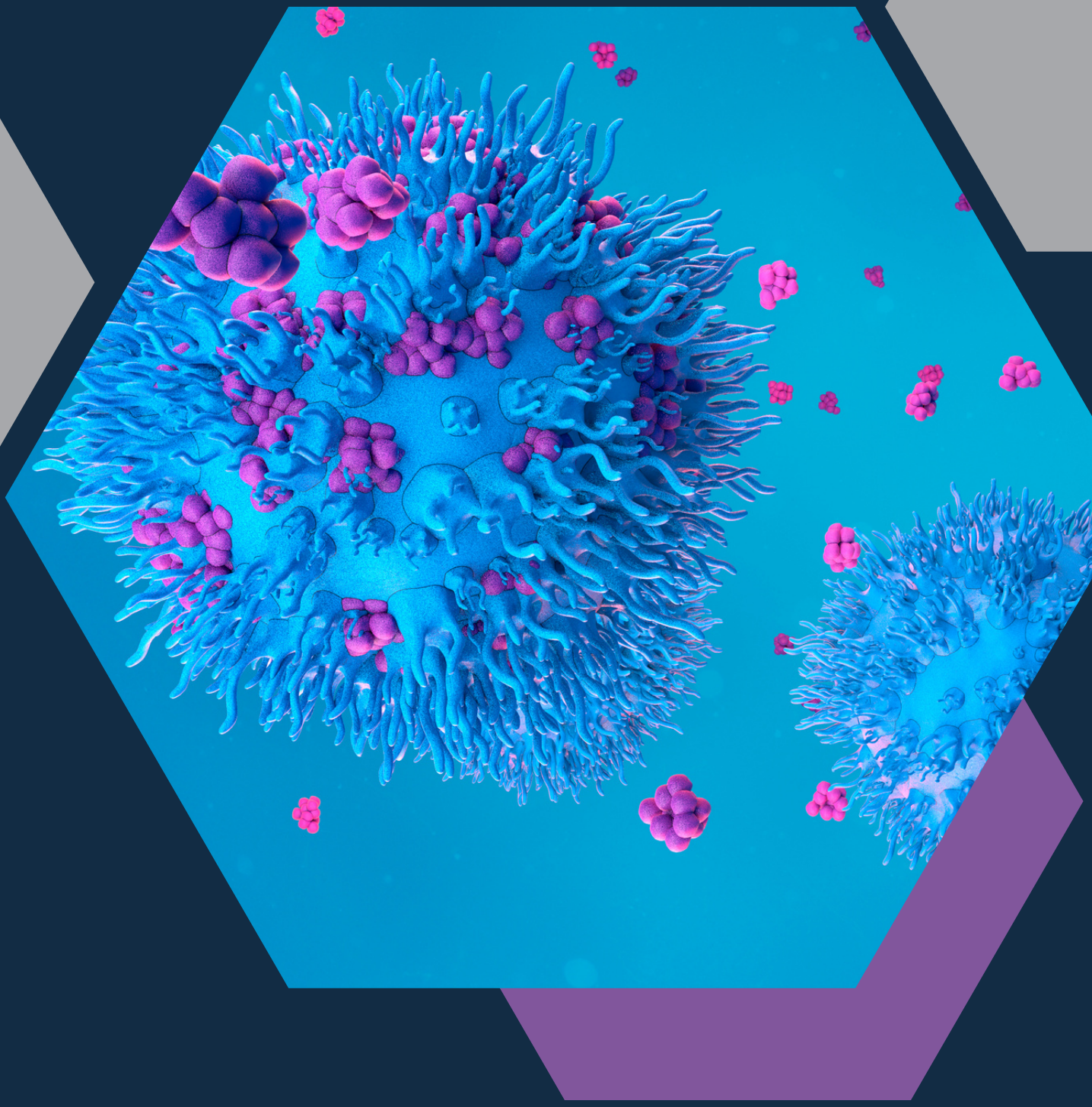
BioTechniques
The International Journal of Life Science Methods



Advancing CAR-T therapy

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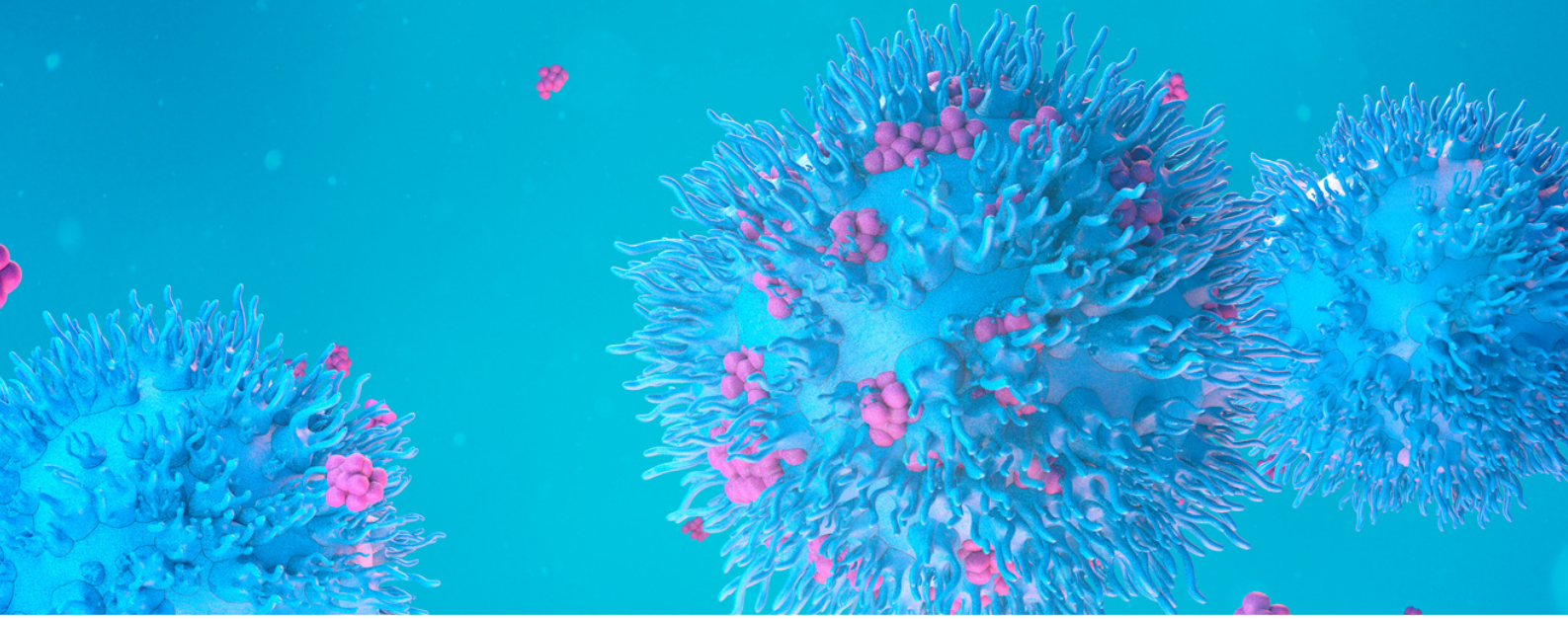

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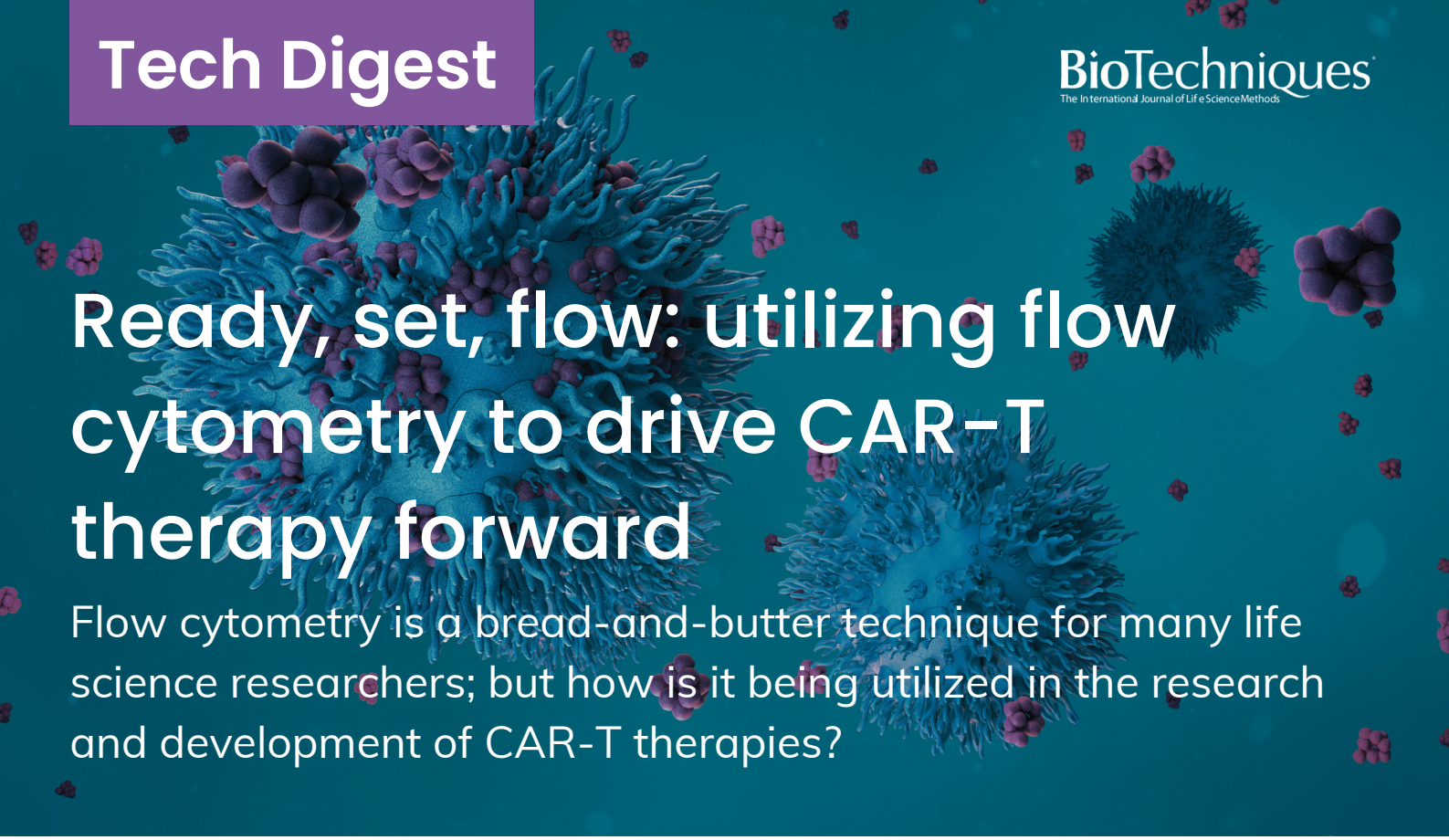

Ready, set, flow: utilizing flow cytometry to drive CAR-T therapy forward

Flow cytometry is a bread-and-butter technique for many life science researchers; but how is it being utilized in the research and development of CAR-T therapies?

