × 10^9 cells were infused.

[101,102,111,44,51,56,65,71–74,91,93]. For studies with sequential administration, the time lag between chemotherapy and CIK therapy infusion varied from two days to one month. Specifically, CIK cell therapy was administered within two weeks after chemotherapy in five studies [58,62,82,94,112], at two weeks in three studies [53,61,92] and after two weeks in six studies [118,121,67,68,80,95].

The number of CIK therapy cycles varied between studies: one cycle in seven studies [53,60,89,94,109,122], two cycles in nine studies [44,46,56,65,69,75,81,93,111], three cycles in 12 studies [117,118,121,52,62,63,71,72,78,88,99,100], four cycles in one study [74], five cycles in two studies [68,85], six cycles in three studies [92,96,101] and 12 cycles in two studies [75,97]. In 11 studies, the cycle number was not set with 160 cycles being the maximal number of cycles infused [103,105,106,112,64,67,73,79,80,91,95]. Each cycle of CIK therapy frequently consisted of multiple CIK cell infusions (Sup. Table 1). The number of CIK cell infusions performed per cycle was one in 12 studies [101,104,117,121,68,74,80,88,91,96,98,99], two in eight studies [100,46,51,71,72,75,78,84], three in 15 studies [102,103,111,52,54,57,60,65,67,70,81,82,85,93,94], four in three studies [44,53,122], five in two studies [89,109], and variable in two studies [73,112].

Four studies reported the number of DC-CIK cell infusions, while 26 studies reported the number of CIK cell infusions (Sup. Table 1). The number of CIK +/− DCs infused per cycle ranged from 1×10^9 cells to 1.6×10^{11} cells, with more than half of the studies infusing 1–9 × 10^9 cells per cycle. A review previously reported that the total number of CIK cells infused to patients ranged from 6×10^6 to 1.5×10^{10} cells per infusion [129].

Infusion protocol and clinical benefit

The potential impact of differing culture and infusion protocols on the clinical benefit of CIK therapy has never been investigated. Challenges arise from the variations in the target patients, including their clinical stage, history of previous systemic treatments, as well as the adjunctive treatment administered with CIK cell therapy across and sometimes within studies. In an attempt to shed light on this, we inspected how these parameters affect OS and ORR in the 19 studies included in our systematic review in which Stage IV CRC patients were treated with chemotherapy with/without CIK therapy in a concurrent manner [100–102,104,114,54,57,59,60,69–71,79,83,84,90,91,96,98] (Sup. Table 2). Estimated hazard ratios (HRs) for OS were reported in seven studies, with all of them reporting favourable outcomes for CIK cell therapy with six of the HRs reaching statistical significance [102,71,83,84,91,96,98]. Within the limitations arising from the small number of studies available, no obvious association was found between the culture or infusion protocol parameters and the estimated OS HR. The reported ORR for the CIK and non-CIK arms were diverse across the 19 studies and it was not possible to make any meaningful assessment on the link between culture and infusion parameters and ORR. Further research is required to identify culture and infusion protocols which achieve the highest level of clinical efficacy. Phenotyping data of the CIK cells was only reported in one study [101] and raises the possibility for variations in the quality and anti-cancer efficacy of CIK cell therapy across studies. Studies involving phenotypic characterisation of CIK cell therapy products before patient infusion and correlating how these relate to patient outcomes are still required. In order for CIK cell therapy to be integrated into mainstream clinical practice, future studies should work towards establishing standard operating procedures for CIK cell therapy production and infusion.

Future perspectives

CIK cell therapy was first described over 30 years ago. There is a renewed interest to investigate how this therapy fits within the current and future immuno-oncology landscape [130]. Having provided an overview of the current production and delivery of CIK cell therapy in CRC treatment, we will now discuss some critical parameters that require further development to facilitate broader adoption.

