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Adoptive cell therapies: advancing cancer treatment for solid tumors

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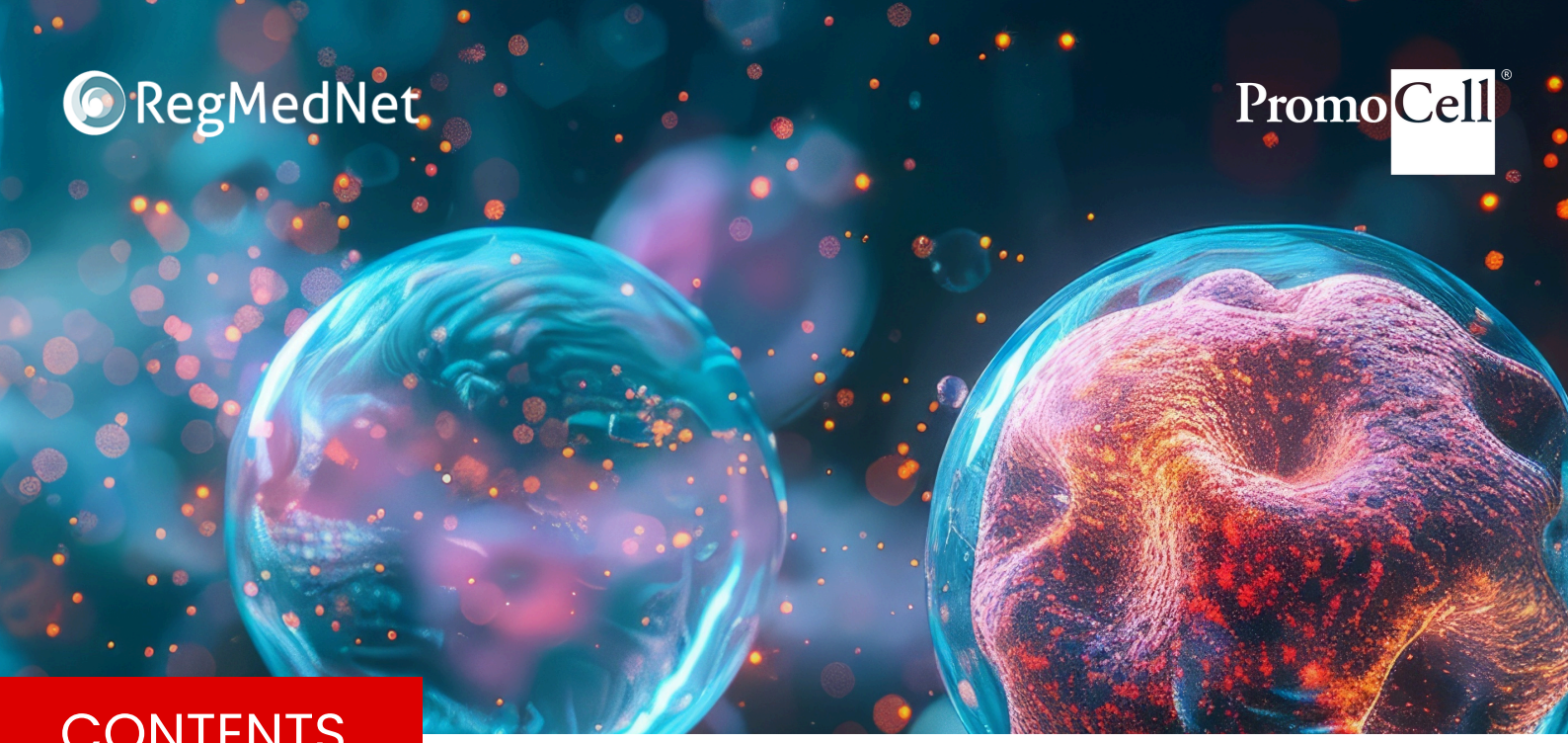

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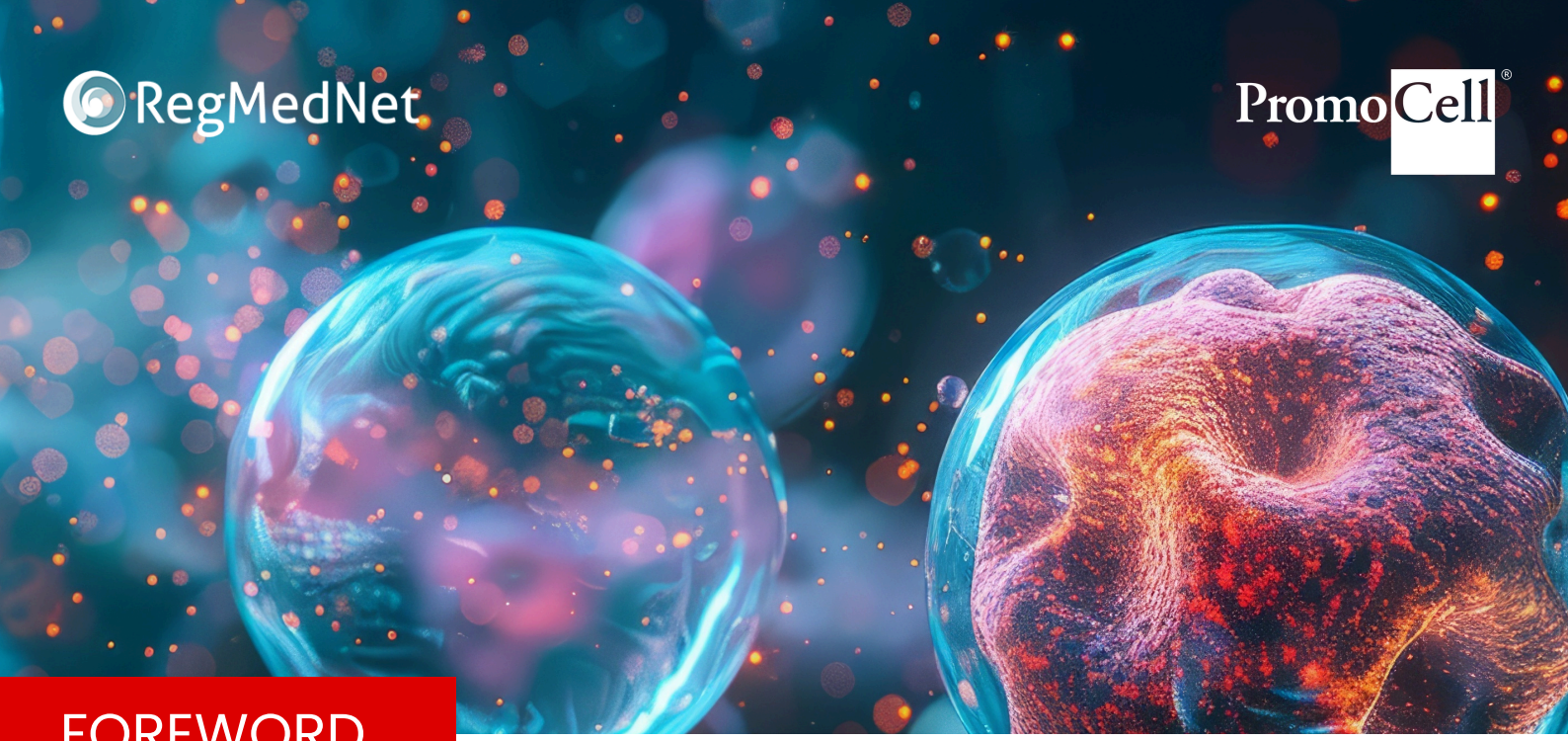

FOREWORD

We are delighted to present this eBook on adoptive cell therapies in solid tumors, showcasing transformative research and clinical perspectives on their application in regenerative medicine.

Adoptive cell therapy harnesses engineered immune cells to target and eliminate cancer. While this approach has already revolutionized treatment for hematopoietic cancers, its effectiveness in solid tumors remains a significant challenge. The hostile tumor microenvironment in solid tumors poses barriers to success, including suppressive signals, physical obstacles, and limited immune cell access. However, recent advancements in gene editing, human leukocyte antigen (HLA) typing, and biomarker identification are reshaping the way adoptive cell therapies are developed and applied in cancer treatment.

This eBook features a curated collection of articles that explore how these advancements are enhancing the fight against cancer, making therapies safer, more effective, and increasingly personalized. Together, these insights provide a comprehensive overview of the current state of the field and its promising future directions.

We hope you enjoy reading these expert insights.

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Therapeutic hurdles in solid tumor management and the promise of adoptive cell approaches: an interview with Leonardo Ferreira

In this interview, Leonardo Ferreira, Assistant Professor of Pharmacology and Immunology at the Medical University of South Carolina (SC, USA), discusses the mechanisms behind solid tumor resistance, exploring some ways in which the tumor microenvironment limits treatment effectiveness. He also touches on how these challenges are influencing research strategies and driving the development of adoptive cell therapies to overcome the resistance mechanisms of solid tumors.

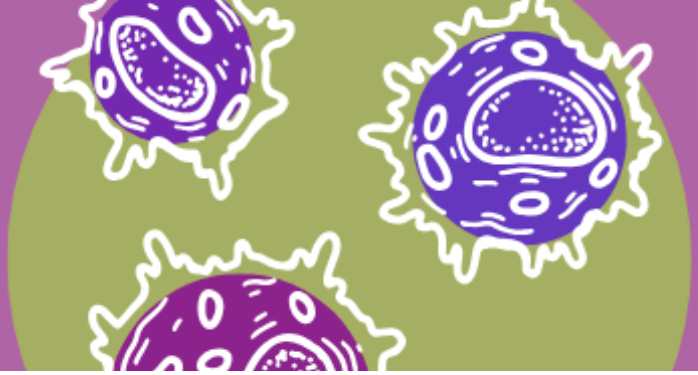
1 What are the main biological and physical barriers within the solid tumor microenvironment that limit the success of adoptive cell therapies?

The reality we face with solid tumors is quite astonishing when compared to liquid tumors. While CAR T-cell therapies result in up to 100% success rates in liquid tumors such as leukemia and lymphoma, they do not work yet in solid tumors. Unfortunately, liquid tumors represent only about 10% of total cancer cases and cancer deaths, with solid tumors like lung, pancreatic, breast and prostate cancer, causing most deaths. It's as if solid tumors have essentially evolved to become better immunologists than we are – they have learned every trick in the book to protect themselves to escape the immune system.

Several key barriers limit cell therapy success in solid tumors. Barrier number one is getting inside. Unlike liquid tumors that circulate in the blood where CAR T-cells can easily find them, solid tumors are difficult to penetrate. Extracellular matrix deposition creates a physical barrier around the tumor, creating a tumor microenvironment that is very inhospitable and hard for immune cells to penetrate. The few T cells that do manage to get inside tumors don't work very well. They become exhausted and dysfunctional.

The second barrier is metabolic in nature. Within this tumor microenvironment, there is limited glucose, glutamine and oxygen availability; an abundance of lactic acid and anti-inflammatory molecules such as interleukin-10 (IL-10) and TGF-beta. Altogether, this environment does not allow for highly effective T-cells. This leads me on to the other barrier, immunological barriers.

The tumor microenvironment breeds suppressive cells, such as myeloid derived suppressor cells (MDSCs) and regulatory T cells (Tregs). So, it's not that solid tumors are devoid of immune cells, it's just that they only have the types that don't attack them and instead suppress immune responses. Additionally, tumor cells can also express Programmed Death Ligand 1, PD-L1, which binds to Programmed Death 1, PD-1, on exhausted T cells, shutting down T-cell receptor (TCR) signaling. When that happens, T cells stop working. We call PD-1 and similar molecules immune checkpoints. If you can block these immune checkpoints, T cells can be reactivated.



Finally, we have target identification challenges. It's not really a physical, metabolic, or immunological barrier – it's what do we target? Unlike liquid tumors with clear targets like CD19, solid tumors lack ideal target antigens. Many potential targets in solid tumors are also expressed in vital normal tissues such as EpCAM, which is expressed highly in cancer, but also, to some extent, in normal tissues. We don't want to be targeting healthy cells too, because there is a chance that damaging them will severely harm the patient. Therefore, we're always looking to find new targets, or take existing ones and see if there is a way to use them in combination, to achieve higher tumor specificity.

2 What role do tumor infiltrating lymphocytes play in reprogramming or reshaping the tumor microenvironment?

The tumor microenvironment has a very special immune composition compared to the blood and the interstitial fluid. Interestingly, cancer cells use a lot of tricks found in pregnancy immunology. Cancer cells adopt several mechanisms from placental cells called trophoblasts. This includes expression of PD-L1 to inhibit T-cell function; indoleamine oxygenase (IDO) to deplete tryptophan, limiting T-cell function; and HLA-G, a non-classical MHC molecule normally only expressed during pregnancy – but some cancers can turn it back on.

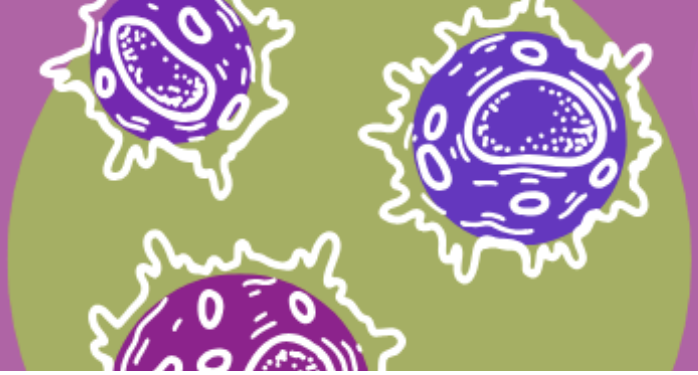
Tumor infiltrating lymphocytes (TILs) play a crucial role in reshaping the tumor microenvironment, as they will encounter all sorts of molecules like the ones mentioned above. Within the tumor microenvironment, TILs exhibit various functional states, including exhausted, regulatory and effector T cells. These can change how immunosuppressive the tumor microenvironment becomes. Too many Tregs and exhausted T cells can secrete inhibitory cytokines that inhibit effector T-cell functions. This can lead to a diminished cytotoxic environment, allowing tumor progression. On the other hand, effector T cells promote a pro-inflammatory tumor microenvironment, increasing cytokine production and activating other immune cells, amplifying anti-tumor responses.