The rise of CAR-T therapies

In recent years, there has been a shift toward the development of more targeted and personalized therapies for many diseases, which are hoped to increase the probability of success for patients [1]. In the treatment of cancer, chimeric antigen receptor (CAR)-T cell therapies have generated exciting results: people with B cell leukemias and lymphomas – who were once deemed untreatable – have been cured, and efforts are underway to extend this success to other cancers [2]. With six CAR-T therapies approved by the US FDA and hundreds more in clinical trial stages, what we once saw as therapies of the future are now therapies of the present.

CAR-T therapy involves modifying a patient's T cells to specifically bind and kill cancer cells. CAR-T cells are typically developed by harvesting T cells from the patient and engineering those T cells to express a synthetic receptor called a CAR, which recognizes a specific antigen found on the surface of cancer cells. Once the CAR-T cells bind to the antigen, they replicate events that occur after T-cell activation, including proliferation, the release of cytolytic granules resulting in cytolysis of cancer cells, tumor necrosis factor (TNF) family member–mediated apoptosis and the secretion of cytokines, interleukins and growth factors [3].

Despite their success, significant challenges remain in developing and optimizing CAR-T therapies, particularly when broadening their use to solid tumors [4]. In addition, while some patients respond well to treatments, others are refractive, and uncovering the basis for these differing outcomes can make them more effective. One technique that can be used to help address these challenges is flow cytometry.

Utilizing flow cytometry

Flow cytometry is a widely used technique that allows for the analysis of multiple cellular characteristics simultaneously. It involves passing cells through a laser beam and measuring the scattered and emitted light to identify and quantify cellular features such as size, shape and fluorescence.

Flow cytometry has a broad range of applications, including cell sorting, the study of

cell proliferation, apoptosis, DNA analysis and the quantification of cell surface and intracellular proteins [5]. As flow cytometry allows for the quantification and characterization of both immune effector and tumor cells at the single-cell level, it has proven to be an important tool in CAR-T therapy studies. For example, using labeling approaches, researchers can distinguish between effector cells, such as T cells, and tumor cells. Cell viability markers can be used to distinguish between live and dead cells. The cells can also be characterized for a number of parameters, including T-cell-dependent cellular cytotoxicity, T-cell phenotype and T-cell activation, degranulation and exhaustion. The evolution of high-throughput flow cytometry platforms, such as the iQue® Advanced Flow Cytometry Platform, extends the capabilities of traditional flow cytometry and enables the concurrent measurement of multiple parameters in a single assay [5]. The platform also allows for reduced sample volumes, rapid sample acquisition, data analysis and ease of use.

These factors make flow cytometry a powerful tool for analyzing T cells, CAR-T cells and tumor cells, and therefore an ideal technique to utilize when addressing some of the challenges associated with CAR-T therapies.

Targeting specific antigens

Although CAR-T therapy has transformed the treatment landscape of hematological cancers, its efficacy in solid tumors is limited. One reason for this is the lack of specific antigens expressed by tumor cells as antigens expressed on the surface of cancer cells are often shared with normal tissues, and because solid tumors are so heterogenous, the antigen may not be expressed by all the cells in the tumor [6]. Most approved CAR-T therapies target CD19, an antigen expressed in both malignant and healthy B cells, and although this means that the CAR-T cells will target healthy B cells as well, B cell aplasia from treatment can be managed. As solid tumors arise from organs or tissues that are indispensable, targeting antigens that aren't specific to cancer cells could harm whole tissues or organs [7]. Therefore, finding and targeting antigens that are specifically expressed by solid tumor cells is vital for developing CAR-T therapies for solid tumors, and flow cytometry can help in these studies.

B7H3 is a transmembrane protein that plays an inhibitory role in adaptive immunity. It has limited expression in normal cells but an abnormally high expression in a range of malignancies, suggesting it could be an attractive target for CAR-T cells [6]. One of the malignancies B7H3 is overexpressed in is pancreatic ductal adenocarcinoma (PDAC). Building on this information, a group of researchers from the University of North Carolina, Chapel Hill (NC, USA) created CAR-T cells targeting B7H3, and tested their efficacy against PDAC cells *in vitro* and in mouse models. They used flow cytometry throughout the study, including *in vitro* experiments to assess the expression of B7H3 in PDAC cell lines and the antitumor activity of B7H3 CAR-T cells against those PDAC cell lines, by co-culturing and quantifying residual tumor cells. The results showed that B7H3 CAR-T cells eliminated PDAC cells from the co-cultures and *in vivo* experiments showed that CAR-T cells effectively controlled PDAC tumor growth in xenograft mouse models. These experiments were replicated for ovarian cancer and neuroblastoma, and their findings supported the clinical development of B7H3 CAR-T cells [8].

The complex tumor microenvironment

The tumor microenvironment (TME) also presents a barrier to broadening CAR-T therapies to solid tumors. The TME is a complex and dynamic entity that influences tumor growth and survival and consists of immune cells, stromal cells, blood vessels, cytokines and

the extracellular matrix. The TME and dense tumor stroma make it difficult for CAR-T cells to penetrate solid tumors and techniques are needed to quantitatively measure the number of T cells in a tissue to assess tumor infiltration.

Flow cytometry represents an optimal technique for T-cell quantitation as it can identify specific cell types in a heterogenous mixture. In a study by researchers at the Mayo Clinic (MN, USA), which looked at the ability of CD126 CAR-T cells to target a range of solid cancers, they used flow cytometry to assess the infiltration of CD126 CAR-T cells into solid tumors in a mouse model. They did this by dissociating the cells and quantifying the proportion of T cells, which were identified by the expression of CD3, and found that the CD126 CAR-T cells were better at infiltrating the tumors than normal T cells [9].

Further personalizing CAR-T therapy

Variability also presents a challenge in CAR-T therapy. Not all patients respond equally to CAR-T therapies and by determining the reasons for these disparate responses, researchers hope to make these treatments more effective for those who do not currently respond well. Recently, a group of researchers from Harvard University (MA, USA) utilized flow cytometry to assess T-cell phenotypes, using this information to significantly enhance the consistency and potency of CAR-T cell products [10].

Although there is widespread knowledge of the differences between a cancer patient's T cells and a healthy individual's T cells, these differences are not taken into account when developing CAR-T therapies. This means the process is the same regardless of variations in T-cell phenotype. In the study, the researchers phenotyped T cells from healthy individuals and from individuals with acute lymphoblastic leukemia or chronic lymphocytic lymphoma via flow cytometry and found differences in T-cell phenotypes.

The researchers then generated a library of CAR-T cells from these samples, using both the standard manufacturing process and a process where they adjusted the amount of T-cell activation – an important step in CAR-T cell production that impacts transduction efficiency, rate of CAR-T cell expansion and differentiation. They found that CAR-T cells derived from healthy samples were more functional than those from patient samples. However, they found altering and matching levels of T-cell activation to T-cell phenotype significantly enhanced the tumor-killing abilities of the patient-derived CAR-T cells *in vitro* and in mouse models [10]. This study shows that taking T-cell phenotype into account in the manufacturing process can help make more personalized CAR-T therapies, making them more effective for individuals.

Future perspectives

While CAR-T therapies have undoubtably changed the cancer treatment landscape, there are still barriers to them becoming widespread, front-line treatments. Flow cytometry presents itself as a powerful tool that can be incorporated into studies of CAR-T therapies aiming to overcome these challenges. In the coming years, with hundreds of CAR-T cells in development against cancers, autoimmune, cardiovascular and infectious diseases, we can hope to see more of these therapies cross the finish line.

Written by Annie Coulson, Digital Editor, *BioTechniques*

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Benchmark

BioTechniques

High transfection efficiency and cell viability of immune cells with nanomaterials-based transfection reagent

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ABSTRACT

Gene manipulation in non-adhesive cells, especially lymphocytes, was difficult due to their low efficiency and high toxicity. Electroporation was reported as a highly efficient method for human and mouse lymphocytes. However, this method requires expensive equipment and causes severe cell damage. Here, the authors present a simple and efficient method to deliver siRNA into lymphocytes with high efficiency and cell viability. This nanomaterials-based transfection reagent was simple and cost-effective and can perform multiple transfections, which further increase the overall efficiency. This method should be applicable for many cell lines and can be used to decipher gene functions of lymphocytes.

METHOD SUMMARY

The authors introduce a nanomaterials-based transfection method to deliver siRNA into lymphocytes with high efficiency and cell viability. The data showed that multiple transfections could significantly augment efficiency.

KEYWORDS:

cell viability • electroporation • lymphocyte • multiple transfection • nanomaterial-based transfection • siRNA

Gene manipulation is commonly achieved via exogenous delivery of genetic materials, such as siRNA, into cells to interrogate gene function and is crucial for biomedical research. However, it is sometimes hampered by the low transfection efficiency or cell toxicity in some cell types, especially suspension cells [1]. T lymphocytes, a typical non-adhesive cell type that plays vital roles in adaptive immunity, have been proved to be difficult to transfect [2].

Several transfection methods have been adopted to elucidate lymphocytic gene function, including non-viral and viral-based delivery systems. However, these methods either are equipment-dependent or have low transfection efficiency and viability. For example, lipid-based transfection reagents were widely used for the delivery of exogenous genetic materials, yet lymphocytes and other immune cells were repelled to lipid-based transfection both *in vivo* and *in vitro* [3]. Therefore, retroviral and lentiviral vectors were generally used for the delivery of genetic materials into T lymphocytes [4,5]. However, the viral vector-based methods were time- and cost-consuming, limited by the package capacity and genomic integration risk [6]. Electroporation was another effective means for the delivery of exogenous genetic materials into cells. Nevertheless, electroporation can often cause remarkable cell death and requires expensive equipment, which further restricts the application for ordinary laboratories [6–8]. To overcome these shortages, a simple and cost–effective method with high transfection efficiency and cell viability should be developed. Here, the authors show a simple and efficient nanomaterials-based transfection method that can achieve high efficiency and cell viability.