Cryopreservation

Given that all CIK cell therapy trials have reported multiple infusions into patients (Table 3), it was surprising to note that cryopreservation strategies were not reported in these studies. Cryopreservation is an integral part of the adoptive cell therapy supply chain to keep cellular products viable and functional. Further, large scale production and subsequent cryopreservation allows the use of aliquots of the same batch of CIK cells over the course of treatment. Established cryopreservation protocols can lead to adoption of CIK cell therapy in treatment centres without cell manufacturing capabilities. Without optimized cryopreservation and thawing protocols, the CIK cells' viability and functionality could be compromised [131]. Cryopreserved PBMCs can be used to generate functional CIK cells [132], and limited studies suggest that CIK

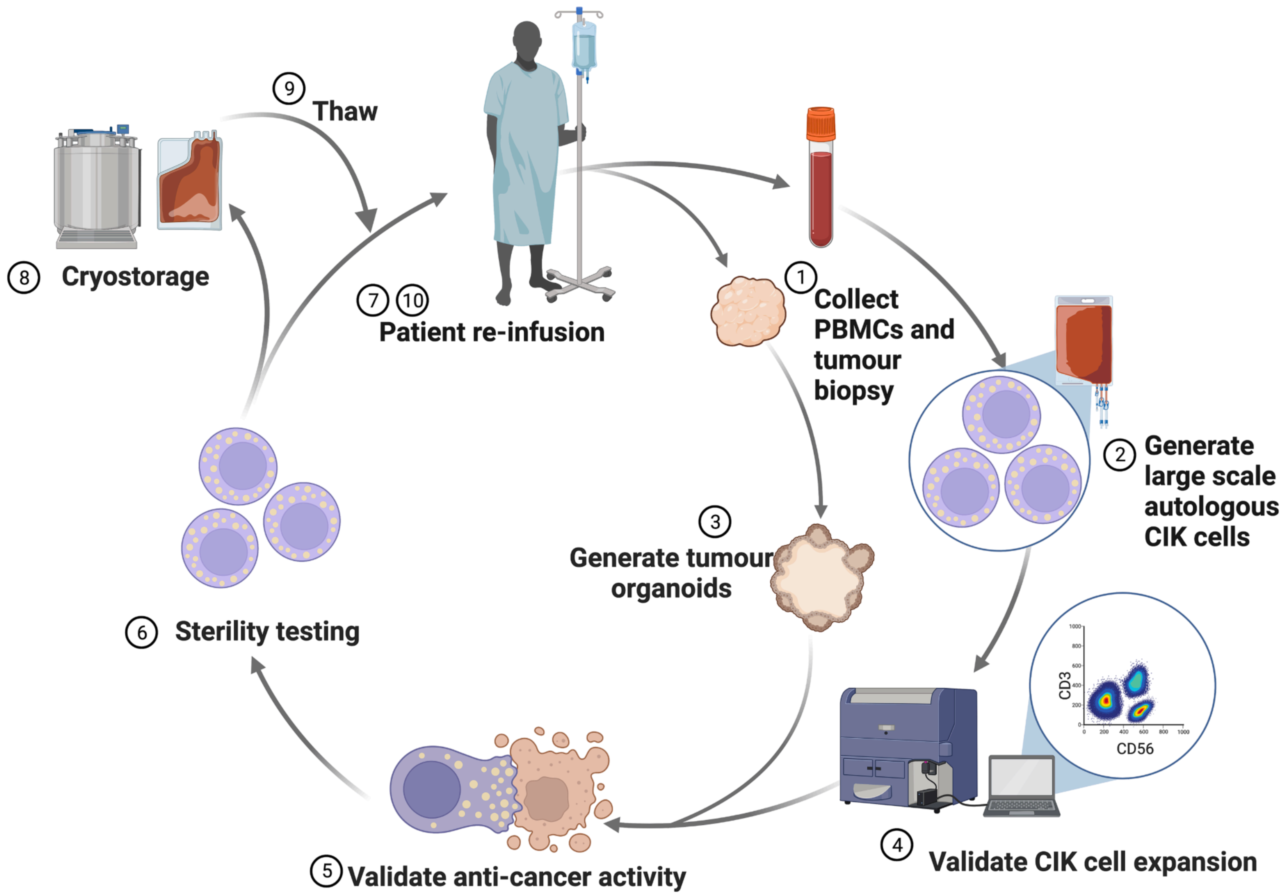

Fig. 3. Proposed autologous CIK cell therapy clinical workflow. (1) Peripheral blood mononuclear cells (PBMCs) and tumour biopsies are collected from the same cancer patient. (2) PBMCs are expanded in vitro using standard large scale CIK culture protocols. (3) Tumour biopsies are cultured to generated patient-derived tumour organoids. Both CIK cell culture and organoids are expected to be ready after 2–3 weeks. Recommended Quality Assurance steps are then performed. (4) CIK cell culture product is validated by expansion of key cellular components i.e., expansion of CD3+ CD56+ NK-like T cells by flow cytometry. (5) CIK cells tested against matched patient-derived tumour organoids. (6) CIK cell therapy products pass sterility and toxicity testing. (7) Freshly cultured CIK cell therapy product is infused into the patient, while (8) remaining products are placed in cryostorage. (9) Cryopreserved CIK cell culture products are thawed for (10) patient infusion as required by the CIK cell therapy treatment plan. Patient or institute dependent variables include cell infusion dose, number of infusions and combination with other therapies.