3 What recent innovations have improved the ability of immune cells to infiltrate and persist within solid tumors?

The challenge of poor infiltration and survival of T cells and CAR-T cells in solid tumor microenvironments has led to several innovative approaches. Some of them are very much on the engineering side. If you can engineer a CAR-T cell along with CRISPR-Cas9 to do a gene knock-out or knock-in, you can delete or introduce any gene you want anywhere you want. In some cases, the knock-out of the PD-1 exhaustion marker from CAR-T cells has been one way to make them become "deaf" to the solid tumor microenvironment exhaustion signal PD-L1.

Another, more sophisticated, way is engineering T cells to deliver "payloads" of inflammatory cytokines upon activation. As I mentioned previously, the tumor microenvironment is enriched in anti-inflammatory cytokines and immunosuppressive cells. Using gene promoters that drive gene expression upon T-cell activation, like NFAT (nuclear factor of activated T cells), to trigger production of interleukins such as IL-2, IL-12, and IL-18 can reshape the immunosuppressive tumor microenvironment and make immune cells more resistant to exhaustion.

Another area of interest is genetically modifying CAR-natural killer (NK) cells. For some cancers like lung cancer, there is an abundance of NK cells, so we can leverage their prevalence and arm them with CAR. Or



we can perhaps modify Tregs, which are normally immunosuppressive and tend to be associated with a bad prognosis, but can acquire inflammatory properties if the CAR affinity is right. These would act as a Trojan horse because tumors naturally attract Tregs.

Looking at what immune cells inside the tumor microenvironment have that immune cells that don't make it don't have, you can go back to the drawing board and figure out how to make immune cells infiltrate better. It's almost like we're learning the algorithm. What buttons do I press to make an immune cell infiltrate the solid tumor? We've learnt and we've gotten very good at modifying conventional T cells, so now, can we also modify NK cells, Tregs and macrophages?

The research is evolving to modify additional immune cells. It's pretty exciting! We have to think beyond just engineering T cells to be resistant to exhaustion, we have to make them more penetrating. Or even trying to repurpose other cell types that naturally live in the tumor microenvironment to see if those cells are better adapted so that we can repurpose them to fight cancer and remodel the tumor microenvironment. It's about finding out whatever approach works best, and it's probably going to be a multi-pronged approach.

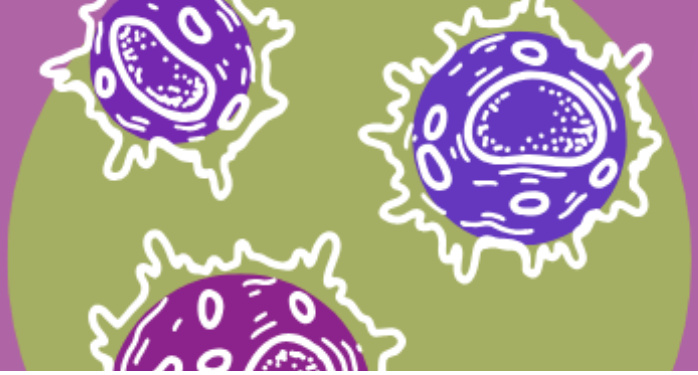
4 How are allogeneic cell therapies currently used to enhance scalability while maintaining efficacy and safety in adoptive cell therapy, and what are its current limitations?

Generally speaking, it can be a pretty laborious process to collect a patient's T cells, isolate, genetically modify, expand and reinfuse them back into the patient. Also, CAR T-cell therapy and cell therapy in general many times is not the first line of therapy, it's not the first treatment that people with cancer receive. But many treatments harm the immune system. If someone has undergone three rounds of different chemotherapy drugs, and then CAR-T is their fourth line of therapy, these person's T cells might not expand as well as someone who is cancer-free.

However, currently, autologous T-cell therapy is the gold standard. Our immune systems are complex and evolved ways to recognize self from non-self. Of course, there are exceptions like in pregnancy where the fetus that is partly non-self gets accepted. Even microbes, they're not part of your cells, but they live in your body and the immune system has also learned ways to keep them as "extended self". There are many systems in place to tell self from non-self. However, cancer cells are "altered self" and, unfortunately for us, just close enough to self to not be rejected right away sometimes.

A big way in which self-nonself recognition happens is through human leukocyte antigen (HLA) mismatch recognition. There are thousands and thousands of HLA alleles, making it virtually impossible to have someone who has the same exact HLA type as someone else, unless they're twins.

When we take T cells from one person, engineer them into CAR-T cells, and then put them into somebody else's body, we run into two problems. First, the recipient's immune system can reject them because they are foreign, and second, those CAR-T cells can attack the recipient's body as these T cells are now in a foreign environment. They are, after all, naturally programmed to fight anything that looks foreign to them. It's a two-way rejection situation that can create complications for treatment.



One way around the first problem is to generate hypoimmune CAR-T cells, that is making the cells "invisible" to the immune system. That means HLA has to go, right? HLA is the number one way through which the immune system tells self from non-self. If you put HLA-A2 positive T cells into an HLA-A2 negative cancer patient, this person's immune system knows HLA-A2 does not belong here. This can lead to the elimination of the donor T-cells. By deleting various genes, you can prevent certain HLA from being expressed, so that foreign therapeutic T cells don't get attacked by the recipient T cells. But there's a catch! The immune system has evolved to recognize these cases. A lot of cancer cells and viruses precisely down-regulate HLA to try to escape. That's when NK cells come in. NK cells kill HLA deficient cells. It's called the missing self-hypothesis. If a cell has no passport, it's very suspicious. To combat this, you can make a cell that is selectively barren for certain HLA alleles and maybe find the right recipe to make the "perfect" hypoimmune cell. But what if this CAR-T cell becomes cancerous or is infected with a virus? Then it would be very hard for the immune system to eliminate it as it usually would.

So those are the strategies being used to implement allogeneic cell therapies. Editing certain genes to make CAR-T cells invisible while making sure they are safe. It's a whole complex web of just too many factors at the moment, with progress but also limitations that remain.

Although autologous CAR T-cell therapy is what works best and all that is approved by the FDA, we're always looking to the future. There are companies focusing precisely on allogeneic T cells. If we can make a centralized bank of CAR-T cells, it would result in much lower costs and more patients being treated, expanding access to cell therapy.

5 Which biomarkers show the most promise for guiding adaptive cell therapy selection and monitoring in solid tumors?

It depends on your patient sample. We're learning a lot and what we're seeing is that in the whole biomarker research field, humans are variable and so are cancers. It can be hard to tell signal apart from noise.

For liquid tumors, you just take blood and the cancer cells themselves are there. But for solid tumors, you need to have indirect ways to assess them, biomarkers. Some biomarkers can be molecules whose levels are very high if you have a big enough tumor, for example hormonal tumors like a pituitary tumor. Or molecules that cancer cells produce to protect themselves like GDF-15. Cell-free DNA derived from dying cancer cells can also be a biomarker. Methylation of this DNA is important because it's a chemical modification on DNA that gives you a hint of whether the gene the DNA fragment is derived from is active or not. Some tumors overexpress growth factor receptors like EGFR, for example. Inflammation markers like IL-6 increase in certain cancers. It really depends on your patient sample.

Don't look just at one biomarker, look at a few hundred. And then, with the right statistics, you can determine what's meaningfully different versus what's not.

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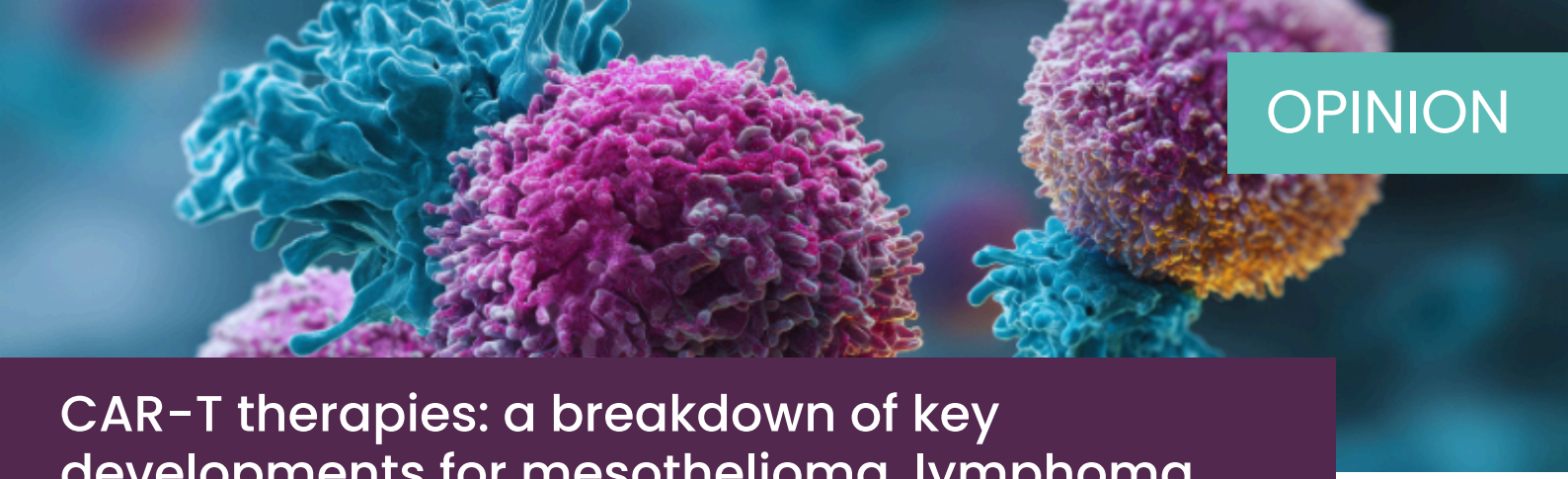


OPINION

CAR-T therapies: a breakdown of key developments for mesothelioma, lymphoma and solid tumors

From next-generation therapies for mesothelioma to dual-targeting approaches for lymphoma, CAR-T therapy is expanding treatment options for aggressive cancers.

FREYA LEASK, CONTRIBUTING EDITOR, ONCOLOGY CENTRAL

CAR-T therapy has made significant strides, moving beyond its initial success in blood cancers to show promise in treating solid tumors. In this listicle, we explore advancements in this field, highlighting novel strategies that address key challenges. We delve into mesothelin-targeted therapies for mesothelioma, review key approvals for lymphomas as well as innovative approaches for solid tumors, including targeted protein degradation and the use of 'on/off' switches to combat T-cell exhaustion.

Mesothelioma

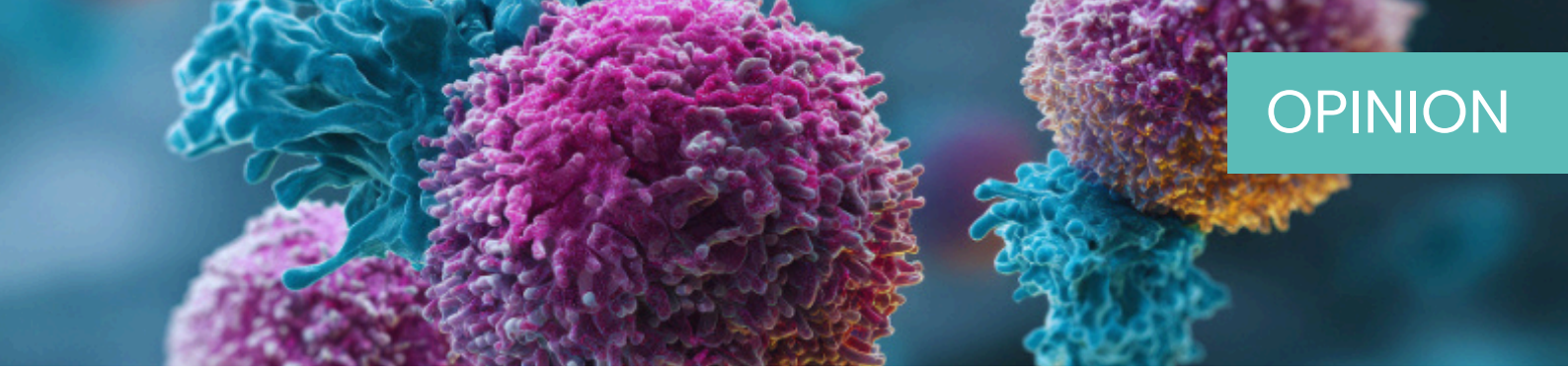
The standard of care for mesothelioma has historically been limited, and patients often have a poor prognosis. However, advancements in CAR-T therapy are providing renewed hope, particularly through mesothelin-targeted CAR-T therapies, which have piqued interest at major oncology meetings.

A few active trials in this area, a Phase I trial is evaluating TNhYP218 CAR T cells, while a Phase I/II trial is investigating A2B694, an autologous logic-gated Tmod™ CAR T-cell product. Both trials focus on establishing the recommended safe dose of the respective therapies.

The landscape of mesothelin-targeted therapies also includes Atara Biotherapeutics (CA, USA) ATA2271, which was granted FDA clearance for an investigational new drug application in 2020. Preclinical testing of ATA2271 demonstrated that it decreased cell exhaustion and improved CAR-T persistence, resulting in effective destruction of cancer cells. Following a fatal serious adverse event, the Phase I trial has not progressed since 2022.