Lymphocytes play vital roles in physiology and pathology; unveiling the gene functions of lymphocytes is crucial for drug discovery [9,10]. Knockdown gene expression by siRNA is a useful method for discovering gene function and validation of potential drug targets [11,12]. To develop a simple and cost-effective method that is accessible by ordinary laboratories to study lymphocyte function, the authors tested siRNA transfection efficiency and feasibility using a commercially available nanomaterials-based transfection reagent. This reagent is composed of 80–100 nm microspheres with specific group modification and can deliver nucleic acids into cells via endocytosis. They first examined cell viability after electroporation. As expected, primary T lymphocytes and dendritic cell mortalities were increased after electroporation using a 4D nucleofector system (Lonza, Switzerland, DN-100) (Supplementary Figure 1). The authors then tested the siRNA delivery efficiency and cell viability of nanomaterials-based transfection reagent in our $CD4^+$ T cells converted double negative T cells (cDNT) system [13]. $CD4^+$ T cells were obtained from lymph nodes and spleens of C57BL/6J mice using a T-cell enrichment column (R&D Systems, MN, USA). $CD4^+CD25^-$ T cells were isolated by depletion of $CD25^+$, $B220^+$, $Ter119^+$, $CD8^+$, $CD11b^+$, $TCR\gamma\delta^+$ and $NK1.1^+$ cells [14]. Mature dendritic cells (mDCs) were separated from lipopolysaccharide (LPS) activated bone marrow cells of C57BL/6J mice [14]. For transfection experiments, 1.0×10^5 $CD4^+CD25^-$ T cells and 2.5×10^4 mDCs were seeded per well in a 96-well

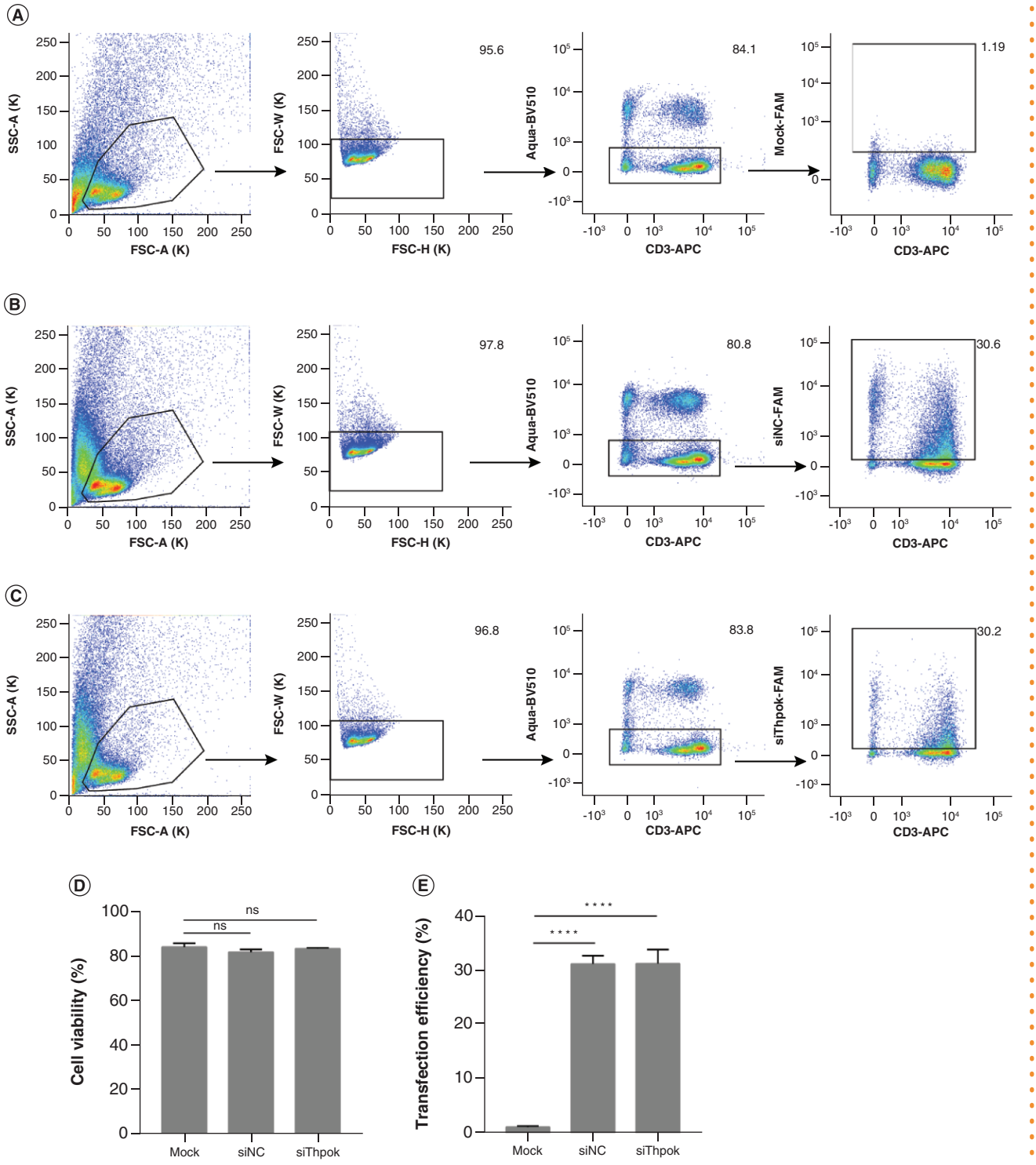

Figure 1. Cell viability and transfection efficiency in T cells and mature dendritic cells 48 h after transfection. (A–C) Flow cytometry analyses of T cells and mature dendritic cells cultured in a 96-well round-bottom plate transfected without siRNAs (Mock) **(A)**, with 0.7 μl (20 μM) fluorescein amidite-labeled negative control siRNAs **(B)** and 0.7 μl (20 μM) fluorescein amidite-labeled *Thpok* targeted siRNAs **(C)** mixed with an equal volume of nanomaterials-based transfection reagent. **(D)** Percentage of cell viability 2 days after transfection with different treatment, one-way analysis of variance. **(E)** Percentage of transfection efficiency 2 days after transfection with different treatment.
****$p < 0.0001$, one-way analysis of variance.
ns: Not significant.

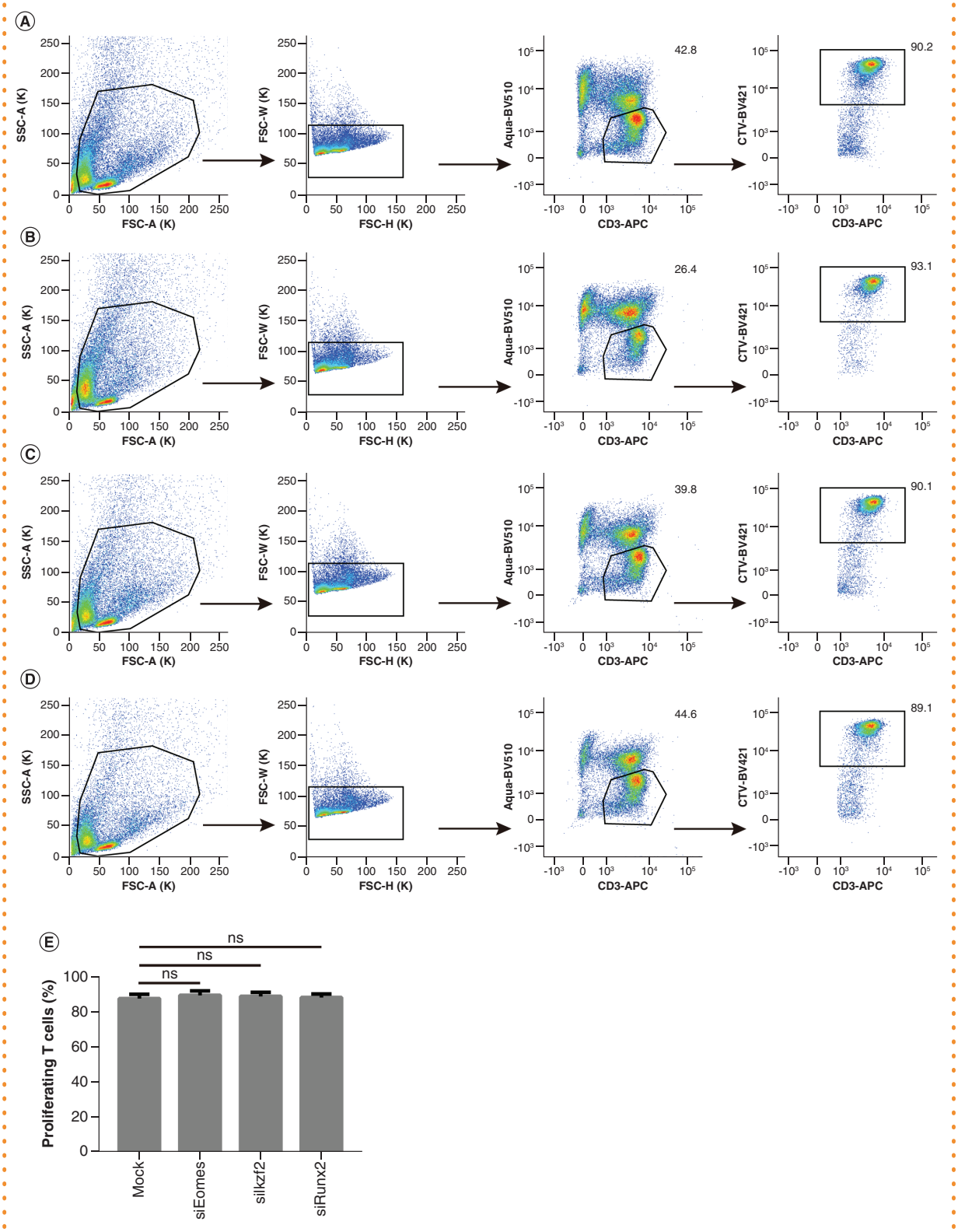

Figure 2. Effect of nanomaterials-based transfection reagent on lymphocyte proliferation 4 days after transfection. (A–D) Representative flow cytometry results show proliferation of lymphocytes in different groups: transfected without siRNAs (Mock) **(A)**, 0.7 μl (20 μM) fluorescein amidite (FAM)-labeled *Eomes* targeted siRNAs (siEomes) **(B)**, 0.7 μl (20 μM) FAM-labeled *Ikzf2* targeted siRNAs (siIkzf2) **(C)** and 0.7 μl (20 μM) FAM-labeled *Runx2* targeted siRNAs (siRunx2) **(D)**. **(E)** Percentage of proliferating T cells 4 days after transfection with different treatment, one-way analysis of variance.

ns: Not significant.