cells can be cryopreserved. One study proposed that thawed CIK cells require re-exposure to IL-2 to restore their cytotoxic capabilities [133]. However, subsequent studies did not report using IL-2 re-exposure, and cryostorage for up to 2 years did not significantly impact on the viability, cellular composition or cytotoxic abilities of these cells [134,135]. Together, these studies suggest that cryopreservation is a viable strategy and should be considered for future deliveries of CIK cell therapies.

Markers for quality assurance and therapeutic efficacy

This review shows that quality assurance strategies for CIK cell delivery in CRC trials are often poorly described and of variable quality. Improvements in this aspect would assure recipients that the therapy is safe and contains clinically effective cellular products. Thus, standardised quality assurance metrics, in particular flow cytometry panels and cytotoxicity screens, could offer insights into therapeutic efficacy prior to patient infusion. In virtually all reports CIK cell therapy doses were counted as total cells without necessarily quantifying the key cellular subsets (Sup. Table 1). Flow cytometry panels could be used to confirm the expansion of the NK-like T cell subpopulation and if immunosuppressive cells are present in the cell culture product. We suggest that dosing based on the number of NK-like T cells rather than total cells could lead to better tumour clearance and be more economical than using sorting-based enrichment strategies. Further, a rigorous well-defined and widely adopted flow cytometry panel could provide valuable data to identify associations between CIK cell composition and therapeutic outcomes.

Cytotoxicity screens have only been used in six studies, with most using cancer cell lines [60,67,97,101,106,109]. Matched patient-derived tumour organoids (PDTO) could be implemented as a personalised screen to determine the cytotoxic efficacy of the CIK cell culture. PDTOs are three dimensional primary cell cultures that resemble their tissue of origin, and are being tested *in vitro* as predictors of cancer immunotherapy responses *in vivo* [136]. In CRC, PDTOs have been used to identify highly potent TILs and their respective TCRs [137]. Further, TIL-PDTO cytotoxicity assays can predict CRC patients with functional anti-cancer immunity who will respond to neoadjuvant chemotherapy and potentially ICIs [138]. Considering that CRC PDTOs can be generated within 2–3 weeks [139], matched CIK-PDTO testing could be integrated to current CIK cell therapy workflows to screen for efficacy prior to infusion (Fig. 3).

Finally, patient biomarkers to predict favourable long-term clinical outcomes from CIK cell therapy are being reported in some cancers, but not CRC. Recent reports suggest that high tumour infiltration of PD-1+ TILs in hepatocellular carcinomas [140] or high PD-L1 expression in breast cancer tissue [141] have led to improved survival outcomes in patients receiving CIK cell therapy. Tumour expression of MHC I-related Chain A (MICA) protein and change in T cell receptor repertoire in response to *ex vivo* T cell expansion have been proposed as additional predictive biomarker candidates in gastric cancer [142,143]. This suggests that investigating both the cancer cells and the tumour microenvironment can potentially lead to new prognostic biomarkers for CIK cell efficacy.

Synergies with monoclonal antibodies

Monoclonal antibodies, including ICIs, are already used in CRC treatment as standard of care [144]. Combining these monoclonal antibodies with CIK cell therapy may be easier to translate to the clinic. As mentioned previously, PD-1/PD-L1 expression in tumours is a positive prognostic predictor for benefit from CIK cell therapy [140,141]. Additionally, ICIs can augment the cytotoxic ability of CIK cells by blocking checkpoint inhibition in CIK cells [145–147]. ICIs such as PD-1/PD-L1 blockade only work in CRCs with high mutational burden or 'hot' tumours characterised by high TILs [148]. Critically, conversion to 'hot' tumours can be initiated by increasing TILs in tumours [149]. Delivery of CIK cell therapies to 'cold' CRCs may drive further inflammation and recruitment of TILs converting them to 'hot' tumours. Thus, this combination could potentially increase the proportion of CRC patients that benefits from ICIs. Together, this suggests that combining CIK cell therapies may synergise with ICIs in CRC treatment.