Another promising approach is IcasM28z, which in a Phase I trial exhibited no signs of therapy-related toxicity whilst providing antitumor activity. Researchers observed tumor regression and a decrease in mesothelin-related peptide in the blood of some patients. The therapy was also combined with anti-PD-1 checkpoint blockade agents to reactivate exhausted CAR T-cells in some patients. The trial has now progressed to Phase I/II, within which the investigators will test the therapy in combination pembrolizumab to assess treatment efficacy for malignant pleural mesothelioma.

Prasad S. Adusumilli of Memorial Sloan Kettering (NY, USA) commented on the Phase I findings that combining these strategies "produced



encouraging results and provides rationale to further investigate this approach in aggressive, therapy-resistant cancers". He concluded that "if this approach is successful, 2 million patients with mesothelin-expressing solid tumors per year in the United States will be eligible for this treatment".

Lymphoma

Immunotherapy has long been the standard of care for various forms of lymphoma, particularly B-cell malignancies. The National Institute for Health and Care Excellence (NICE; London, UK) has already approved four personalized CAR-T therapies in this space; Yescarta® for diffuse large-B-cell lymphoma, and Tecartus for relapsed or refractory B-cell acute lymphoblastic leukemia, Breyanzi® for refractory large B-cell lymphomas and Kymriah® for B-cell acute lymphoblastic leukemia.

In the ZUMA-7 trial, Yescarta was reported as superior to the current standard of care as a second-line treatment for relapsed or refractory large B-cell lymphoma. The trial demonstrated a 60% reduction in the risk of disease progression or death for patients receiving Yescarta, with a median follow-up of 2 years. The lead investigator of the ZUMA-7 trial, Frederick L. Locke (Moffitt Cancer Center; FL, USA) commented that the results "paint the picture of a potential paradigm shift in the treatment of large B-cell lymphoma".

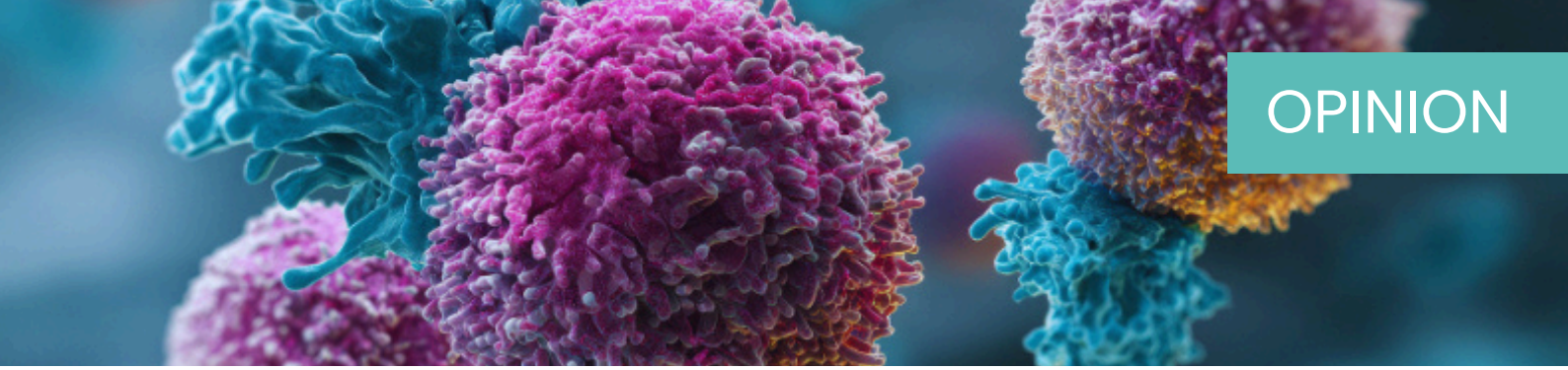
Another promising development for lymphomas are CAR-T therapies that use a bilateral attack to target two proteins, CD19 and CD20, simultaneously. In early results from a small trial for people with non-Hodgkin's B-cell lymphoma, four out of five patients had a complete metabolic response and are in ongoing complete remission. The trial's lead author, Sanaz Ghafouri (UCLA Jonsson Comprehensive Cancer Center; CA, USA), expressed hope that work will "provide patients… a chance at a possible cure or at the very least a lasting long-term remission".

Solid tumors

Solid tumors have often proved a challenging target for CAR-T therapy due to several factors, including antigenic heterogeneity and the immunosuppressive nature of the tumor microenvironment.

One innovative approach in this cancer type uses targeted protein degradation to create "on/off" switches for CAR-T therapy. According to Stefan Pfister, (Hopp Children's Cancer Center Heidelberg, Germany) this method can reverse T-cell exhaustion and improve the efficacy of combination immunotherapies. This technique also allows for the targeting of previously "undruggable" oncoproteins, such as transcription factors, which are often drivers of childhood cancers.

A major player in this space is AstraZeneca (Cambridge, UK), which is investigating therapies that can shift the immune system from the 'ignore' or 'defend' states to the 'attack' state. Therapies, including immune-cell engagers, are being designed to overcome the 'ignore' state by creating artificial immune synapses to trigger T-cell activation. For the 'defend' state, which is characterized by immune suppression, new



approaches include developing bispecific antibodies to block multiple inhibitory checkpoints simultaneously. The company is also working to expand the use of cell therapies to more patients by developing “off-the-shelf” CAR-T and TCR-T therapies.

Promising results have also been achieved when CAR-T products are combined with other agents, for example an mRNA vaccine. One such combination, BNT211, showed early signs of efficacy in patients with CLDN6-positive solid tumors. Developed by BioNTech (Germany), BNT211 is currently being tested in a randomized Phase II clinical trial, expected to end this year.

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REVIEW

Pitfalls and strategies of CAR-T therapy in solid tumors and implications for chordoma treatment

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ABSTRACT

In recent years, CAR-T cell therapy has emerged as a promising immunotherapeutic approach, demonstrating remarkable therapeutic efficacy in hematologic malignancies such as leukemia and lymphoma. However, its effectiveness in treating solid tumors remains limited, with challenges such as low response rates, poor therapeutic persistence, and high recurrence rates. The unique and complex immune microenvironment of solid tumors, characterized by a dense extracellular matrix, an abundance of immunosuppressive cells, and cytokines, is considered a major factor impeding CAR-T cell infiltration, antitumor activity, and persistence, significantly hindering the clinical potential of this therapy. To address these challenges, various strategies have been developed to optimize CAR-T cell functionality and adaptability. As a rare and highly complex solid tumor, chordoma presents with several challenges for CAR-T cell therapy, including the lack of tumor-specific antigens, rich extracellular matrix, and enrichment of immunosuppressive factors such as TGF-β. This review summarizes the key challenges and corresponding strategies to enhance CAR-T cell therapy in solid tumors, with a particular focus on underlying its therapeutic potential for the treatment of chordoma.

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KEYWORDS
CAR-T therapy; pitfalls; solid tumor; chordoma; combination therapy

1. Introduction

Chordoma is a rare spinal malignant tumor with an incidence of 0.08/100,000 cases per year. It is resistant to chemotherapy and radiotherapy due to its poor blood supply and inefficient drug delivery. Surgery remains the only available treatment option. However, the rate of local recurrence following surgical removal of the primary tumor is extremely high, accounting for 50% of patients [1]. Additionally, about 30–40% of patients present with metastatic disease, mainly localized to lung and liver [2]. Few clinical studies have reported the limited efficacy of immunotherapeutic approaches, including the use of PD-1 inhibitors, thus underlying the urgent need of developing novel effective treatment options for chordoma patients [3]. In recent years, various clinical trials have been initiated to explore novel therapeutic strategies for this disease. As summarized in Table 1, these trials encompass a range of approaches including targeted therapy, immune checkpoint inhibitors, chemotherapy-based regimens, and emerging immunotherapies. Of the total registered trials, 29 have been completed, while 21 are currently recruiting, and 8 are active but not recruiting. A smaller number are listed as not yet recruiting (4), terminated (5), or have unknown (5) status. Three trials have been withdrawn, marked as no longer available, or available, respectively. Regarding study type, the majority (58 trials) are interventional, aiming to assess the safety and efficacy of investigational treatments. Observational studies account for 15 trials, and expanded access programs for 2 trials. These data reflect a growing research focus on improving therapeutic outcomes in chordoma and underscore the importance of ongoing efforts to develop innovative approaches such as immunotherapy.

In this clinical scenario, chimeric antigen receptor (CAR)-T cell therapy represents a promising approach, even though it has not been largely explored yet. CARs are recombinant proteins designed to specifically recognize and bind target antigens expressed on tumor cells. The cytotoxic mechanism of CAR-T cells does not rely on the major histocompatibility complex (MHC) class I-mediated antigen presentation. This feature makes them an ideal therapeutic approach to target tumors characterized by defective expression of MHC class I molecules. Indeed, many tumors, including chordoma, actively downregulate MHC class I molecule expression to escape T cell immune surveillance. CAR T cell therapy has revolutionized the treatment of hematologic malignancies; Kymriah, the first FDA-approved CAR-T therapy, was developed for children and young adults with relapsed or refractory acute B-cell lymphoblastic leukemia (ALL), marking a significant milestone in immunotherapy [4] (Figure 1). As

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Article highlights

- The review systematically summarizes the key obstacles limiting CAR-T cell efficacy in solid tumors, including antigen heterogeneity, immunosuppressive microenvironments, poor infiltration, and treatment-related toxicity.
- A range of innovative strategies are discussed to overcome these limitations, such as multi-target CAR designs, immune modulation techniques, infiltration enhancers, and toxicity-reducing approaches.
- Chordoma, as a rare malignant tumor, is examined in depth with regard to its unique immune features and its limited but emerging suitability for CAR-T therapy.
- The review outlines both barriers and solutions specific to chordoma, bridging general CAR-T challenges and tumor-specific translation opportunities.

today, six CAR T cell products are currently approved by FDA for the treatment of patients with relapsed/refractory hematologic malignancies, including CD19 and B cell mature antigen (BCMA)-targeted CAR-T [5]. CAR-T cell efficacy has been demonstrated promising preclinical results in several types of solid tumors including lung cancer, hepatocellular carcinoma, gastric cancer, breast cancer, and chordoma [6]. Additionally, this immunotherapeutic approach has generated encouraging clinical outcomes [7–9]. As of April 2025, data from ClinicalTrials.gov indicate a total number of 182 CAR-T cell clinical trials targeting solid tumors (6 completed, 15 active/not recruiting, 98 actively recruiting or enrolling by invitation, 41 with unknown status, 9 suspended/terminated/withdrawn, and 14 not yet recruiting) [10]. In the published results, the average overall response rate (ORR) and (Disease Control Rate) DCR is around 50% and 75% [11,12], respectively.

However, CAR-T cells present several limitations with solid tumors, such as the difficulty to infiltrate the tumor, extracellular matrix in chordoma, low T cell recruitment to the tumor site due to the massive release of inhibitory soluble factors by tumor cells, and the immunosuppressive tumor microenvironment (TME) [13–17]. Other limitations including cytokine-release syndrome (CRS) and on-target off-tumor toxicities (OTOT) restrain the therapeutic index [18]. Big effort in developing strategies to enhance CAR-T efficacy include combinatorial strategies, novel CAR designs and localized delivery. In this review, we will summarize major challenges of CAR-T cell therapy in solid tumors with particular focus on its application for chordoma.

2. Key pitfalls limiting CAR-T cell therapy with solid tumors

2.1. Antigen heterogeneity and antigen Loss

Selecting appropriate tumor-specific antigens (TSAs) is essential for CAR-T therapy. These antigens must be highly expressed on tumor cells but minimally present on normal tissues. However, fewer than 10% of cellular proteins are found on the tumor cell membrane, severely limiting the pool of potential targets [19]. As a result, CAR-T strategies often rely on tumor-associated antigens (TAAs) instead, such as epidermal growth factor receptor (EGFR), human epidermal growth factor receptor 2 (HER2), human endogenous retrovirus-H long terminal repeat-associating protein 2 (HHLA2), mesothelin (MSLN) and disialoganglioside GD2 [20]. Tumor cells, however, are highly heterogeneous – genetic mutations and microenvironmental changes can cause different cells within the same tumor to express varying antigen profiles. In some regions, antigen expression may be too low or entirely absent, leading to resistance or recurrence [21]. Antigen escape can occur through a variety of mechanisms, such as the selection of preexisting antigen-negative clones, spontaneous antigen loss triggered by cellular stress, acquired genetic mutations, splicing site variations, T-cell trogocytosis, and lineage switching from a lymphoid to a myeloid phenotype. Additional pathways include impaired surface antigen presentation and epitope masking, among others [21,22]. Furthermore, surviving tumor cells may downregulate antigen expression by altering the regulatory networks of upstream transcription factors, enabling them to evade CAR-T targeting.

2.2. Hostile tumor immune microenvironment

The tumor microenvironment (TME) of solid tumors, including chordoma, is highly immunosuppressive and poses significant barriers to effective CAR-T cell therapy. Key cellular components such as tumor-associated macrophages (TAMs), regulatory T cells (Tregs), and myeloid-derived suppressor cells (MDSCs) accumulate in large numbers and impair CAR-T cell activity and persistence. These cells secrete immunosuppressive cytokines – such as TGF-β and IL-10—and express immune checkpoint molecules like PD-L1 and CTLA-4 [23,24]. TAMs can express PD-L1, directly engaging PD-1 on CAR-T cells and contributing to their functional exhaustion. Tregs suppress effector T-cell activity through secretion of TGF-β and IL-10, while MDSCs further impair CAR-T cell proliferation and function through the release of reactive oxygen species (ROS) and granulocyte-macrophage colony-stimulating factor (GM-CSF), both of which are known to blunt immune responses [25,26].