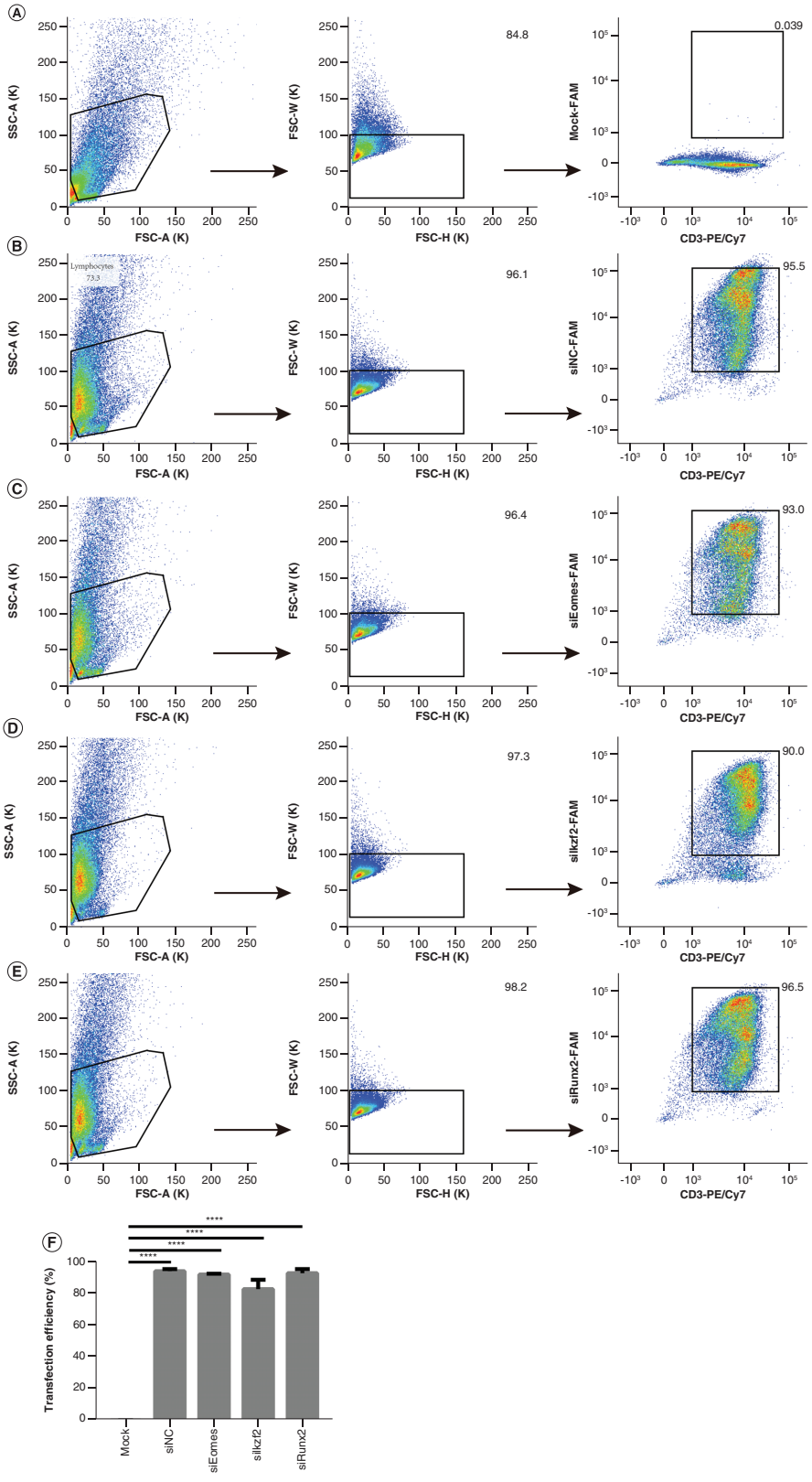

Figure 3. Cell transfection efficiency in T cells 1 week after twice transfection. **(A–E)** Flow cytometry analyses of T cells and mature dendritic cells cultured in a 96-well round-bottom plate on day 7. Transfections were performed on day 0 and day 4 with different groups: **(A)** mock, **(B)** siNC, **(C)** siEomes, **(D)** silkzf2 and **(E)** siRunx2. **(F)** Percentage of transfection efficiency 7 days after transfection with different treatment.
****$p < 0.0001$, one-way analysis of variance.

round-bottomed plate. Then 0.7 μl (20 μM) fluorescein amidite (FAM)-labeled negative control siRNAs (siNC) and *Thpok* targeted siRNAs (siThpok) were mixed thoroughly with an equal volume of nanomaterials-based transfection reagent (BioRainTech, Beijing, China, EC0003, www.bioraintech.com) and incubated for 10 min at room temperature. Next, the transfection complexes were added to each cell-containing well and mixed gently. Cell viabilities were assessed 48 h post-transfection via Zombie Aqua™ fluorescent dye (BioLegend, CA, USA, 423101). The flow cytometry analysis showed that nanomaterials-based transfection reagent does not compromise the viability of $CD4^+CD25^-$ T cells and mDCs, and at least 80% of cells were still alive, which was similar to the non-transfection group (Figure 1). Moreover, efficient transfection was observed in the living $CD4^+CD25^-$ T cells and mDCs with a minimum of 30% efficiency (Figure 1). The knockdown efficiencies of siRNAs were assessed by quantitative real-time PCR (Supplementary Figure 1). Taken together, these data suggest that this nanomaterials-based transfection reagent has little cell toxicity and could deliver siRNA into $CD4^+CD25^-$ T cells and mDCs to a moderate degree.

Though nanomaterials-based transfection reagent does not affect lymphocyte viability, the authors further examined its possible interference on T-cell proliferation. To test this possibility, they used CellTrace™ Violet (CTV) Cell Proliferation Kits (Thermo Fisher Scientific, MA, USA, C34571) to measure lymphocyte proliferation [15]. Cells were collected and resuspended in 1× Dulbecco's phosphate-buffered saline (DPBS) at a maximum density of 2×10^6 cells/ml after co-culture of $CD4^+CD25^-$ T cells and mDCs for 4 days. Besides co-culture, cells were transfected with siRNAs that targeted *Eomes*, *Ikzf2* and *Runx2*, respectively. Then cells were mixed with an equal volume of 4 μm CTV and incubated for 20 min at 37°C. After that, five volumes of MACS buffer (500 ml DPBS, 2.5 g bovine serum albumin [BSA], and 0.5 M ethylenediaminetetraacetic acid [EDTA]) were added into the medium and incubated for another 5 min. Then cells were collected and prepared for flow cytometry analysis. The data confirmed that nanomaterials-based transfection reagent does not affect lymphocyte proliferation (Figure 2). In summary, this nanomaterials-based transfection reagent is safe for the delivery of siRNAs into lymphocytes

Owing to the non-adhesive characteristic of immune cells, the transfection was accomplished by simply adding the transfection complex into the culture medium. Therefore, the authors next tested whether multiple transfections could increase the overall transfection efficiency. They transfected siRNAs that targeted *Eomes*, *Ikzf2* and *Runx2* accordingly on day 0 and day 4 and determined the efficiency on day 7. As expected, the transfection efficiencies were significantly augmented compared with single transfection (Figure 3). Importantly, these augments were universal and highly efficient (Supplementary Table 1). These data suggest that twice transfection could efficiently deliver siRNAs into the majority of lymphocytes.

In summary, the authors demonstrated that nanomaterials-based transfection represents a simple and efficient technique for the delivery of siRNAs into lymphocytes and mDCs. This method has several advantages compared with other approaches. First, it's cost–effective and very efficient to deliver siRNAs into T cells and mDCs. Second, it retains high cell viability and does not affect lymphocyte proliferation compared with electroporation. Besides, it does not require additional, expensive equipment and is commercially available to ordinary laboratories. Third, it's simple and extremely time-saving to perform the experiments compared with viral vector-based methods and thus and is suitable for high-throughput screens. Fourth, it may deliver siRNAs into other suspension cells besides lymphocytes and dendritic cells. Importantly, efficient genome or epigenome editing could be achieved by transfection of gRNA only using Cas9 or nuclease-deficient Cas9 (dCas9) transgenic mice derived primary cells [16,17]. Overall, nanomaterials-based transfection is an ideal choice for lymphocyte or other suspension cell research.

Future perspective

Gene manipulation in lymphocytes is important for basic and translational research. This study presents a relatively simple and efficient approach to the delivery of siRNAs into immune cells, which may provide a powerful tool for precision medicine. For example, chimeric antigen receptor (CAR) T cell-based therapy provides a new avenue for cancer treatment [18]. Although the retrovirus-based gene transfer method is widely used for CAR T-cell generation, nonviral gene delivery approaches should be developed to overcome safety concerns and high cost demands. This nanomaterials-based transfection method presents a simple and efficient means of lymphocytic gene delivery with high efficiency and cell viability and has the potential for CAR T-cell generation.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/suppl/10.2144/btn-2022-0024

Author contributions

S Wang designed the study. S Wang and D Tian performed the experiments. S Wang wrote the manuscript and analyzed the data. All authors read and approved the manuscript.

Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

Adult male mice were used in this study and housed at the animal facility of Beijing Friendship Hospital. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Beijing Friendship Hospital.

Open access

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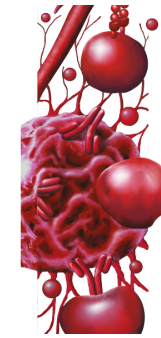
Review

Immunotherapy



Reversing chemokine/chemokine receptor mismatch to enhance the antitumor efficacy of CAR-T cells

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Currently, the antitumor efficacy of chimeric antigen receptor T cells in solid tumors is modest. Both chemokines and their receptors play a key role in the proliferation of cancer cells, tumor angiogenesis, organ-selective metastasis and migration of immune cells to solid tumors. Unfortunately, frequent chemokine/chemokine receptor 'mismatch' between effector cells and the tumor microenvironment results in inefficient T-cell infiltration and antitumor efficacy. Thus, reversing the 'mismatch' of chemokines and chemokine receptors appears to be a promising method for promoting T-cell infiltration into the tumor and enhancing their antitumor efficacy. In this review, we discuss functions of the chemokine/chemokine receptor axis in cancer immunity and the current understanding, challenges and prospects for improving the effect of chimeric antigen receptor T cells by reversing the mismatch between chemokines and chemokine receptors.

Plain language summary: Chimeric antigen receptor T (CAR-T) cell therapy is emerging as a promising therapeutic approach for cancers in the blood. The success of CAR-T cells has also sparked interest in applying this strategy to solid tumors; however, attempts have been disappointing, with only a few patients achieving a partial response. The chemokine/chemokine receptor axis plays a key role in the growth and spread of tumors. Unfortunately, chemokines and chemokine receptors in immune cells and the environment surrounding the tumor do not always match, which results in inefficient immune cell trafficking and infiltration. Therefore, reversing the 'mismatch' of chemokines and chemokine receptors appears to be a promising method for potential treatment. In this review, we discuss functions of the chemokine/chemokine receptor axis in cancer immunity and the current understanding, challenges and prospects for improving CAR-T cell therapy by reversing this mismatch.

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Keywords: antitumor effect • chemokine/chemokine receptor • chemotactic functions • chimeric antigen receptor • immunosuppressive tumor microenvironment • infiltration • solid tumor • trafficking

Chimeric antigen receptor T (CAR-T) cell therapy has resulted in a quantum leap in preclinical and clinical practice and is emerging as a promising therapeutic approach for hematological malignancies [1–3]. The remarkable antitumor effect of CAR-T cells resulted in the approval of four CD19 CAR-T cell products – Kymriah, Yescarta, Tecartus and Breyanzi – by the US FDA for the treatment of refractory or recurrent B-cell leukemia or lymphoma [4–6]. The success of CAR-T cells in hematological malignancies has also sparked interest in applying this strategy to solid tumors. Recently, a series of studies have investigated the efficacy and safety of CAR-T cells in a variety of solid tumors [7–15]. To date, more than 200 clinical trials that investigate CAR-T cells targeting antigens for solid tumors have been launched around the world, including human EGF receptor 2 (HER2), glypican-3 (GPC3),

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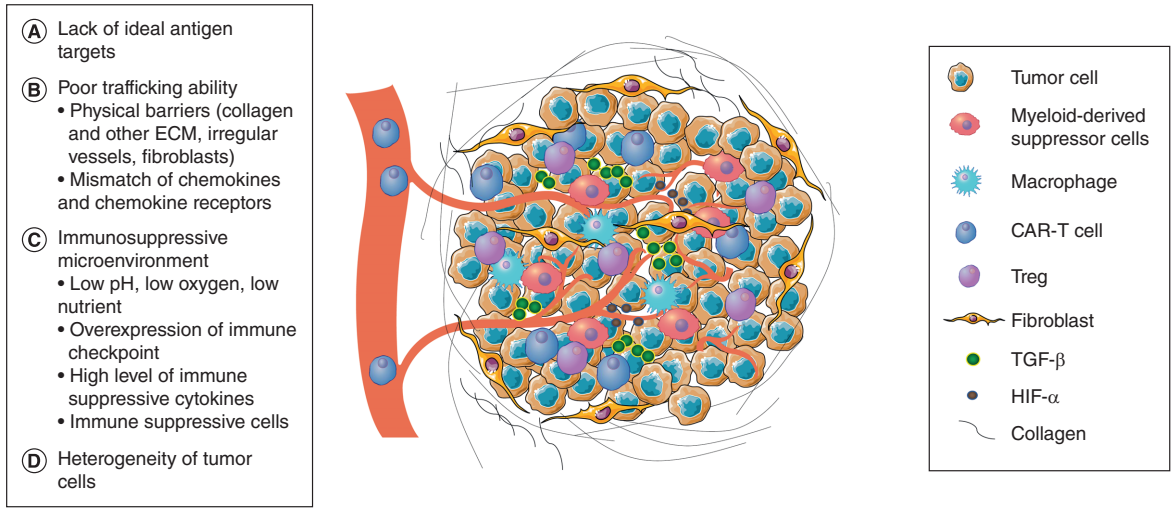

Figure 1. Key challenges of application of CAR-T cells to solid tumors.

carcinoembryonic antigen (CEA), prostate-specific membrane antigen (PSMA) and claudin 18.2 [16–24]. However, attempts to apply CAR-T cells to solid tumors have been disappointing, with only a few patients achieving a partial response and no significant effects for most patients [8,14,15,25,26].