Therapeutic monoclonal antibodies also target growth factor pathways that are highly dysregulated in CRC [144]. Receptor binding antibodies against epidermal growth factor receptor (EGF, Cetuximab and Panitumumab for wild-type RAS CRC), vascular endothelial growth factor (VEGFR, Ramucirumab), mesenchymal-epithelial transition factor (c-MET, Onartuzumab) and human EGF receptor type 2 (HER2, Trastuzumab and Pertuzumab for HER2 positive CRC) have shown efficacy in often specific populations of metastatic CRC. Interestingly, by binding to these receptors that are typically overexpressed in CRC cells, these antibodies have potential to induce ADCC mediated tumour clearance [144]. Co-administration with CIK cell therapy could therefore improve clinical outcomes by acting as a tag for CD16+ CIK cell subsets capable of ADCC [33,34]. Extending this concept, bi-specific T-cell engagers (BiTE) are artificial monoclonal antibodies that contain two distinct antigen binding domains, one specifically designed to bind to the TCR while the other binds to a tumour antigen. This binding promotes T cell engagement to cancer cells leading to tumour cytotoxicity. There are multiple BiTEs in development for CRC [150]. Similarly, natural killer cell engagers (NKCE) which are bi-specific antibodies that bind to both an NK cell receptor and a tumour antigen are under development [151]. Considering that CIK cells have both functional TCRs and NK receptors, we hypothesise that these technologies will be suitable for combination with CIK cell therapy.

Final thoughts and concluding remarks

In this review, we have highlighted the variability in the current practices for CIK cell therapy of CRC, and highlighted key areas for improvement so that a standardised CRC CIK cell therapy protocol can be developed (Fig. 3). However, CIK cell therapy is still an understudied adoptive cell therapy and many questions remain that could significantly improve its utility. CRC patients have reported minor adverse events when treated with CIK cell therapy [19]. More broadly, CIK cell therapy causes minimal side effects or graft versus host disease, making it ideally suited for the development of allogeneic adoptive cell therapies [152–154]. As a consequence, CIK cells have been proposed for allogeneic CAR T cell development [155]. Thus, we can envisage that future CRC CIK cell therapies could be available 'off the shelf' using blood from allogeneic donors. CIK cells may potentially be improved by incorporation of CARs. Studies have also attempted to improve CIK cultures by changing the cytokines used for cell culture [135,156]. However, we believe that we must first fully understand the cellular components in CIK cell therapy, in particular the identity of precursors that are responsible for longevity of these cells *in vivo* and the key effectors cells that infiltrate the tumours and mediate cytotoxicity. While there is some knowledge about these cell subsets, recent advances in single cell analytical techniques such as single-cell RNA sequencing (scRNA-seq) [157] and high dimensional flow cytometry [158] will allow us to identify key CIK cellular subsets to target for further enrichment in culture and patient transfer. With scRNA-seq we can identify the cell subsets present and the key signalling pathways and transcription factors that drive their differentiation. This should lead to more informed decisions on how to base our quality assurance screens for patient transfers and for potential modifications in the therapeutic product.

In conclusion, results from clinical trials of CIK cell therapy in CRC show that clinical benefit can be obtained despite diverse cell culture and treatment strategies. A standardised protocol is likely to increase the uptake of this therapy. By summarising the current strategies employed, many not easily accessible to the English language audience, this review serves as a first step to address the challenge of improving the utility of CIK cell therapy for the treatment of CRC.

Ethics approval and consent to participate

Not applicable.

Consent for publication

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CRediT authorship contribution statement

Celine Man Ying Li: Funding acquisition, Writing – original draft, Conceptualization, Writing – review & editing. **Runhao Li:** Writing – review & editing. **Paul Drew:** Supervision, Writing – review & editing. **Timothy Price:** Supervision, Resources. **Eric Smith:** Writing – review & editing. **Guy J. Maddern:** Supervision, Resources. **Yoko Tomita:** Supervision, Writing – review & editing, Conceptualization. **Kevin Fenix:** Writing – original draft, Conceptualization, Resources, Writing – original draft, Writing – review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

All data generated or analysed during this study are included in this published article and its supplementary information files.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ctrv.2023.102665.

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