Hypoxia is another defining feature of the chordoma TME. Furthermore, hypoxia alters immune cell phenotypes: it suppresses CD8+ T cell cytotoxicity, promotes FOXP3 expression (driving Treg development), induces Th2 skewing of CD4+ T cells, and enhances M2 polarization of macrophages – all of which suppress antitumor immunity [27,28].

Additionally, the metabolic profile of chordomas contributes to immune evasion. Accumulation of lactate, prostaglandins, and reduced extracellular pH within the TME has been shown to inhibit CAR-T cell proliferation, reduce cytokine secretion, and even trigger CAR-T cell apoptosis or dysfunction [29–31].

2.3. Low rate of CAR-T infiltration

Before CAR-T cells can recognize and bind to tumor-specific antigens, they must first successfully infiltrate the tumor tissue. However, in solid tumors – including chordoma – this step remains a major hurdle. It is estimated that only about 2% of intravenously administered CAR-T cells reach the tumor microenvironment (TME), largely due to the dense stroma and

Table 1. Clinical trials for chordoma.

NCT Number	Study Title	Study Status	Study Type
NCT05041127	Cetuximab for the Treatment of Advanced Unresectable or Metastatic Chordoma	RECRUITING	INTERVENTIONAL
NCT06794645	Pembrolizumab and Pemetrexed for Progressive Chordoma	RECRUITING	INTERVENTIONAL
NCT06957327	A Study of ERAS-601 in People With Chordoma	RECRUITING	INTERVENTIONAL
NCT06787664	A Study of BL-B01D1 in Patients With Locally Advanced or Metastatic Chordoma	RECRUITING	INTERVENTIONAL
NCT03623854	Nivolumab and Relatlimab in Treating Participants With Advanced Chordoma	COMPLETED	INTERVENTIONAL
NCT03910465	Children and Adults With Chordoma	RECRUITING	OBSERVATIONAL
NCT03955042	Pemetrexed for the Treatment of Chordoma	COMPLETED	INTERVENTIONAL
NCT04486820	Immunohistochemical Study of Chordomas to Improve Their Diagnosis and Prognosis Care	COMPLETED	OBSERVATIONAL
NCT00341627	Genetic Aspects of Chordoma: A Collaboration With SEER Registries to Identify Chordoma Families	COMPLETED	OBSERVATIONAL
NCT03595228	BN Brachyury and Radiation in Chordoma	COMPLETED	INTERVENTIONAL
NCT01407198	Nilotinib With Radiation for High Risk Chordoma	ACTIVE_NOT_RECRUITING	INTERVENTIONAL
NCT03110744	CDK4/6 Inhibition in Locally Advanced/Metastatic Chordoma	COMPLETED	INTERVENTIONAL
NCT00496119	Proton Beam Therapy for Chordoma Patients	ACTIVE_NOT_RECRUITING	INTERVENTIONAL
NCT00150072	Efficacy and Safety of Imatinib in Chordoma	COMPLETED	INTERVENTIONAL
NCT03083678	Afatinib in Locally Advanced and Metastatic Chordoma	COMPLETED	INTERVENTIONAL
NCT02986516	Sacral Chordoma: Surgery Versus Definitive Radiation Therapy in Primary Localized Disease	RECRUITING	INTERVENTIONAL
NCT00410670	Chordoma Family Study	COMPLETED	OBSERVATIONAL
NCT02383498	QUILT-3.011 Phase 2 Yeast-Brachyury Vaccine Chordoma	COMPLETED	INTERVENTIONAL
NCT02989636	Nivolumab With or Without Stereotactic Radiosurgery in Treating Patients With Recurrent, Advanced, or Metastatic Chordoma	ACTIVE_NOT_RECRUITING	INTERVENTIONAL
NCT01175109	Study of Imatinib, a Platelet-derived Growth Factor Receptor Inhibitor, and LBH589, a Histone Deacetylase Inhibitor, in the Treatment of Newly Diagnosed and Recurrent Chordoma	UNKNOWN	INTERVENTIONAL
NCT00797602	Proton Therapy for Chordomas and/or Chondrosarcomas	COMPLETED	OBSERVATIONAL
NCT00713037	Hypoxia-positron Emission Tomography (PET) and Intensity Modulated Proton Therapy (IMPT) Dose Painting in Patients With Chordomas	COMPLETED	INTERVENTIONAL
NCT01449149	Proton Radiation for Chordomas and Chondrosarcomas	ACTIVE_NOT_RECRUITING	INTERVENTIONAL
NCT05861245	Hypofractionated Protontherapy in Chordomas and Chondrosarcomas of the Skull Base	RECRUITING	INTERVENTIONAL
NCT01811394	Ion Irradiation of Sacrococcygeal Chordoma	ACTIVE_NOT_RECRUITING	INTERVENTIONAL
NCT02802969	Improvement of Local Control in Skull Base, Spine and Sacral Chordomas Treated by Surgery and Protontherapy Targeting Hypoxic Cells Revealed by [18F]FAZA) PET/CT Tracers	COMPLETED	INTERVENTIONAL
NCT06463262	Exploration of Personalized Biomarkers During Neoadjuvant Radiation Therapy for Spinal and Sacral Chordoma	RECRUITING	OBSERVATIONAL
NCT00592748	Charged Particle RT for Chordomas and Chondrosarcomas of the Base of Skull or Cervical Spine	COMPLETED	INTERVENTIONAL
NCT06140732	Apatinib Combined With Camrelizumab in Treating Participants With Advanced Chordoma	RECRUITING	INTERVENTIONAL
NCT05519917	Study to Evaluate the Efficacy of Afatinib in Skull Base Chordoma	NOT_YET_RECRUITING	INTERVENTIONAL
NCT01200680	Genetic Clues to Chordoma Etiology: A Protocol to Identify Sporadic Chordoma Patients for Studies of Cancer-Susceptibility Genes	RECRUITING	OBSERVATIONAL
NCT01182779	Trial of Proton Versus Carbon Ion Radiation Therapy in Patients With Chordoma of the Skull Base	UNKNOWN	INTERVENTIONAL
NCT05888064	Multi-parametric Imaging in Personalized Radiotherapy	RECRUITING	OBSERVATIONAL
NCT05707767	A Prospective Study of Surgical Treatment Strategies for Chordoma	UNKNOWN	INTERVENTIONAL
NCT03647423	QUILT-3.091 NANT Chordoma Vaccine vs Radiation in Subjects With Unresectable Chordoma.	WITHDRAWN	INTERVENTIONAL
NCT04246671	TAEK-VAC-HerBy Vaccine for Brachyury and HER2 Expressing Cancer	COMPLETED	INTERVENTIONAL
NCT06029218	Analysis of the Toxicity and Efficacy of Daily 1 vs 2 Beam Proton Therapy	RECRUITING	INTERVENTIONAL
NCT03242382	Trial of Palbociclib in Second Line of Advanced Sarcomas With CDK4 Overexpression.	RECRUITING	INTERVENTIONAL
NCT01346124	High Dose Intensity Modulated Proton Radiation Treatment ± Surgical Resection of Sarcomas of the Spine, Sacrum and Base of Skull	ACTIVE_NOT_RECRUITING	INTERVENTIONAL
NCT04042597	Anlotinib Hydrochloride Versus Imatinib Mesylate in Locally Advanced, Unresectable or Metastatic Chordoma	UNKNOWN	INTERVENTIONAL
NCT04832620	Image Assisted Optimization of Proton Radiation Therapy in Chordomas and Chondrosarcomas	RECRUITING	OBSERVATIONAL
NCT02838602	Randomized Carbon Ions vs Standard Radiotherapy for Radioresistant Tumors	RECRUITING	INTERVENTIONAL
NCT02520128	A Study of IMRT in Primary Bone and Soft Tissue Sarcoma	COMPLETED	INTERVENTIONAL
NCT05033288	Comparing Carbon Ion Therapy, Surgery, and Proton Therapy for Management of Pelvic Sarcomas Involving the Bone	RECRUITING	OBSERVATIONAL
NCT04087902	Long-Term Longitudinal QoL in Patients Undergoing EEA	RECRUITING	OBSERVATIONAL
NCT02936102	A Study of FAZ053 Single Agent and in Combination With PDR001 in Patients With Advanced Malignancies.	TERMINATED	INTERVENTIONAL
NCT03886311	Talimogene Laherparepvec, Nivolumab and Trabectedin for Sarcoma	RECRUITING	INTERVENTIONAL
NCT01347307	Stereotactic Body Radiotherapy for Spine Tumors	COMPLETED	INTERVENTIONAL
NCT03058289	A Phase 1/2 Safety Study of Intratumorally Dosed INT230–6	COMPLETED	INTERVENTIONAL
NCT03190174	Nivolumab (Opdivo®) Plus ABI-009 (Nab-rapamycin) for Advanced Sarcoma and Certain Cancers	COMPLETED	INTERVENTIONAL
NCT06535997	Descriptive Cohort of French Patients Treated with Carbonetherapy Since October 2010 Outside PHRC-ETOILE	NOT_YET_RECRUITING	OBSERVATIONAL
NCT05286801	Tiragolumab and Atezolizumab for the Treatment of Relapsed or Refractory SMARCB1 or SMARCA4 Deficient Tumors	RECRUITING	INTERVENTIONAL
NCT04091295	BLESSED: Expanded Access for DNG64 for Advanced Pancreatic Cancer, Sarcoma and Carcinoma of Breast	AVAILABLE	EXPANDED_ACCESS
NCT05407441	Tazemetostat+Nivo/Ipi in INI1-Neg/SMARCA4-Def Tumors	RECRUITING	INTERVENTIONAL
NCT04416568	Study of Nivolumab and Ipilimumab in Children and Young Adults With INI1-Negative Cancers	RECRUITING	INTERVENTIONAL
NCT00003926	Amifostine to Protect From Side Effects of PSCT in Treating Patients With Solid Tumors	TERMINATED	INTERVENTIONAL
NCT07005297	Clinical Genetics Branch Eligibility Screening Survey	NOT_YET_RECRUITING	OBSERVATIONAL
NCT00349024	Polyvinylpyrrolidone-Sodium Hyaluronate Gel in Reducing Pain From Oral Mucositis in Young Patients With Cancer	UNKNOWN	INTERVENTIONAL
NCT00931931	HSV1716 in Patients With Non-Central Nervous System (Non-CNS) Solid Tumors	COMPLETED	INTERVENTIONAL

(*Continued*)

Table 1. (Continued).

NCT Number	Study Title	Study Status	Study Type
NCT02601950	A Study of Tazemetostat in Adult Participants With Soft Tissue Sarcoma	COMPLETED	INTERVENTIONAL
NCT00464620	Trial of Dasatinib in Advanced Sarcomas	COMPLETED	INTERVENTIONAL
NCT06625190	Alpha/Beta T and B Cell Depletion With Zoledronic Acid for Solid Tumors	NOT_YET_RECRUITING	INTERVENTIONAL
NCT00919269	Collecting and Storing Tissue, Blood, and Bone Marrow Samples From Patients With Rhabdomyosarcoma or Other Soft Tissue Sarcoma	COMPLETED	OBSERVATIONAL
NCT01344356	Stereotactic Body Radiotherapy for Head and Neck Tumors	COMPLETED	INTERVENTIONAL
NCT05355753	A Study to Assess the Safety and Tolerability of CFT8634 in Locally Advanced or Metastatic SMARCB1-Perturbed Cancers, Including Synovial Sarcoma and SMARCB1-Null Tumors	TERMINATED	INTERVENTIONAL
NCT01924689	Safety Study of Intratumoral Injection of Clostridium Novyi-NT Spores to Treat Patients With Solid Tumors That Have Not Responded to Standard Therapies	COMPLETED	INTERVENTIONAL
NCT01567046	Studying Genes in Tissue Samples From Younger and Adolescent Patients With Soft Tissue Sarcomas	COMPLETED	OBSERVATIONAL
NCT01046487	Imatinib Mesylate And Cyclophosphamide In Metronomic Administration: Dose Escalation Study Of Imatinib Mesylate	COMPLETED	INTERVENTIONAL
NCT04965753	FHD-609 in Subjects With Advanced Synovial Sarcoma or Advanced SMARCB1-Loss Tumors	TERMINATED	INTERVENTIONAL
NCT04670679	A Dose Escalation/Expansion Study of ERAS-601 in Patients With Advanced or Metastatic Solid Tumors	ACTIVE_NOT_RECRUITING	INTERVENTIONAL
NCT03874455	Tazemetostat Expanded Access Program for Adults With Solid Tumors	NO_LONGER_AVAILABLE	EXPANDED_ACCESS
NCT01682746	Photodynamic Therapy (PDT) for Recurrent Pediatric Brain Tumors	COMPLETED	INTERVENTIONAL
NCT00154388	Phase II Study of Imatinib Mesylate in Patients With Life Threatening Malignant Rare Diseases	COMPLETED	INTERVENTIONAL
NCT02834013	Nivolumab and Ipilimumab in Treating Patients With Rare Tumors	ACTIVE_NOT_RECRUITING	INTERVENTIONAL
NCT01966809	Photodynamic Therapy (PDT) For Recurrent High Grade Gliomas	TERMINATED	INTERVENTIONAL