Why are solid tumors more challenging to treat using CAR-T cells? The modest efficacy of CAR-T cells in solid tumors is primarily attributed to the intrinsic biological characteristics of solid tumors, which include several factors. First, a lack of tumor-specific antigens is regarded as one of the primary reasons for the poor efficacy of CAR-T cells. To date, targets of CAR-T cells for solid tumors are mostly tumor-associated antigens, and the specificity of these antigens is not high enough. Second, barriers to immune cell trafficking and infiltration into tumor sites are regarded as another limitation to treatment efficacy. Hematological malignancies are easily captured by CAR-T cells because they usually present in the blood. In contract, solid tumor cells are encased by stromal cells and extracellular matrix (ECM), making it difficult for T cells to be recruited. Third, finally, the immunosuppressive tumor microenvironment (TME) also acts as a barrier to treatment. The TME is composed of ECM, tumor vasculature, tumor cells, stromal cells and immune cells. Meanwhile, multiple molecules, such as chemokines and cytokines, and an abnormal metabolic environment, such as hypoxia, are also regarded as key components in the TME (Figure 1). The TME suppresses the function of CAR-T cells through several mechanisms. As mentioned previously, the physical barrier in the TME, such as collagens, inhibits immune cell trafficking and infiltration into tumor sites. Meanwhile, tumor cells express high levels of ligands of inhibitory immune checkpoints, which inhibit the function of T cells. However, the TME also recruits inhibitory immune cells, which interfere with the cytotoxic function of effector T cells. In addition, a low pH, hypoxia and a reduced bioenergetic status caused by abnormal metabolism of tumors also plays important roles in influencing T-cell activity [27–29].

Chemokines are small, secreted proteins that are best known for their roles in mediating immune cell trafficking and lymphoid tissue development. Recently, it has been demonstrated that the chemokine/chemokine receptor axis plays a significant role in several processes of cancer development, including proliferation, tumor angiogenesis and organ-selective metastasis. Moreover, the chemokine/chemokine receptor axis has also been demonstrated to play a key role in the trafficking and infiltration of T cells into solid tumors [30]. Physiologically, T cells induce the expression of chemokine receptors that are unique for chemokines in each tissue, which guarantees the appropriate trafficking of T cells into target tissues [31,32]. However, it is unlikely that all chemokine receptors expressed by T cells match the chemokines present in the TME, which makes the chemokine/chemokine receptor axis a promising target for improving the antitumor efficacy of CAR-T cells [33]. In this article, we review current understanding of the chemokine/chemokine receptor axis in cancer development and immunity, and focus particularly on the development, challenges, and future prospects of enhancing the antitumor effect of CAR-T cell therapy by targeting the chemokine/chemokine receptors axis.

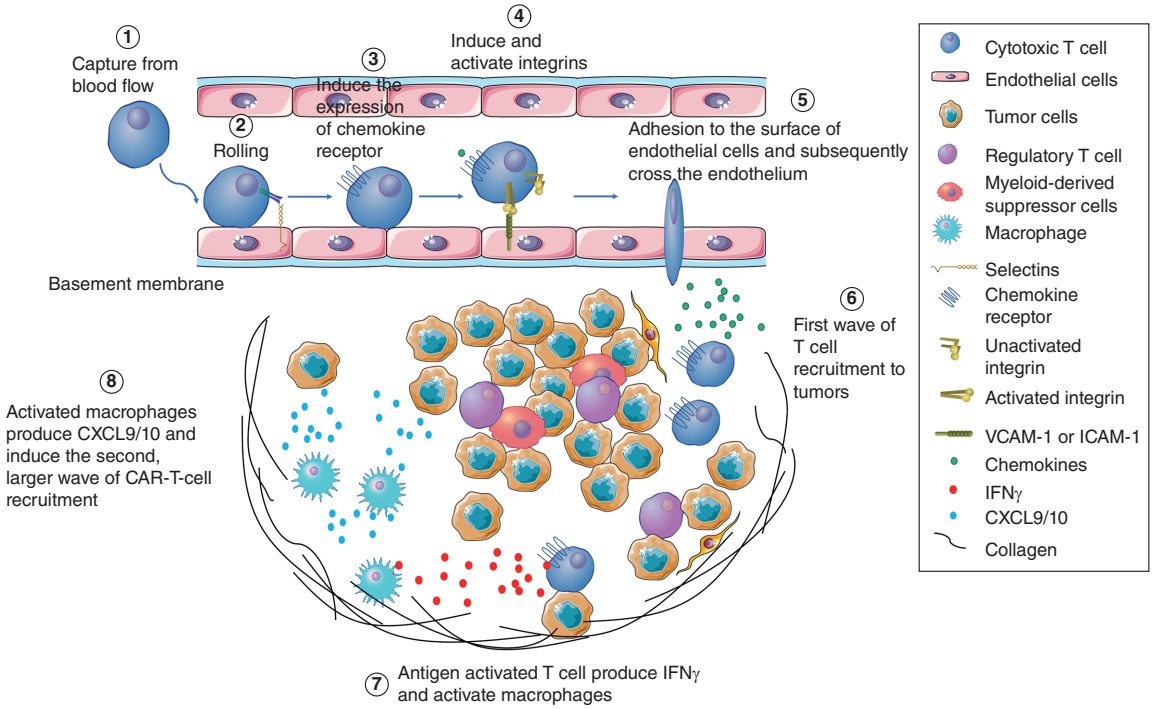

Figure 2. T-cell recruitment cascade.

Chemokine/chemokine receptors axis & their function in tumor

Chemokine/chemokine receptors

Chemokines belong to a family of small (8–14 kDa) cytokines that process chemotactic functions. Currently, more than 50 chemokines and more than 20 corresponding G protein-coupled receptors have been identified. Chemokines are grouped into four categories according to the number and position of conserved cysteine residues, including C- (two cysteine residues and one disulfide bond), CC- (four cysteine residues, with two cysteine residues adjacent to each other), CXC- (four cysteine residues, with an amino acid between the cysteine residues) and CX3C- (three amino acids separate the first two cysteine residues) chemokines (Table 1) [34]. Chemokines are widely expressed by a number of different cell types, including tumor, stromal and immune cells, and the function of chemokines depends on their binding to cognate receptors expressed on the surface of migrating cells (Figure 2).

In addition to their chemotactic functions, the chemokine/chemokine receptor axis has been demonstrated to play a significant role in the proliferation and survival of cancer cells, tumor angiogenesis and organ-selective metastasis of cancer (Figure 3) [32,35,36]. In addition, chemokines are involved in multiple steps of cancer therapy, mediating sensitivity or resistance to treatments based on chemotherapy, radiotherapy and targeted therapy [37–40]. Unlike normal cells, the homeostasis among chemokines and chemokine receptors in tumor cells is destroyed in TME, and chemokines and their receptors have been demonstrated to be involved in tumor cell proliferation, survival and progression [41]. For instance, a number of chemokines, including CXCL12, CCL5, CXC3L1, CCL28 and CCL2, have been found to be overexpressed in breast cancer and implicated in tumor cell proliferation and survival [42–45]. Moreover, chemokine receptors such as CXCR4, CXCR7 and CCR5 were also found to be overexpressed in cancer cells, which renders the tumor cells responsive to their cognate ligands and promotes the growth of tumor cells [43,46,47]. Angiogenesis represents another rate-limiting step in the development of malignant tumors [48]. In the past decades, chemokines and their receptors have been demonstrated as essential elements for the regulation of tumor angiogenesis [49–51]. Based on the presence of glutamic-leucine-arginine (Glu-Leu-Arg, ELR) motif at the N-terminus, CXC chemokine can be divided into two groups: ELR+ chemokines and ELR- chemokines. In general, ELR+ CXC chemokines, which included CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7 and CXCL8, have been demonstrated to promote tumor angiogenesis and endothelial cell survival. On the contrary, most ELR- cytokines, such as CXCL4, CXCL9, CXCL10, CXCL11 and CXCL14, have been shown to inhibit tumor angiogenesis by inhibiting chemotaxis to endothelial cells [52,53]. However, CXCL12, an ELR- chemokine,

Table 1. The functions of chemokine and their receptors in the tumor microenvironment.