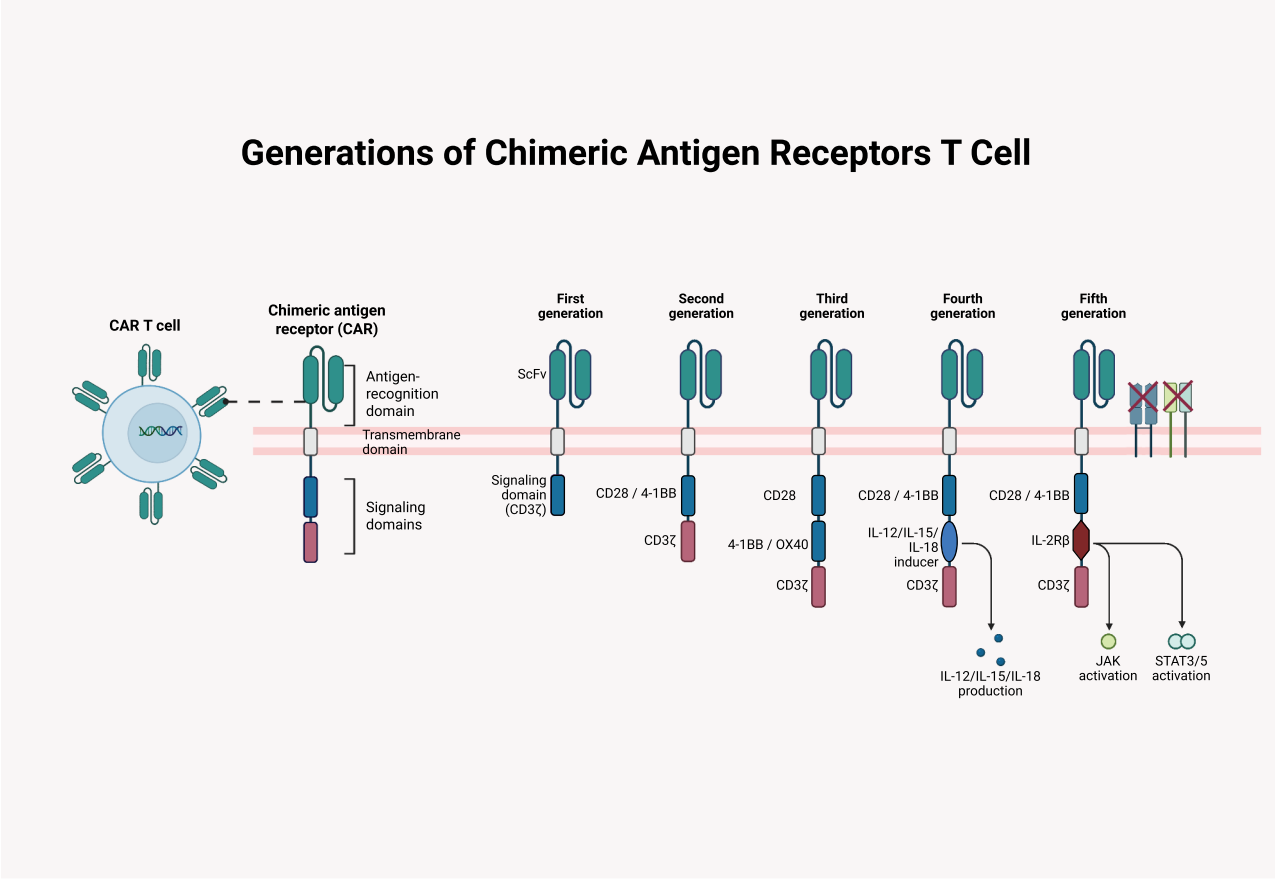

Figure 1. Generations of chimeric antigen receptors T cell. Figure created with Biorender.

abnormal vasculature characteristic of solid tumors[29]. The trafficking of CAR-T cells into tumors is regulated by a complex cascade of processes – rolling, adhesion, extravasation, and chemotaxis – all of which are tightly influenced by the local microenvironment and chemokine gradients produced by both tumor and immune cells [32].

Low oxygen levels not only contribute to stromal fibrosis by promoting the differentiation of fibroblasts into cancer-

associated fibroblasts (CAFs) via TGF-β, PDGF, FGF, IL-6, and IL-1β signaling, but also impair CAR-T cell function [33,34]. The latter have been shown to increase tumor mesenchymal density through the secretion of extracellular matrix components, further limiting CAR-T infiltration and migration [35,36].

In addition to these cellular barriers, the extracellular matrix (ECM) of chordoma presents unique structural and biochemical challenges [37,38]. ECM is dynamically regulated by intrinsic mechanisms such as the secretion of matrix metalloproteinases (MMPs), heparanase, and cathepsins, activation of the uPA/uPAR system, and immune signaling [39]. The chordoma ECM is highly abundant and rich in glycosaminoglycans, which imbibe water and contribute to a dense, hydrated matrix that physically impedes lymphocyte infiltration [40]. Hypoxia-induced fibrosis increases ECM deposition and mesenchymal density, worsening the physical barrier. Moreover, due to the high sulfate content, the ECM is highly negatively charged. This negative charge may further restrict CAR-T cell penetration and function, as lymphocytes themselves carry a net negative surface charge. Evidence suggests that negatively charged environments can directly inhibit lymphocyte activity, potentially compounding the immunosuppressive effects of the chordoma TME [41]. Collectively, these features form a formidable barrier to CAR-T cell trafficking, persistence, and cytotoxic function within chordoma tumors. Moreover, chordomas are known to exhibit increased interstitial fluid pressure, which reduces convective transport and further limits immune cell entry into the tumor core.

These combined factors – the physical density and charge of the ECM, elevated tumor pressure, hypoxia, and immunosuppressive metabolic byproducts – form a formidable barrier that significantly impairs CAR-T cell trafficking, infiltration, and efficacy in chordoma.

2.4. The immune-related adverse events

CAR-T therapy can induce a variety of immune-related adverse events, such as cytokine release syndrome (CRS), immune effector cell-associated neurotoxicity syndrome (ICANS) and on-target off-tumor toxicity (OTOT) [42]. CRS is the most common side effect of CAR-T cell therapy and is characterized by over-activation of the immune system and the release of large amounts of inflammatory cytokines. Clinical manifestations include hyperthermia, fatigue, myalgia, nausea, capillary leakage, tachycardia, and hepatic and renal dysfunction [43]. CRS occurs in virtually all patients receiving CAR-T cell therapy and its severity is closely related to tumor burden. Severe CRS can be managed with the use of immunosuppressive agents such as corticosteroids and tocilizumab (IL-6 monoclonal antibody) [44]. Early use of tocilizumab has been shown to reduce the incidence of severe CRS without compromising therapeutic efficacy [45]. ICANS is another common toxic reaction, the underlying mechanisms are not fully understood and possibly associated with or independent of CRS [46]. Clinical manifestations include delirium, expressive aphasia, decreased alertness, and seizures. In some clinical trials, neurotoxicity has resulted in patient deaths, mainly due to cerebral edema. A study reported by Neelapu et al. reported that five patients died because of developing cerebral edema after CAR-T cell therapy, two of them were treated with cyclophosphamide alone and three patients were treated with a combination of cyclophosphamide and fludarabine [42]. In addition, CAR-T cell therapy can cause other adverse effects, such as B-cytopenia (due to CAR-T cells attacking normal CD19-expressing B cells) and prolonged haematopenia [43]. As patients have usually received immunosuppressive and lymphocyte-depleting chemotherapy before treatment, the addition of the above adverse effects can increase the risk of serious infections [44]. CAR-T cells may recognize and kill normal cells expressing the target antigen, leading to OTOT toxicity. Although the amount of TAA expressed in normal cells is much lower than that in tumor cells, CAR-T cells can still respond to low-expressed antigens due to their high sensitivity. For example, CAIX-targeted CAR-T cells can recognize normal epithelial cells in the lining of the bile ducts, leading to discrete cholangitis in patients with renal cell carcinoma [47]. Additionally, OTOT toxicity has also been associated with HER2-targeted CAR-T cells in pancreatic cancer patients, who experienced mild skin pruritus and severe upper gastrointestinal bleeding [48] (Figure 2).

3. Potential strategies to overcome pitfalls of CAR-T therapy for solid tumors

To enhance the tumor-killing effect of CAR-T cells, scientists have proposed various innovative strategies, including combination therapy, multi-target therapy, and modification of the structural domains of CARs, which have achieved remarkable progress [49,50] (Figure 3).

3.1. Development and improvement of multi-target CAR-T cells

Single-target CAR-T cells often have limited therapeutic efficacy. Multi-target strategies including bi/dual-target CAR-T cells, tandem-specific CAR-T cells (tanCARs) and triple-target CAR-T cells (triCARs), have achieved significant efficacy in the treatment of solid tumors [51].

The combination of multiple CAR-T cell products offers an effective solution for overcoming challenges associated with low or heterogeneous antigen expression or antigen loss in tumor cells [52]. The combination of CAR-T cells targeting EGFR and CD133 significantly improved the therapeutic efficacy of cholangiocarcinoma treatment [53]. The co-expression of two CARs on the same CAR-T cell has been shown to have better killing efficacy and fewer antigens escape variants than the combination of two single-antigen CAR-T cell products. Other structure like the tanCAR, may enhance killing effect by altering steric interactions with tumor antigens [54]. Nanobodies – heavy-chain-only antibodies naturally found in sharks and camelids – have recently been proposed as substitutes for scFvs, offering the potential to create compact tanCAR and precisely defined affinity [55]. TanCAR-T cells targeting CD70 and B7-H3 exhibit enhanced antitumor functionality in the treatment against solid tumor and melanoma [56]. Another research for glioblastoma showed a reduced immune escape response after the

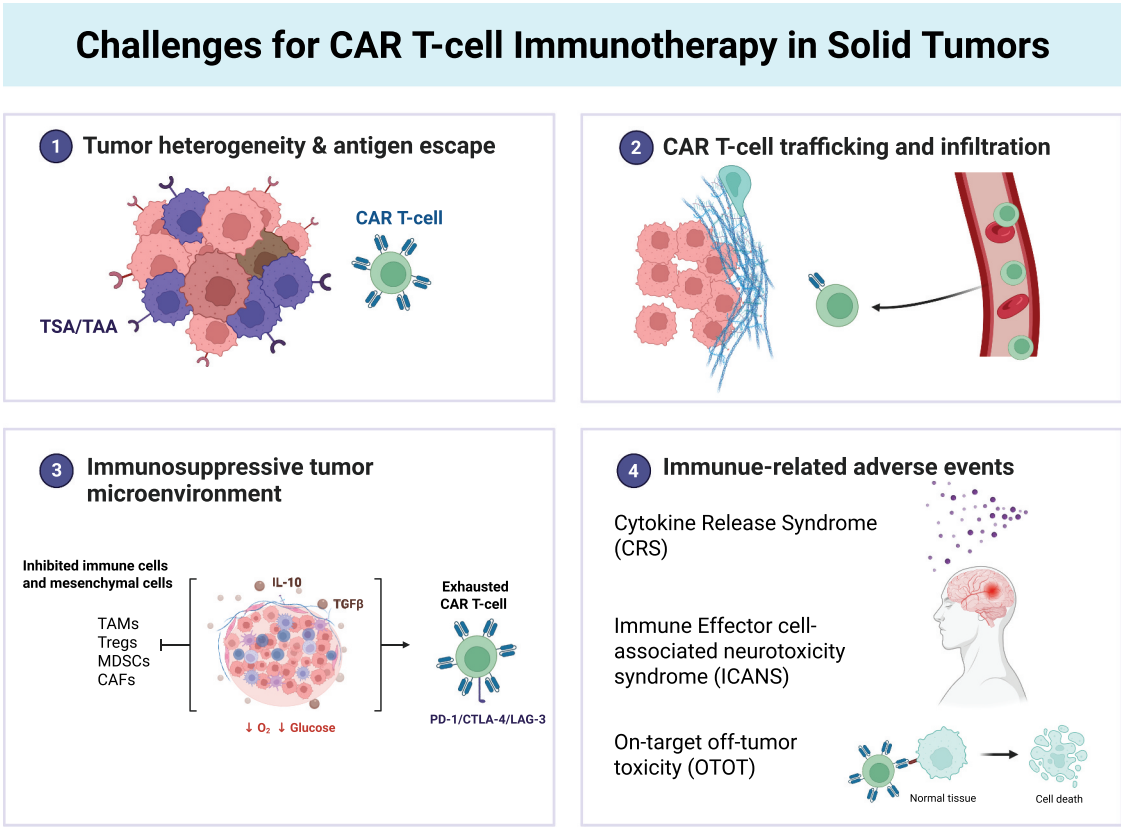

Figure 2. Challenges for CAR T-cell immunotherapy in solid tumors. Figure created with Biorender.

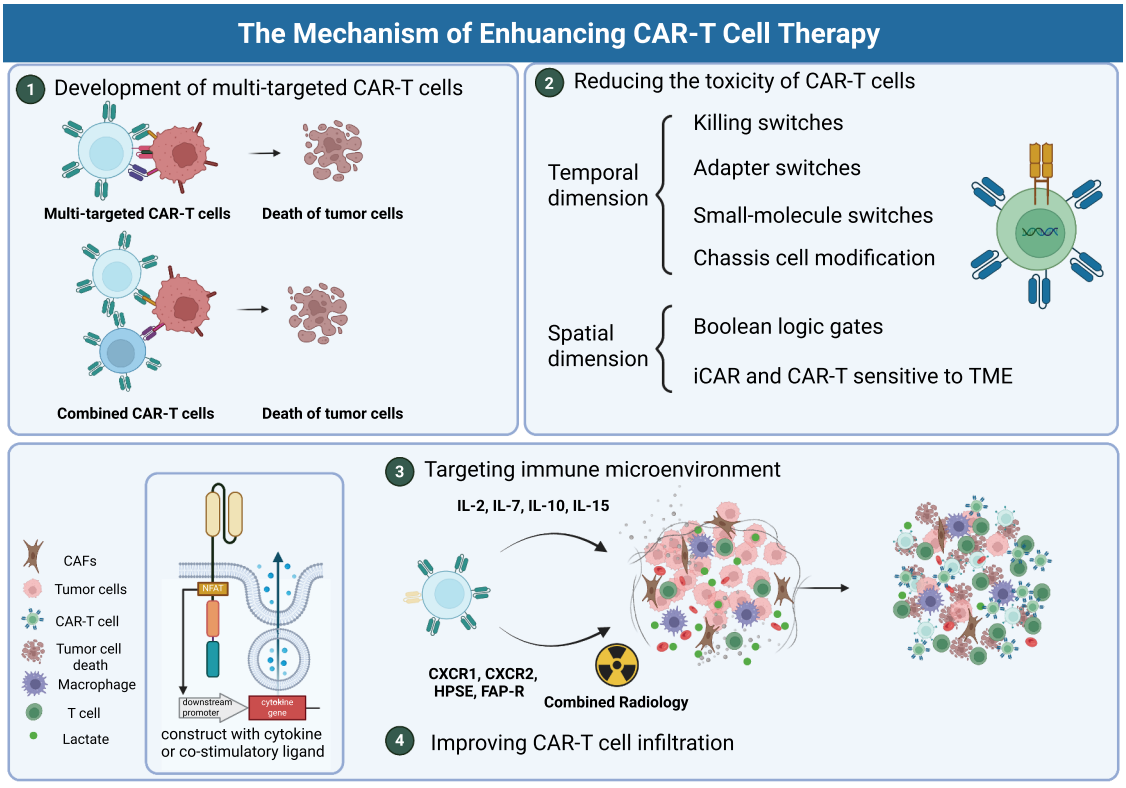

Figure 3. The mechanism of enhancing CAR-T cell therapy. Figure created with Biorender.

application of tanCAR-T cells recognizing IL13Rα2 and HER2 [57]. The combination of CAR-T cells targeting prostate stem cell antigen (PSCA) and Mucin1 (MUC1) also improved the therapeutic efficacy of non-small-cell lung cancer (NSCLC) treatment [58,59].

The inclusion of OR GATE in the design of bi-specific CAR constructs helps to prevent antigen escape: indeed, OR GATE CAR-T cells recognize two distinct TAAs and are activated when encountering either antigen A or antigen B (or both) [60]. This design prevents CAR-T cells from losing efficacy as antigens are often downregulated in the TME [61]. In vitro cytotoxicity assays indicated robust therapeutic potential, and in vivo studies in mice validated its capacity to markedly prolong overall survival [62]. For instance, the effective combinatorial use of CAR-CD19-CD28-T cells and CAR-CD19- 4-1BB-T cells illustrates how Trogocytosis in CAR-T cells can be mitigated using the CARpool method, which employs an "OR" logic strategy. On the other hand, the "AND" logic gating strategy enhances the therapeutic precision of CAR-T cells by requiring the concurrent recognition of two distinct tumor-associated antigens for full activation, thereby improving antigen specificity and reducing the risk of on-target off-tumor cytotoxicity. Dual-CAR-T cells targeting ErbB2 and MUC1 have shown safer and greater anti-tumor efficacy in vitro than the combination of two single-targeted CAR-T cells [63,64]. In addition, Wittsten et al. developed the synthetic notch (synNotch) "AND" Gate T Cells, which can induce target gene expression after detecting a ligand bound to the cell surface. SynNotch can recognize a TAA and stimulate the production of a CAR, which then activates the T cell after recognizing a second TAA [65]. CAR-T cells can be engineered to perform "AND-NOT" logic operations to reduce off-tumor toxicity. This strategy involves the co-expression of an activating CAR that target tumor-associated antigens (TAAs) and an inhibitory chimeric antigen receptor (iCAR) that recognizes antigens selectively expressed on normal tissues, thereby suppressing T cell activation in the presence of normal cells [66].

3.2. Strategies to overcome limitations of the suppressive immune microenvironment

The introduction of co-stimulatory molecules into the CAR construct is one of the strategies that has been showed to help counteract the immunosuppressive tumor microenvironment. Specifically, CD28 co-stimulation can overcome TGF-β-induced proliferation arrest in T cells, ultimately enhancing their resistance to Treg-mediated immunosuppression [67]. The integration of mechanisms targeting immunosuppressive cytokines, such as TGF-β, within CAR constructs represents a promising strategy to significantly augment CAR-T cell antitumor activity [68]. In addition, the activation of cytokines such as IL-2, IL-7, IL-10, and IL-15 can antagonize immunosuppressive factors, which can in turn improve the antitumor efficacy of CAR-T cells [69]. IL-10 has been shown to maintain mitochondrial integrity and function, leading to elevated oxidative phosphorylation and increased proliferative capacity of CAR-T cells [70]. IL-12 has been shown to promote apoptosis of antigen-negative cancer cells, thereby inhibiting immune escape, while also enhancing T cell persistence by recruiting and activating macrophages and other innate immune cells, ultimately improving the efficacy of immunotherapy [71]. Mauro et al. have demonstrated that IL-18-secreting CAR-T cells secreting IL-18 can enhance antitumor immunotherapy efficacy by activating the endogenous immune system in mice [72]. CAR-T co-expressing IL-15 and IL-21 exhibit superior proliferative capacity and persistence, leading to enhanced in vivo antitumor activity [73]. Furthermore, the tumor-fighting capacity of CAR-T cells co-expressing IL-23 and IL-36 has been validated in prostate cancer and lymphoma models, respectively [74,75]. Koneru et al. developed armored CAR-T cells, also known as T cells, redirected for universal cytokine-mediated killing (TRUCKs), which secrete pro-inflammatory cytokines to reshape the tumor microenvironment and enhance efficacy in human ovarian cancer xenografts [76]. Suarez et al. further engineered CAR-T cells to secrete anti-PD-L1 antibodies, resulting in suppressed tumor progression, enhanced NK cell recruitment and infiltration, and improved antitumor responses [77].

The combination therapy can alleviate immunosuppression within the tumor microenvironment (TME) and potentiate the anti-tumor efficacy of CAR-T cells. The combination of CAR-T therapy with immune checkpoint inhibitors including anti-PD -1 and anti-CTLA-4 antibodies, is another promising strategy to enhance therapeutic efficacy, as evidenced by several preclinical studies [78]. Furthermore, combining CAR-T cell therapy with chemotherapy can alleviate immunosuppression within the tumor microenvironment (TME) increase the infiltration of T cells by upregulating chemokines including CCL5, CXCL9, and CXCL10 [79]. Radiation modulates the tumor immune microenvironment by inducing the release of inflammatory mediators that recruit and regulate immune cell activity. NKG2D-based CAR-T cells have shown potent anti-tumor efficacy, sustained persistence, and synergistic therapeutic effects when combined with radiotherapy in murine glioma models [80].

3.3. Enhancing CAR-T cell infiltration

As noted, the immunosuppressive tumor microenvironment (TME) of solid tumors severely restricts CAR-T cell chemotaxis and infiltration. Tumor-secreted chemokines such as CXCL5 impair T cell trafficking [81]. Thus, engineering CAR-T cells to express chemokine receptors matching the tumor milieu represents a viable strategy [82]. For instance, CAR-T cells co-expressing CXCR2 show improved metastasis toward melanomas [83]. CAR-T cells expressing both CXCR1 and CXCR2 displayed enhanced infiltration and persistent tumor killing in glioblastoma models, resulting in complete tumor regression [84]. Similarly, CCR4 expression in CAR-CD30 T cells promotes trafficking to Hodgkin lymphoma by recognizing CCL17 secretion [85]. Adhesion molecules such as integrins and their ligands have been used in conjunction with chemotactic factor receptors to enhance CAR T cell infiltration [82].

In contrast to hematological tumors, targeting solid tumors for local injection of CAR T cells may result in better infiltration [86]. The major component of the stroma in solid tumors is heparan sulfate proteoglycan (HSPG), which is the

first step for CAR T cells to pass through the stroma-rich solid tumors [87]. Caruana et al. engineered CAR-T cells expressing heparanase (HPSE) to promote the degradation of HSPG, thereby enhancing their infiltration and anti-tumor ability [88]. Ruocong Zhao et al. engineered CAR-T cells secreting hyaluronic acid synthases (HAS). sPH20-IgG2 could enhance the infiltration and anti-tumor activity of CAR-T cells by promoting CAR-T cell infiltration against gastric cancer [89]. Targeting complex fibrous tissues and vascular networks, fibroblast activation protein (FAP)-targeted CAR-T cells and VEGF receptor-2-targeted CAR-T cells have also been shown to be effective in reducing tumor mesenchymal density, thereby promoting the infiltration and migration of CAR-T cells and other immune cells and enhancing the immunotherapeutic effect [35,90]. Radiotherapy can achieve microenvironmental remodeling by stromal cells and enhance the expression of TAA to increase the killing efficiency of CAR T cells [91,92]. In addition, some researchers have explored local injection or controlled release systems to deliver CAR-T cells directly to the tumor site to reduce systemic adverse effects and enhance local anti-tumor effects [93].

These synergistic strategies are designed to overcome the immunosuppressive barriers of solid tumors and enhance the infiltration efficiency of CAR-T cells [94].

3.4. Reducing the toxicity of CAR-T cells

Various strategies have been developed to improve the safety of CAR-T cell therapy. Studies have shown the strategy that targeting multiple TAAs minimizes the potential of antigen escape and effectively targeting tumor subclones [95].

Modulating the affinity of the CAR allows for the selective targeting of tumor cells while minimizing off-target toxicity to normal tissues expressing low levels of the antigen. Inhibitory CAR (iCAR) therapeutic strategies can also reduce excessive off-target toxicity. For example, iCAR cells with PD-1 and CTLA-4 inhibitory capabilities have been shown to protect normal cells inducing negative immune checkpoint signaling in a mouse model [66]. CAR-T cells can sense to hypoxia-inducible factor 1 alpha (HIF1 alpha) through the hypoxic environment and then induce CAR expression to recognize antigens. CAR-T cell stops its function rapidly as if the hypoxic environment signal is removed [96].

By incorporating killing switches into the CAR structure, activation of the killing switches is induced by specific small molecules that initiate programmed cell death pathways leading to irreversible cessation of CAR-T killing. The incorporation of killing switches is one of the most direct and efficient ways to improve the controllability of CAR-T cells. Based on their different underlying mechanisms, killing switches can be classified into three categories: inducible caspase-9 suicide gene system (iCas9) switches, ganciclovir-activated switches, and antibody-dependent cell-mediated cytotoxicity (ADCC) switches [97]. Adapter switches, small molecule switches, and chassis cell modification are other temporal regulators that would allow CAR-T cells to switch between different steady states and self-regulate [98]. Combining synthetic receptors with different functions can create Boolean logic gels. Functions can create Boolean logic gates such as the “AND” gate and “NAND” gate, thereby enabling precise tumor tissue targeting and reducing OTOT toxicity, which is a safety guarantee for CAR-T cells in the spatial dimension [99,100]

4. Implications for chordoma treatment

While the majority of solid tumors discussed in CAR-T cell therapy are carcinomas, sarcomas – malignancies arising from mesenchymal tissues – represent another important group with distinct therapeutic challenges. Among them, chordoma is a rare, locally aggressive tumor that arises from embryonic notochordal remnants within the axial skeleton, accounting for approximately 20% of primary malignant spinal tumors [101]. It is classified by the World Health Organization (WHO) under bone and soft tissue tumors.

Although chordoma progresses more indolently than many carcinomas, it shares several resistance mechanisms commonly seen in sarcomas, including poor vascularization, immune exclusion, and resistance to checkpoint inhibitors. These features compromise effective drug delivery and reduce the therapeutic efficacy of both radiotherapy and systemic treatments [102]. To date, there have been no significant breakthroughs in immunotherapy for chordoma, including immune checkpoint inhibitors (ICIs) and molecular targeted therapies (MTTs) [103,104].

Given these challenges, exploring CAR-T cell therapy in chordoma is meaningful, especially by drawing on insights from both sarcoma and broader solid tumor treatment experiences. Novel strategies targeting tumor-specific antigens and overcoming the tumor microenvironment may help advance treatment options in this rare and refractory disease.

4.1. Potential CAR targets for the treatment in chordoma

Tumor-associated antigens including B7H3, vascular endothelial growth factor receptor (VEGFR), and chondroitin sulfate proteoglycan 4 (CSPG4) are main reported targets of CAR-T therapy, which means there are more risks of OTOT. The limited blood supply and dense tumor capsule of chordoma affect the efficacy of intravenous infiltration of CAR-T cells, so postoperative local injection by ommaya reservoir system may be effective in reducing the recurrence rate of chordoma and prolonging progression-free survival (PFS). It also induces the complication rate of CRS and ICANS because the dural keeps the CAR-T cells away from the central neural system (CNS).

B7-H3 is an immune checkpoint of the B7 family of molecules, and has emerged as a promising target for CAR-T cell therapy in chordoma due to its high expression in tumors and limited expression in normal tissues [69,91,105]. Preclinical studies have demonstrated that B7-H3 CAR-T cells exhibit significant antitumor activity against a range of solid tumors, including glioblastoma, neuroblastoma, and osteosarcoma [106]. Ten Clinical trials are currently to evaluate the safety and efficacy of B7-H3-targeted CAR-T therapies for solid tumors.

CSPG4 has become another novel target for chordoma. According to Andrew, 72% of CSPG4 IHC staining was positive, correlating with a higher risk of metastasis [107]. Preclinical studies have demonstrated that CSPG4 CAR-T cells exhibit significant antitumor activity against a range of solid tumors, including melanoma, breast cancer, head, and neck squamous cell carcinoma, and glioblastoma [108,109]. However, there is only a clinical trial of CSPG4 CAR-T for head and neck squamous cell carcinoma.