Ligands	Receptors	Effects on cancer immunity	Direct and indirect effects on tumor cells
CCL1 (I309)	CCR8	Attracts Treg cells Increases myeloid cells in peripheral blood and cancer	Promotes the stemness of cancer cells
CCL2 (MCP1)	CCR2, CCR5	Recruits monocytes, NKT cells and monocytic MDSCs	Promotes proliferation, stemness and survival of tumor cells Promotes the formation of new vessels, cancer invasion and metastasis
CCL3 (MIP1α)	CCR1, CCR4, CCR5	Recruits monocytes and macrophage	Promotes cancer invasion and metastasis
CCL4 (MIP1β)	CCR1, CCR3, CCR5	Recruits Treg and macrophage (pro-tumorigenic effect) Recruits CTL, macrophage and DCs (antitumor effect)	Induces VEGF-C expression and lymphangiogenesis
CCL5 (RANTES)	CCR1, CCR3, CCR4, CCR5	Recruits Treg, monocytes, DCs and macrophage Promotes intratumoral T-cell infiltration	Promotes cancer invasion and metastasis
CCL7 (MCP3)	CCR1, CCR2, CCR3	Attracts monocytes and neutrophils	Promotes cancer migration, invasion and metastasis
CCL11 (eotaxin 1)	CCR3	Attracts eosinophils and Treg	Promotes survival of cancer cells
CCL17 (TARC)	CCR4	Recruits Treg	Promotes proliferation of tumor cells Promotes cancer migration, invasion and metastasis
CCL19 (MIP3β)	CCR7	Recruits T cells and DCs	Suppresses proliferation, migration, motility and invasion of cancer cells
CCL20 (LARC, MIP3α)	CCR6	Promotes Treg, Th17 recruitment and DC maturation	Promotes cancer chemotherapy resistance Promotes proliferation, migration, invasion and metastasis of cancer cells
CCL21 (SLC)	CCR7	Recruits T cells and DCs	Promotes migration, EMT and stemness of cancer cell
CCL22 (MDC)	CCR4	Recruits Treg Inhibits intratumoral T-cell function	–
CCL24 (eotaxin 2)	CCR3	Recruits eosinophils	–
CCL25 (TECK)	CCR9	Recruits T cells and MDSCs	Promotes chemoresistance, proliferation, migration, invasion and metastasis Suppresses apoptosis of cancer cells
CCL27 (CTACK)	CCR10	Recruits macrophage and T cells	Promotes migration and invasion of cancer cells
CCL28 (MEC)	CCR4, CCR10	Recruits Treg Inhibits intratumoral T-cell function	Promotes growth, migration, invasion and metastasis of cancer cells Promotes angiogenesis
CXCL1 (GROα, MGSA)	CXCR1, CXCR2,	Recruits MDSCs and neutrophils	Promotes angiogenesis, proliferation of tumor cells and metastasis
CXCL2 (GROβ, MIP2α)	CXCR2	Recruits MDSCs and neutrophils	Promotes stemness and resistance Inhibits proliferation and promotes apoptosis
CXCL3 (GROγ, MIP2β)	CXCR2	Recruits monocytes	Promotes stemness, proliferation, migration and metastasis
CXCL4 (PF4)	CXCR3	–	Inhibits angiogenesis and tumor growth
CXCL5 (ENA78)	CXCR2	–	Promotes proliferation, migration, metastasis, PD-L1 expression, angiogenesis and resistance
CXCL7 (NAP2, PBP)	CXCR1, CXCR2	Recruits macrophages	Promotes proliferation and invasion
CXCL8 (IL-8)	CXCR1, CXCR2	Recruits neutrophils and granulocytic MDSCs	Promotes stemness, invasion and migration of tumor cells Promotes resistance to hypoxia Promotes angiogenesis Increases immunogenicity of the tumor
CXCL9 (MIG)	CXCR3	Recruits T cells and NK cells	Inhibits tumor progression and angiogenesis
CXCL10 (IP10)	CXCR3	Recruits T cells and NK cells	Inhibits tumor progression and angiogenesis
CXCL11 (ITAC)	CXCR3, CXCR7	Recruits T cells	Promotes proliferation, migration, angiogenesis and EMT
CXCL12 (SDF1)	CXCR4, CXCR7	Recruits B cells, MDSCs, DCs and Treg	Promotes proliferation, stemness and survival of cancer cells Promotes invasion, metastasis and angiogenesis
CXCL13 (BLC)	CXCR5	Recruits B cells and T cells	Promotes proliferation, invasion and metastasis
CXCL14 (BRAK)	?	Recruits DCs	Promotes invasion (pro-tumorigenic effect) Inhibits proliferation, invasion and metastasis Increases apoptosis (antitumor effect)
CXCL15 (Lungkine)	?	–	–
CXCL16 (SRPSOX)	CXCR6	Recruits monocytes and macrophages	Promotes angiogenesis, proliferation, migration and metastasis
CXCL17	?	Recruits MDSCs	Promotes angiogenesis
XCL1	XCR1	Recruits T cells	Promotes cell growth and migration
CX3CL1	CX3CR1	Recruits T cells and NK cells	Inhibits tumor progression

CTL: Cytotoxic T cell; DC: Dendritic cell; EMT: Epithelial–mesenchymal transition; MDSCs: Myeloid-derived suppressor cells; NKT: Natural killer T; VEGF-C: Vascular endothelial growth factor C.

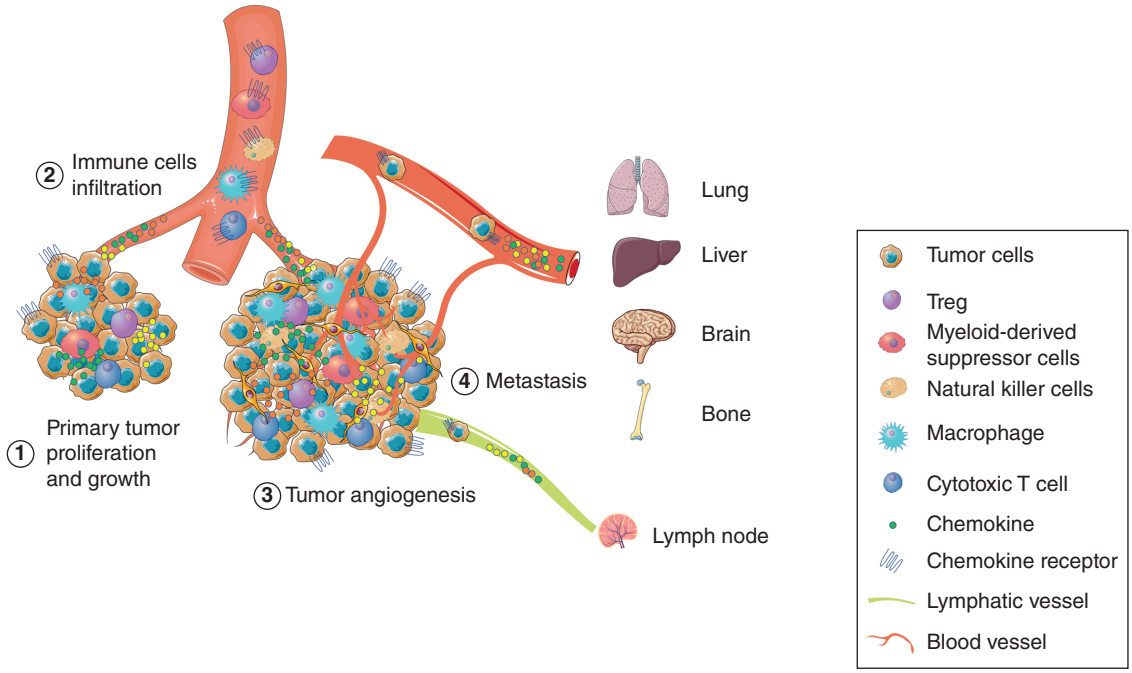

Figure 3. Multifaceted roles of the chemokine/chemokine receptor axis in the development of cancer.

has been regarded as the most potent angiogenic chemokine [54]. Besides CXC chemokines, CC chemokines, such as CCL2, CCL11, CCL16 and CCL18, can also promote tumor angiogenesis and endothelial cell survival [55]. Metastasis, which is an essential feature of malignancies and represents the leading cause of cancer-related death, refers to the dynamic process of cancer cell dissemination from the site of primary tumor to distant organs [56]. It has been largely reported that the chemokine/chemokine receptors axis plays a critical role in the process of organ-selective metastasis. Tumor cells can express selected chemokine receptors, whereas the sites of metastasis, such as lymph nodes, bone marrow, lung and liver, produce specific chemokines that attract circulating tumor cells into specific anatomic sites and form the metastases [57]. For example, CCR7 has been reported to be highly expressed in triple-negative breast cancer. Moreover, the expression of ligands of CCR7, including CCL19 and CCL21, was significantly increased in lymph nodes of breast cancer patients, which promote the metastasis of breast cancer cell to lymph nodes [58]. CXCR4 and its ligand CXCL12 have also been demonstrated as another key regulator involved in metastasis of different types of tumors, such as breast cancer, prostate cancer and lung cancer [59]. In addition to the critical role in the process of tumor proliferation and metastasis, chemokines are also involved in multiple steps of cancer therapy, which mediates the sensitivity or resistance to treatments. Chemotherapy and radiotherapy are the most common treatment options for cancers. Recently, it has been demonstrated that the therapeutic efficacy of these treatments may rely on the specific immune contexture, which includes the presence of specific chemokines in the TME [60,61]. In a preclinical fibrosarcoma model, Ma *et al.* demonstrated that treatment with anthracycline can induce the expression of CCL2 and the therapeutic efficacy of anthracycline are CCL2 dependent [62]. CCL12 could recruit CD11b + CD11c + Ly6C^{high} cells into the tumor sites, thus enhancing the antitumor effect of T cells. Meanwhile, these immune cells were also demonstrated as the major source of CCL2, which generate a positive feedback loop for optimal antitumor responses. Despite chemotherapy, chemokines are also implicated as key regulators for radiotherapy. A study by Lim *et al.* demonstrated that the expression of CXCL10 was correlated with the response to radiotherapy [63]. CXCL10 secreted by myeloid cells could recruit CD8$^+$ effector T cells, which were considered as major effector cells in radiotherapy.

Chemokine/chemokine receptor mismatch between T cells & the TME limits the trafficking & infiltration of T cells into solid tumors

Trafficking and infiltration into solid tumors are the first steps to achieving the antitumor effects of CAR-T cells, and patients with inadequate therapeutic response frequently experience inefficient tumor infiltration by the transferred

cells [30]. A cascade of events is required for the infiltration of immune cells into tumors, which relies on the sequential involvement of selectins, chemokine receptors and integrins. Initially, circulating selectin-dependent lymphocytes coordinate rolling and adhesion to the endothelial surface, which also triggers the consequent expression of chemokine receptors on the surface of lymphocytes. Upon activation by chemokines, chemokine receptors induce the expression and activation of integrins, such as leukocyte function-associated antigen 1 (LFA-1) and very late antigen 4 (VLA-4). These integrins bind to endothelial receptors, including vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1). This process enables T-cell adhesion to the surface of endothelial cells. Then, T cells cross the endothelium and patrol peripheral tissues following a chemotactic gradient [64,65]. During the process of immune cell trafficking and infiltration, chemokines and their receptors play a key role through their matching. Chemokines enable the formation of chemotactic gradients that play an essential role in guiding and trafficking the cells expressing the matching chemokine receptors. Specifically, certain chemokines expressed in the TME positively correlate with tumor infiltration by cytotoxic T cells expressing cognate receptors [31,32]. CXCL9, CXCL10 and CXCL11 are ligands of CXCR3 and are strongly associated with attracting tumor-specific T cells that express CXCR3 into tumors [66,67]. Several recent studies have demonstrated that these chemokines improve antitumor efficacy by inducing the recruitment of a series of effector cells, including NK cells, $CD4^+$ Th1 cells and $CD8^+$ cytotoxic T lymphocytes (CTLs), into tumor sites [68–72]. CCL5 is another chemokine involved in the recruitment of immune cells with antitumor activity upon binding to its cognate receptor, CCR5. In a mouse model, $CCR5^+$ $CD4^+$ T cells and $CD8^+$ T cells exert protective immunity against tumors, and CCR5 deficiency significantly enhances the proliferation of transplantable cancers [73]. CXCL12 and its receptor, CXCR4, are expressed at high levels in several types of cancers. The CXCL12/CXCR4 axis promotes immunosuppression by increasing fibrosis of tumor cells and decreasing the infiltration of T cells. In a mouse breast cancer model, blockade of CXCR4 signal transduction significantly reduced fibrosis and increased CTL infiltration, which attenuated immunosuppression in the TME [74]. However, chemokines can be categorized as constitutive or inducible based on their expression patterns [31]. Therefore, 'sequence' or hierarchies existing with chemokines are additional essential factors needed for effective trafficking. In a recent study, House *et al.* found that CXCL9 and CXCL10 were significantly upregulated in the TME following dual PD-1/CTLA-4 blockade. When CXCR3 was neutralized, the therapeutic efficacy of dual PD-1/CTLA-4 blockade was abrogated. The primary source of CXCL9 is macrophages, and expression of CXCL9 is induced by the production of IFN-γ. The depletion of these cells abrogated $CD8^+$ T-cell infiltration and the therapeutic efficacy of dual PD-1/CTLA-4 blockade [75]. Another study by Dangaj *et al.* identified an interesting sequence of events involving constitutive CCL5 and inducible CXCL9. They found that CCL5 was constitutively expressed by tumor cells and initially attracted T cells to the tumor sites. Upon encountering tumor antigens, these cells begin producing high levels of IFN-γ, which induces local tumor-associated macrophages and dendritic cells (DCs) to secrete CXCL9. Secretion of CXCL9 subsequently attracts another wave of infiltrating T cells. These results reveal a hierarchy of chemokines that regulate the trafficking and infiltration of T cells [76]. Overall, these studies provide evidence that chemokines within the TME guide the trafficking of immune cells and induce increased tumor-infiltrating lymphocytes (TILs) in the tumor.

As mentioned previously, the chemokine/chemokine receptor axis plays an essential role in mediating the directed migration of immune cells. T-cell trafficking into the TME depends on a 'match' between chemokines and their receptors expressed on effector cells and on tumor cells. Unfortunately, frequent chemokine/chemokine receptor 'mismatches' between effector cells and tumor cells in solid tumors result in insufficient antitumor efficacy [77]. The expression of chemokine receptors is substantially variable in patients with cancers and correlates with the levels of lymphocytes infiltrating into the TME. One study demonstrated that overexpression of CXCL8 in melanoma was not sensed by TILs with low expression of the corresponding chemokine receptors (CXCR1 and CXCR2). Engineered expression of CXCR1 enhanced the migration of TILs toward melanoma [78]. Another study used a transplanted tumor model of B16 melanoma cells to demonstrate that the trafficking and infiltration of antigen-specific $CD8^+$ T cells was significantly reduced by pretreatment with a CXCR3-blocking antibody or in the case of CXCR3 deficiency [33]. In addition, for some chemokines highly expressed in TME, the corresponding receptors were expressed on subsets of tumor-associated T cells but far from the majority of TILs. In previous studies, CXCL13 was demonstrated to be highly expressed in tumor tissue of non-small-cell lung cancer (NSCLC) patients [79]. CXCR5, the corresponding receptor to CXCL13, is mainly expressed on B cells and follicular helper T (Tfh) cells, but these cells do not have a direct cytotoxic effect [80,81]. However, in some types of primary tumors, the level of chemokines are not always sufficient to form chemotactic gradients, which are essential for

the chemoattraction of immune cells. Overall, these results indicate that the chemokine/chemokine receptor axis is a candidate for genetic manipulation to enhance the trafficking of adoptively transferred T cells to improve the antitumor efficacy of CAR-T cells.

Targeting the chemokine/chemokine receptor axis to enhance the antitumor efficacy of CAR-T cells

Genetic modification of CAR-T cells with chemokine receptors

Based on the concept that the expression of certain chemokine receptors correlates with the levels of lymphocyte infiltration into the TME, engineered expression of cognate receptors of chemokines in the TME to enhance the antitumor efficacy of CAR-T cells has been recently investigated. IL-8 is overexpressed in certain types of cancer, including glioblastoma, breast cancer, colon cancer, gastric cancer, lung cancer, melanoma and hematological malignancies [82]. Previous studies have demonstrated that IL-8 promotes the development of cancer by enhancing the recruitment of tumor-associated myeloid-derived suppressor cells, which are involved in epithelial-mesenchymal transition and enhance tumor invasion, resistance, stemness and angiogenesis [83,84]. CXCR1 (also known as IL-8RA) and CXCR2 are two receptors of IL-8. Both of these receptors bind IL-8 with high affinity [85]. Genetic manipulation of TILs to express these two receptors has been tested in several recent studies and was shown to significantly improve the antitumor efficacy of TILs by enhancing T-cell migration to tumor sites [77,86–88]. Successful co-expression of CXCR1or CXCR2 in TILs encouraged the transfer of the method to the field of CAR-T cell therapy. A preclinical study by Whilding *et al.* modified the original integrin αvβ6-CAR T cells with CXCR2 to enhance CAR-T cell trafficking. Integrin αvβ6-CAR T cells modified to express CXCR2 elicit superior antitumor activity because these cells migrate more efficiently toward tumor-produced IL-8, indicating that modification of CAR-T cells with CXCR1 or CXCR2 may be a promising method for improving the therapeutic activity of CAR-T cells against solid tumors [89]. Several subsequent and similar studies were performed to modify CAR-T cells to overexpress CXCR1 or CXCR2 for other targets. Two studies showed that CAR-T cells overexpressing CXCR1, CXCR2 or both can significantly improve antitumor efficacy in a series of cancers [90,91].

CCL2 (MCP-1) has been demonstrated to be a potent chemoattractant that recruits several types of immune cells, including NK cells, memory T cells and immature DCs [92]. CCL2 performs its function by binding to CCR2. In addition to CCL2, CCR2 can bind other chemokines, including CCL7 (MCP-2), CCL8 (MCP-3) and CCL13 (MCP-4), with high binding affinity [93]. Overexpression of CCL2 has been detected in a number of cancer types, including breast cancer, ovarian cancer and gastric cancer, and was shown to promote tumor growth and metastasis [92,93]. A preclinical study by Craddock *et al.* modified GD2 CAR-T cells with CCR2b to enhance the tumor trafficking of CAR-T cells in a human neuroblastoma model. GD2 CAR-T cells expressed high levels of CCR2b, which significantly enhanced the migration of CAR-T cells *in vitro* and *in vivo*, improving their antitumor activity [94]. Another similar study transduced meso-targeted CAR-T cells with CCR2b and achieved a 12.5-fold increase in T-cell tumor infiltration, which was associated with a significant increase in antitumor activity [95].

Furthermore, CCR4 has been demonstrated to be overexpressed in a wide range of hematologic malignancies. For example, Reed-Stemberg cells overexpress CCL17 and CCL22, which attract Th2 cells and Tregs with high expression of CCR4 [96]. However, in the TME, $CD8^+$ effector T cells are rarely detected due to a lack of CCR4 expression. A preclinical study by Di Stasi *et al.* engineered the expression of CCR4 by CD30 CAR-T cells, enhancing their cytotoxic function and cytokine secretion *in vitro* and improving the antitumor efficacy of CAR-T cells in a mouse model of Hodgkin lymphoma [97].

Engineering CAR-T cells to deliver chemokines

Engineering CAR-T cells to deliver chemokines is another promising strategy for improving the 'mismatch' of chemokines and chemokine receptors. IL-7 and CCL19 produced by T-zone fibroblastic reticular cells have been demonstrated to play a critical role in the recruitment of T cells and DCs from the peripheral blood [98,99]. A recent preclinical study by Adachi *et al.* developed CAR-T cells that expressed IL-7 and CCL19 (7 × 19 CAR-T cells), which recruit T cells and DCs to tumor tissues to enhance the antitumor effects of CAR-T cells. As expected, 7 × 19 CAR-T cells achieved a better response in a mouse tumor model and significantly prolonged the survival of mice compared with animals treated with conventional CAR-T cells. Investigation of the mechanisms of the effect indicated that infiltration of DCs and T cells into the tumor tissues was considerably higher in the 7 × 19 CAR-T cell group [100]. A similar study by Luo *et al.* engineered CLDN18.2 CAR-T cells to co-express IL-7 and CCL21 (7 × 21 CAR-T), which significantly improved the proliferation and chemotaxis of CAR-T cells. In an

animal model, 7 × 21 CAR-T cells exhibited superior antitumor effects compared with conventional CAR-T cells or previously reported 7 × 19 CAR-T cells. Consistent with the results obtained using 7 × 19 CAR-T cells, co-expression of IL-7 and CCL21 significantly enhanced the trafficking and survival of CAR-T cells and DCs in the TME. Moreover, inhibition of angiogenesis was regarded as another mechanism for the antitumor effect of 7 × 19 CAR-T cells [101].

Delivery of chemokines via oncolytic virus

Oncolytic viruses (OVs), including adenoviruses, herpes viruses, measles viruses and coxsackie viruses, have been engineered to selectively replicate in and lyse tumor tissues but not normal cells [102]. The antitumor effects of OVs include two approaches. First, OVs directly infect tumor cells to lyse tumor cells and release tumor antigens. Second, OVs can be equipped with immune system-activating agents, such as GM-CSF, to enhance the antitumor effect of the immune system [103]. Despite enhanced antitumor effects, OVs expressing immune system-activating agents may restrict the secretion of these agents in the TME to potentially reduce side effects. Thus, delivery of chemokines via OVs may be another promising method for enhancing the effect of CAR-T cells. A preclinical study by Nishio *et al.* investigated the antitumor efficacy of a combination of CAR-T cells with OVs armed with the chemokine RANTES and the cytokine IL-15. Infection of neuroblastoma cells with Ad5D24 armed with RANTES and IL-15 resulted in the production of high levels of RANTES and IL-15 both *in vitro* and *in vivo* to significantly promote efficient trafficking of CAR-T cells into the TME and increase antitumor functions [104]. GD2 CAR-T cells combined with Ad5D24 armed with RANTES and IL-15 significantly prolonged the survival of mice in a mouse neuroblastoma model compared with monotherapy. Another preclinical study by Moon *et al.* modified CAR-T cells to deliver CXCL11 (CAR/CXCL11) into tumors armed with oncolytic vaccinia virus (VV) to produce CXCL11 (VV.CXCL11). Then, the antitumor effects of CAR/CXCL11 and CAR-T cells combined with VV.CXCL11 were compared. Both treatment methods significantly increased the levels of CXCL11 in the TME. However, VV.CXCL11 recruited a higher number of total and CAR-T cells into the tumors, which significantly enhanced the antitumor efficacy compared with that achieved by treatment with CAR/CXCL11, indicating that combining CAR-T cells and OVs armed with chemokines is a potential approach to augment adoptive T-cell transfer or vaccine immunotherapy [105].

Clinical trials targeting the chemokine/chemokine receptor axis to enhance the antitumor effect of CAR-T cells

Although targeting the chemokine/chemokine receptor axis has been demonstrated as a promising method for enhancing the antitumor effect of CAR-T cells in several preclinical trials, very few early clinical trials have evaluated the effect of homing T cells to metastatic tumors by targeting the chemokine/chemokine receptor axis. In 2019, Qian *et al.* initiated a single-arm, open-label prospective study to investigate the safety and efficacy of IL-7 and chemokine (C-C motif) ligand 19-expressing CD19-CAR-T therapy in refractory/relapsed B-cell lymphoma patients. The estimated completion date is 30 April 2022 [106]. Another single-center, single-arm, open-dose climbing study investigating the safety and efficacy of anti-EGF receptor (EGFR) CAR-T cells modified by C-X-C chemokine receptor type 5 (CXCR5) in patients with advanced adult NSCLC was sponsored by Sun Yat-sen University Cancer Center. The study will be completed by December 2022 [107]. Another similar study evaluating the effect and safety of anti-EGFR CAR- T cells modified by CXCR-5 in patients with advanced adult NSCLC is also recruiting patients at Second Affiliated Hospital of Guangzhou Medical University [108]. Although there are few clinical studies on improving the antitumor effect of CAR-T cells by targeting the chemokine/chemokine receptor axis, with the gradual deepening of research in the field, an increasing number of clinical trials will be performed in the near future.

Limitations & potential combined strategies

CAR-T cell therapy has emerged as a promising therapeutic approach for hematological malignancies. However, the antitumor efficacy of CAR-T cells in solid tumors is modest. For example, glioblastoma represents one of the severest malignancies. To date, IL-13 receptor alpha 2 (IL13-Ra2), HER2, and epidermal growth factor receptor variant III (EGFRvIII) have been tested as targets of CAR-T cell therapy for glioblastoma, the results of which are disappointing [109,110].