VEGFR particularly is a key mediator of tumor angiogenesis and is highly expressed in various solid tumors, making it a promising target for CAR-T cell therapy. Preclinical studies have shown that VEGFR-2 CAR-T cells exert potent antitumor effects in murine models of melanoma, colon carcinoma, and renal cancer, demonstrating enhanced infiltration and tumor suppression [90]. The stronger killing effect has been verified, which is attributed to the optimized sequence of TGF-β scFv linked with VEGFR CAR in the treatment of chordoma [68]. In addition, VEGF Inhibitor can normalize vasculature inside the tumor, which improved CAR-T cell infiltration and distribution in TME. For example, anti-VEGF antibody B20 significantly enhanced the infiltration and antitumor efficacy of EGFRvIII CAR-T cell in glioblastoma models [110].

In addition, there are more potential targets for CAR-T therapy like HER, PD-L1, PDGFR-α (75%), EGFR (83%), c-MET (77%), and ALCAM which are verified high expression on the surface of the chordoma cell by IHC staining [105,111,112] (Table 2).

4.2. The main pitfall and its solution for CAR-T therapy in chordoma

The bottlenecks mentioned above were also encountered during CAR-T treatment of chordoma. The specific TME is a key factor limiting the efficacy of immunotherapy. The single cell sequencing data published by Chen et al. showed that most chordoma cells had dysfunctional MHC molecules, such ystematically summari as NK cells, macrophages, DC cells. Cytotoxic T cells were the most common effector cells, which impair antigen presentation causing tumor immune escape and greatly affects the efficacy of immunotherapy [113]. The TME of chordoma has been relatively understudied [114,115]. An analysis of T cell subsets in chordoma demonstrated that the CD8+/Foxp3+ ratio cell correlates with patient outcomes and contributed to the establishment of an immune cell-based prognostic risk prediction model [116,117]. Pathological evidence shows that TIM3+ T-cell infiltration in the immune microenvironment of chordoma has been reported, as well as high expression of CTLA4 in tumors and T cells [118]. In addition, tumors can release a variety of immunological markers. In addition, tumors can release a variety of immunosuppressive cytokines such as TGF-β, IL-10, PGE2. They can also recruit immunosuppressive cells such as Treg or activate immune-negative regulatory pathways such as PD-1/PD-L1, CTLA4/CD80 [119]. Under these multiple pathways, anti-tumor T-cells are difficult to infiltrate and exhausted, resulting in a cold tumor [120]. Given the limitations of T-cell infiltration and dysfunction, traditional T cell-based immunotherapies, including immune checkpoint blockade and CAR-T cell therapy, often fail to deliver optimal outcomes. Therefore, integrating strategies to modulate the TME with CAR-T cell approaches represents an emerging and highly promising area of research.

The challenge of local infiltration of effective T cells into chordoma, due to physical barriers like fibrous septa and mucin components, is a well-recognized issue. The idea of locally injecting B7-H3-specific CAR-T cells into tumors, as mentioned, is supported by recent research. In glioblastoma models, B7-H3 CAR-T cells have shown promise in overcoming tumor barriers and enhancing T-cell infiltration. One study demonstrated that B7-H3 CAR-T cells could successfully target tumor lesions in both immunocompromised and immunocompetent GBM models, leading to improved T-cell infiltration and prolonged survival [121,122]. Additionally, B7-H3-targeted CAR-T cells have been investigated in various solid tumors, including osteosarcoma and basal cell carcinoma, with promising results in terms of both efficacy and safety. These findings support the potential for B7-H3-specific CAR-T cells to penetrate the tumor microenvironment and overcome barriers like fibrous septa and mucin [123,124]. Thus, the approach of injecting B7-H3 CAR-T cells locally could indeed offer a promising strategy to tackle the ECM in chordoma, enhancing CAR-T cells infiltration and therapeutic efficacy.

Combination strategies are becoming an important direction to improve killing efficacy of CAR-T cell therapy. Researchers have explored a variety of combined measures to enhance the antitumor activity of CAR-T cells. Wu et al. optimized the sequence of TGF-β scFv to the CAR structure, which greatly reduces TGF-β in the TME and increases the killing effect of VEGFR CAR-T [68]. By co-expressing interleukin-7 (IL-7) in B7-H3 CAR-T cells, Wu et al. also reported that this modification enhanced the antitumor activity of B7H3 CAR-T cells. The expression of IL-7 not only improved the proliferation and survival of CAR-T cells but decreased the expression of immune checkpoint molecules, which increased the killing effect on chordoma cells [69]. Wang et al. demonstrated that radiation therapy (IR) upregulates the expression

Table 2. Exist and potential effective targets for CAR-T therapy in chordoma.

Target	Characteristics	Expression in normal tissue	Disadvantages
B7H3	A member of the B7 family of immunoregulatory proteins	Low expression in normal tissue	–
VEGFR	A receptor tyrosine kinase	Widely expressed in normal tissue	No in vivo experiments
CSPG4	A cell surface glycoprotein	Low expression in normal tissue	–
HER3	A receptor tyrosine kinase	Low expression in normal tissue	–
PDGFR-α	A receptor tyrosine kinase	Expression in normal tissue	Off-target toxicity
PD-L1	Type 1 transmembrane checkpoint protein	Widely expressed in normal tissue	Off-target toxicity
EGFR	A receptor tyrosine kinase	Widely expressed in normal tissue	Off-target toxicity
c-MET	A receptor tyrosine kinase	Low expression in normal tissue	Off-target toxicity
ALCAM	A type-I membrane protein	Widely expressed in normal tissue	Off-target toxicity

of B7-H3 on the surface of chordoma cells and enhanced the killing effect of targeting and killing efficacy of B7-H3 CAR-T cells. In vitro and vivo models, the combination of radiotherapy and CAR-T cells showed synergistic antitumor effects on radioresistant chordoma stem and tumor cells [91].

Collectively, these findings highlight that combinatorial approaches may offer a viable strategy to address the therapeutic challenges of chordoma, improve the clinical effectiveness of CAR-T cell therapy, and potentially extend patient survival.

5. Future perspective

Although CAR-T cells have demonstrated significant potential to surpass conventional therapies due to their ability to migrate to and proliferate at tumor sites. However, their application in solid tumors, particularly rare entities like chordoma, still faces substantial challenges. Chordoma is characterized by a lack of tumor-specific antigens, a highly immunosuppressive microenvironment, and significant tumor tissue heterogeneity, all of which hinder CAR-T cell infiltration, activation, and persistence. While the success of CAR-T therapy in hematological malignancies has provided valuable insights for solid tumor applications, further systematic research is required to optimize target selection, remodel the TME, enhance T-cell persistence, and refine T-cell engineering for effective clinical translation in chordoma.

Recent advances in multi-target CAR designs, localized delivery approaches, and the incorporation of immune checkpoint inhibitors have yielded encouraging preclinical outcomes in chordoma models. Meanwhile, the growing number of clinical trials investigating CAR-T therapy in solid tumors worldwide is providing crucial opportunities to better understand and address therapeutic bottlenecks. However, constructing CAR-T cells that are stable, expandable, toxicity-controlled, and precisely targeted remains a major hurdle limiting their broader clinical application in chordoma. Moving forward, the integration of multidisciplinary technologies, the establishment of sophisticated preclinical models, and the optimization of cell manufacturing processes will be pivotal in driving meaningful clinical breakthroughs for CAR-T cell therapy in solid tumors, including chordoma.

Author contributions

Review concept and design: Maoyang Qi, Joseph Schwab, Ferrone CR and Zan Chen. Data collection: Zhang L, Camillo C, Quattrocchi E, Eduardo TP. Writing the manuscript: Maoyang Qi and Cattaneo G. All authors approved the final version of the submitted manuscript.

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Human Leukocyte Antigen

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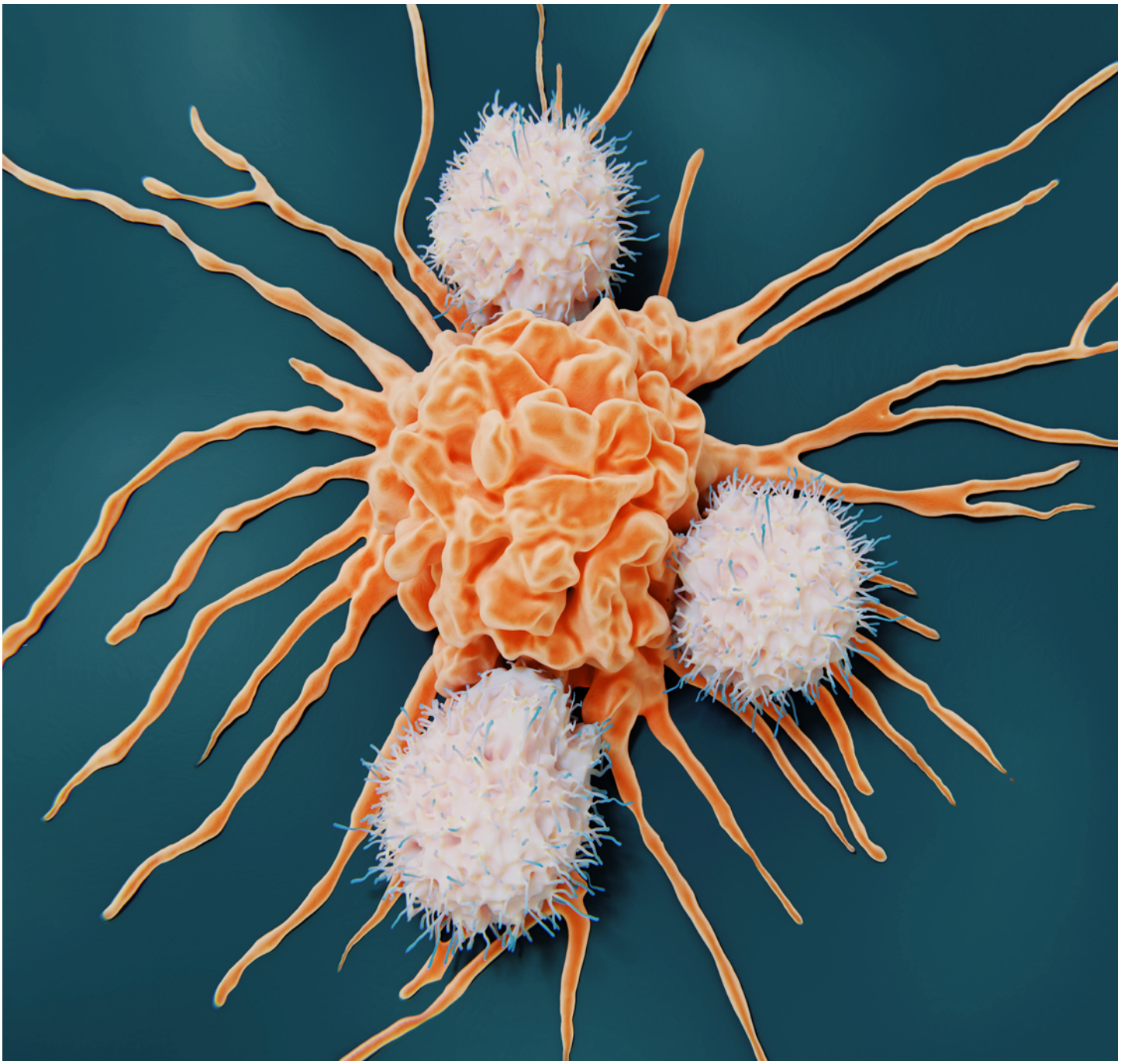
The importance of HLA for the development of cell therapies



What do puzzles and immunity have in common? The human leukocyte antigen (HLA) system of cell-surface proteins help to regulate the immune system. Yet particular antigens can make a crucial difference. Patients with certain HLA types are more likely to have autoimmune diseases, contract infectious disease or develop cancer. Now, through better understanding of HLA function, scientists are piecing together new cancer immunotherapies.