In recent years, a series of novel methods for improving the antitumor efficacy of CAR-T cell therapy have been widely explored, which include the following. The modification of T cells to secrete cytokines, such as IL-12, IL-15,

has been explored [111,112]. Synchronous blockade of PD-1 and CTLA-4 via genetic modification has also been shown to improve the antitumor activity of CAR-T cells [113,114]. Bispecific CAR-T cells, or targeting metabolism, have also been investigated in recent studies [115]. In addition, significant progress has been made in extending the CAR platform from T cells to alternative leukocytes, such as macrophages and other cells of the myeloid lineage. Macrophages are innate immune cells with potent phagocytic and cytotoxic capabilities, which can initiate and potentiate an adaptive immune response via T-cell recruitment, antigen presentation, co-stimulation and cytokine secretion. In addition, unlike lymphocyte-based therapies, macrophages readily localize to and persist within the TME [116,117], which makes CAR-macrophages (CAR-M) a novel promising method for the treatment of solid tumors. As mentioned in this review, the accumulating data suggested that chemokine or chemokine receptor engineering of CAR-T cells is a promising strategy for improving homing and the potential response to treatment with CAR-T cells. Some potential limitations remain despite the great success of CAR-T cells modified to express chemokines or chemokine receptors. First, chemokines or chemokine receptors are quite different in different tumor types; therefore, it is critical to select the most appropriate chemokines/chemokine receptors for different tumor types. Mcp-1 has been demonstrated to be expressed at higher levels in NSCLC tumor tissues than in noncancerous tissues. In addition, the receptors Mcp-1, CCR2b and CCR4 are expressed on activated T cells at low levels. In a recent study, Wang *et al.* generated Msln-CAR T cells modified with CCR2b or CCR4. They found that Msln-CCR2b-CAR and Msln-CCR4-CAR T cells increased migration to tumor supernatants with a high level of Mcp-1 *in vitro*. In an NSCLC CDX model, they found that co-expression of CCR2b enhanced infiltration into tumor tissue and antitumor function of CAR-T cells [118]. In another similar study, Jin *et al.* identified that CCL 20 is highly expressed in lung cancer. Forced expression of CCR6, the receptor of CCL20, can significantly enhance trafficking and infiltration of CAR-T cells toward CCL20-secreting tumors *in vitro* and *in vivo*, which led to effective tumor clearance and longer survival of tumor-bearing mice [119]. Second, several chemokines, such as CCL2 or IL-8, are expressed at high levels in tumor cells; however, these chemokines are also widely expressed in normal tissues, which may increase side effects. Third, previous studies have demonstrated that T cells modified to express chemokine receptors, such as CCR2, may induce high levels of intracellular calcium, which can lead to accelerated T-cell hypofunction [120,121]. Fourth, in addition to chemokines/chemokine receptors, several other factors, such as ECM barriers and irregular tumor vessels, are critical for the migration of immune cells [122]. Therefore, combination with other strategies, such as targeting fibroblasts to overcome ECM barriers or targeting tumor angiogenesis to form regular vascular flux, may further improve the homing of CAR-T cells into the TME. However, genetic manipulation of CAR-T cells with chemokine receptors can enhance the trafficking of CAR-T cells into the TME, but improvement of the direct killing ability of CAR-T cells by these methods is limited. Thus, combinatorial approaches for improving the direct antitumor effect of CAR-T cells, such as combining CAR-T cells with PD-1 inhibitors to decrease the immunosuppressive influence of the TME, are urgently needed. Another potential limitation that should be mentioned is the animal models used in these studies. In several studies, human T cells have been used in mice with human tumor xenografts. Although some mouse chemokines can bind and activate human chemokine receptors (i.e., CXCR4 and CXCL12) and *vice versa*, some do not. Fortunately, they used human tumor cells with high levels of certain chemokines and modified human T cells to express their cognate receptors, which made the results of these studies reasonable. However, chemokines in the TME are not only expressed by tumor cells, and a hierarchy exists in chemokines that regulate the trafficking and infiltration of T cells. Although chemokines secreted by tumor cells can recruit the first wave of T-cell infiltration in these models, chemokines secreted by antigen-activated human T cells may not induce the activation and secretion of macrophages or DCs in the TME due to species specificity, which blocks the second larger wave of T-cell recruitment. Overall, these models may not reflect the actual tumor environment, and humanized animal models with complete immune systems are urgently needed in future studies.

Conclusion & future perspective

CAR-T cells have recently emerged as a promising treatment option for patients with hematological malignancies. Despite success in the treatment of hematological malignancies, the effect of CAR-T cell therapy on the treatment of solid tumors has yet to be realized. Therefore, improving the antitumor effect of CAR-T cells in solid tumors is urgently needed. Efficient trafficking of T cells to the TME is regarded as a critical step for CAR-T cell therapy, and chemokines and chemokine receptors play a key role in this process. In addition to their roles in the trafficking of immune cells, chemokines and chemokine receptors play an important role in several other tumor processes. In this review, we first discussed the function of chemokines and chemokine receptors in cancer immunity. Subsequently,

we reviewed current studies that aimed to improve the antitumor efficacy of CAR-T cells by targeting chemokines or chemokine receptors. Fortunately, modification of chemokine receptors or chemokines on CAR-T cells can overcome the obstacles of poor trafficking to the TME. In addition, we summarized the limitations of CAR-T cells combined with targeting chemokines or chemokine receptors and other potential combination strategies. Overall, modification of CAR-T cells to express chemokines or chemokine receptors has yielded encouraging results in preclinical studies; however, the approaches described in this review remain to be validated in clinical trials. Despite several limitations, targeting the chemokine/chemokine receptor axis represents an alternative strategy for enhancing the trafficking and antitumor effect of CAR-T cells.

Executive summary

Chemokine/chemokine receptor axis plays a key role in cancer immunity

- Chemokines belong to a family of small cytokines with chemotactic functions.
- The chemokine/chemokine receptor axis has been demonstrated to play a key role in the proliferation of cancer cells, tumor angiogenesis and organ-selective metastasis.
- Chemokines also involved in mediating the sensitivity or resistance to chemotherapy, radiotherapy and targeted therapy.

Chemokine/chemokine receptor mismatch between T cells & TME results in insufficient infiltration of T cells & antitumor efficacy

- Expression of chemokine receptors is substantially variable in patients with cancers and correlates with the levels of lymphocytes infiltrating into the TME.
- Frequent chemokine/chemokine receptor 'mismatches' between the effector cells and tumor cells in solid tumors results in insufficient antitumor efficacy.
- The chemokine/chemokine receptor axis is regarded as a promising candidate to enhance the trafficking of adoptively transferred T cells to enhance the antitumor effect of CAR-T cells.

Targeting the chemokine/chemokine receptor axis to enhance the antitumor effect of CAR-T cells

- Engineered expression of cognate receptors of chemokines in the TME in CAR-T cells has been shown to significantly improve the antitumor efficacy of CAR-T cells by enhancing T-cell migration to tumor sites.
- Engineering CAR-T cells to deliver chemokines is another promising strategy to improve the 'mismatch' of chemokines and chemokine receptors and significantly enhanced the trafficking and survival of CAR-T cells.
- Delivery of chemokines via oncolytic viruses significantly promotes efficient trafficking of CAR-T cells into the TME and increases antitumor functions.

Author contributions

PF Zhang and Q Li were responsible for the conception. PF Zhang, Q Li, C Wang and L Zhang were responsible for literature collection, draft writing and editing.

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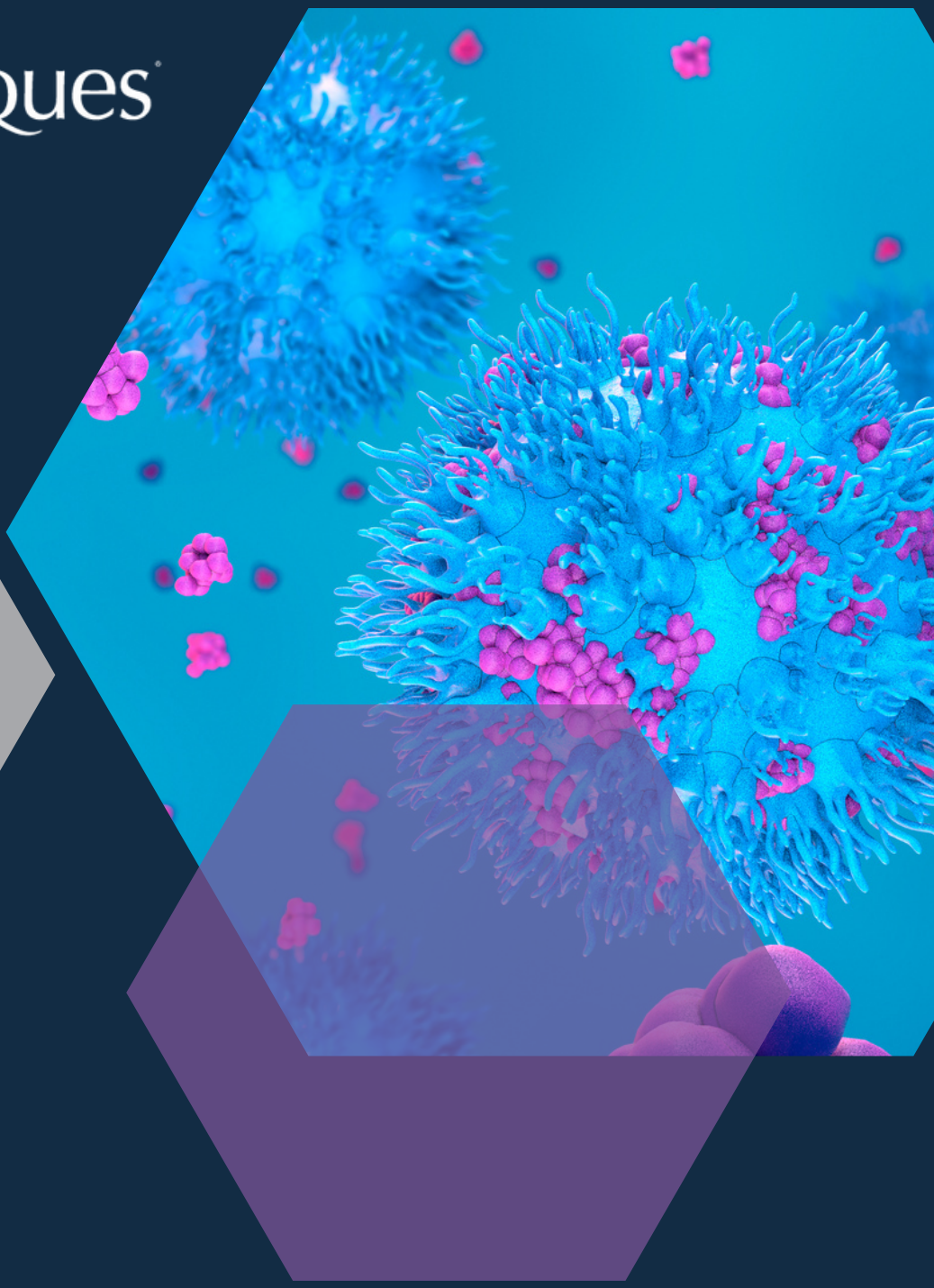

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