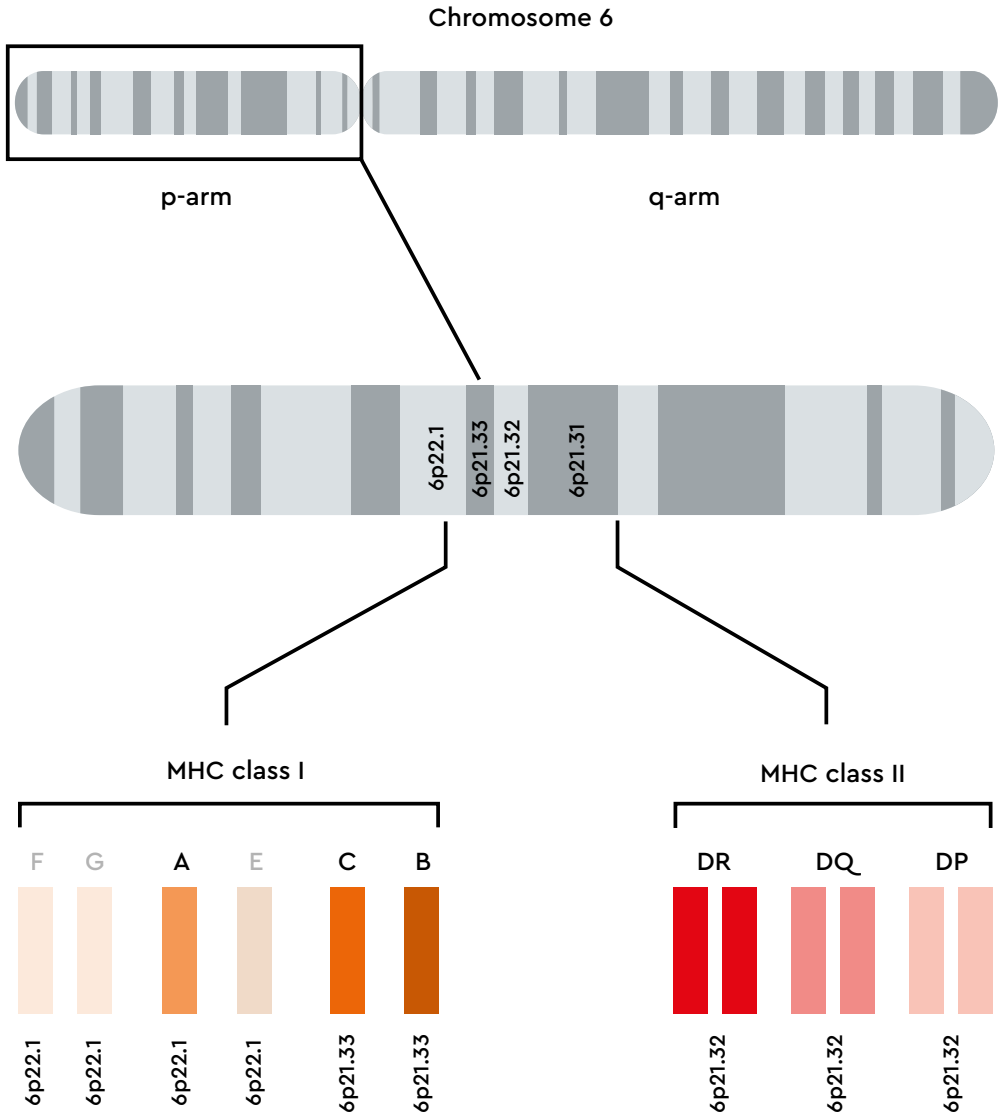


Figure 1: *Coding the HLA molecules. More than 250 genes on chromosome 6 are responsible for encoding MHC class I and II molecules.*

In a complicated puzzle, just one piece will fit at each specific place. This is similar to what happens when the cells of our immune system, the T cells, meet an antigen. If they recognize it as a "fitting", normal antigen, nothing will happen. However, if this is a foreign, or pathologic antigen, T cells will be activated. To be recognized by T cells, antigens must be loaded on human leucocyte antigen (HLA) molecules. HLA is the human version of the major histocompatibility complex (MHC) of vertebrates, a group of cell-surface proteins coded by more than 250 genes on chromosome 6. These are the essential puzzle pieces of the immune-response mechanisms.

All of us inherit many different HLA genes from our parents. HLA types aren't evenly distributed among the population, as some HLA types are more frequent than others. Our individual HLA type is for example what will reject tissue following organ or stem cell transplantation. "If the HLA type of the donor doesn't match the HLA type of the recipient, the recipient's immune system will recognize the transplanted organ, or the transplanted stem cells, as foreign and reject them," explains Lisa Hamelmann, Product Manager at PromoCell. "HLA are also pivotal in cancer immunotherapy, as cancer-specific antigens are presented on HLA molecules. When T cells recognize the antigens of cancer cells, they attack and kill them." This is why researchers who perform cell culture experiments should know the HLA type of the cells, or risk discovering that the T cells have eliminated the intruders.

HLA restriction: finding and binding

More than 60 years ago, researchers observed that blood serum from one person reacted with white blood cells of another person. This led them to identify the first HLA molecule, HLA A-2 (Dausset, 1958). By 1968, so many HLA antigens had been identified that the HLA Nomenclature Committee was founded, with the aim of giving official names to new HLA molecules (Thorsby, 2009). In the 1970s, the Nobel-prize winners Rolf Zinkernagel and Peter Doherty showed that MHC enabled T cells to recognize antigens. However, researchers needed another decade to discover the rules for the interactions between HLA proteins and T cells, and how to explain the phenomenon of HLA restriction (Bjorkman et al., 1987; Rammensee et al., 1986). In fact, T cells can recognize peptides only if they are bound to HLA molecules. These peptide-HLA complexes interact only with HLA-matched T cells that have the corresponding receptors. But there are many more pieces to this complicated puzzle. Two main classes of MHC molecules, MHC class I (HLA-A, HLA-B, and HLA-C) and MHC class II (HLA-DP, HLA-DR, and HLA-DQ) exist. Located between the genes coded for MHC class I and II proteins on chromosome 6, is a cluster of genes for MHC class III proteins. These proteins are not involved in antigen presentation, but mainly serve as signals for intercellular communication. MHC class I proteins are expressed on all cells of the body, except for red blood cells, and these proteins bind peptides from within the cells. MHC class II proteins are usually on the cell surface of antigen-presenting cells (APCs) and bind antigens that have been phagocytosed and processed from outside the body.

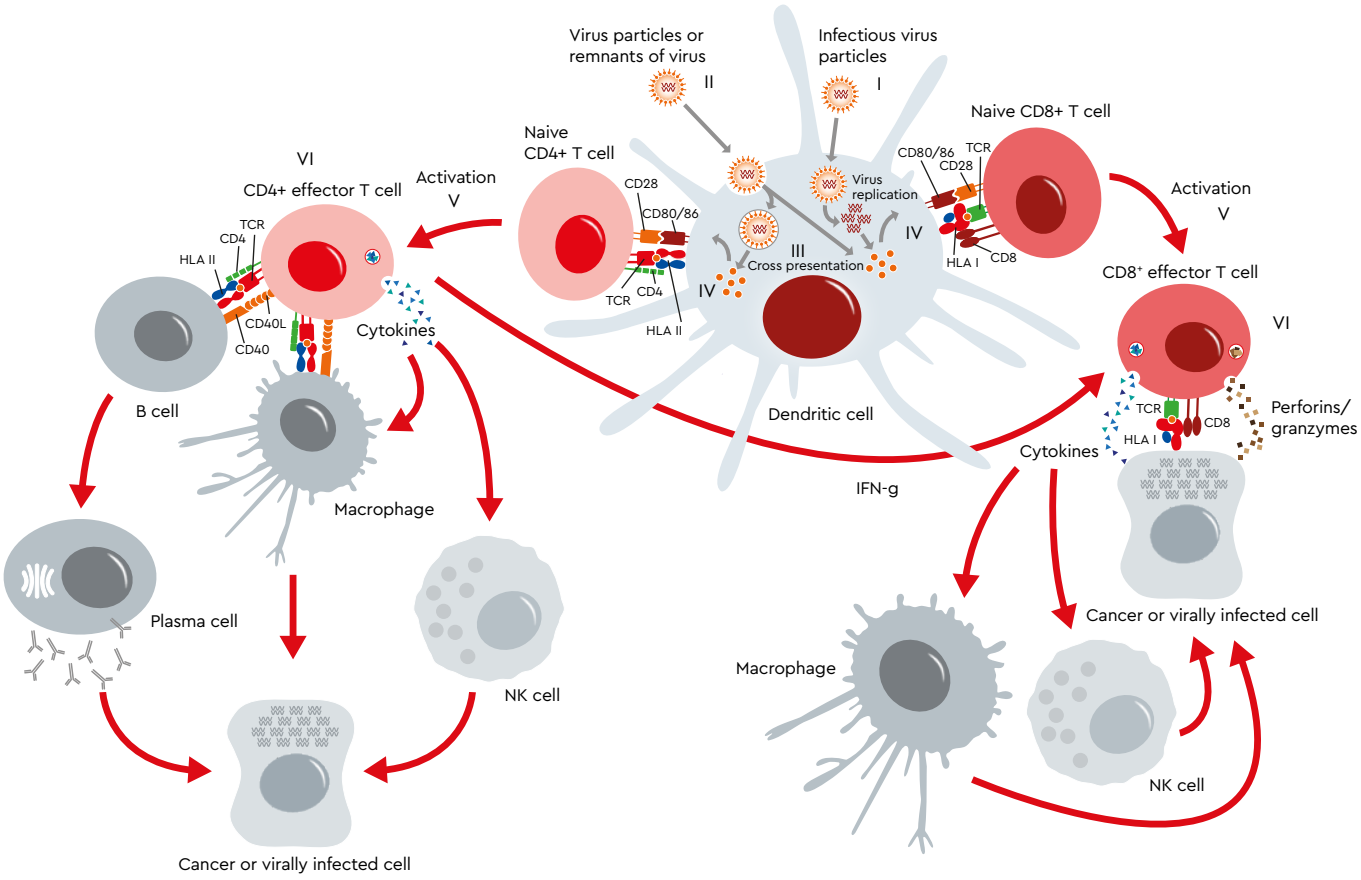


Figure 2: *A finely-tuned response system. Cytotoxic CD8⁺ T cells and helper CD4⁺ T cells work side by side to defend the body from viral infections and to eliminate cancer cells.*

The structure of both MHC classes displays a groove where antigens, that is, processed peptides, can bind. T cells recognize the MHC-antigen complex and interact with it via the T cell receptor. Then APCs such as dendritic cells load proteins from extracellular sources, for example, loading infectious virus particles onto MHC I. When this MHC I / antigen complex binds to the appropriate T cell receptor, this activity induces the differentiation of naive CD8⁺ T cells into CD8⁺ cytotoxic effector T lymphocytes. When these effector T cells are activated, they secrete lytic proteins that induce cell lysis or apoptosis of virus-infected cells. This action deprives the virus of the environment it needs to reproduce and survive. Effector T cells also produce cytokines, primarily TNF-α and IFN-γ, which activate other immune cells as macrophages or natural killer cells. And APCs can present peptides on MHC class II to CD4⁺ T helper cells, which activate other cells of the immune system such as B cells, macrophages, natural killer cells, and CD8⁺ T cells, for example by secreting cytokines.

HLA and diseases: activating autoimmune responses

Yet the immune response can backfire and having a specific HLA allele can hang like the sword of Damocles over a person. "It is true that HLA molecules are key for a functioning immune system. Yet, certain HLA alleles are associated with distinct pathological conditions," remarks Hamelmann. Autoimmune diseases including rheumatoid arthritis, ankylosing spondylitis, type 1 diabetes, and Graves' disease are related to certain HLA class I molecules. When cytotoxic T cells join in, they can spark the onset of the disease or worsen it. Variations in HLA class I genes can trigger autoimmunity, in particular, following bacterial or viral infections. Then the vicious circle becomes more vicious.

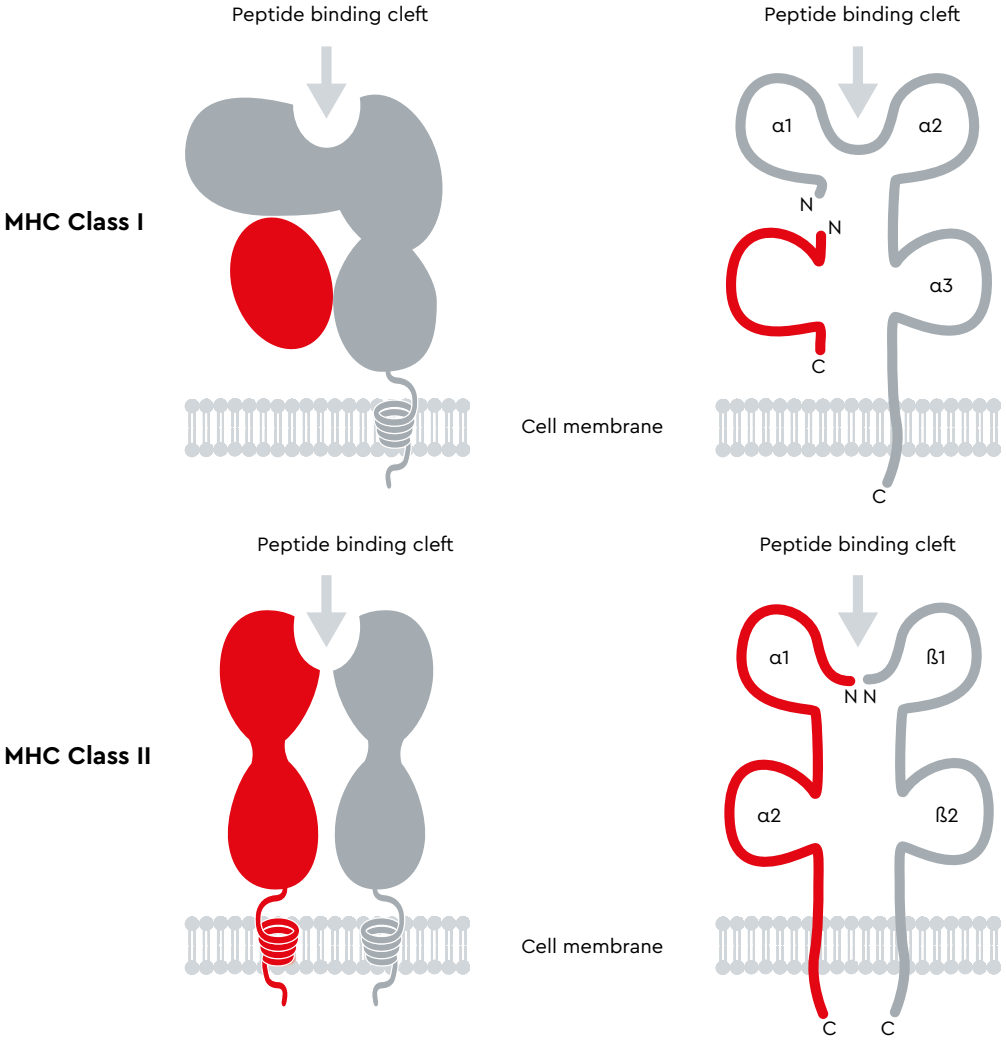


Figure 3: *Presenting antigens to T cells. The two major histocompatibility complex (MHC) or human leukocyte antigen (HLA) classes are expressed on the surface of cells and form a groove where peptides can bind.*

Microbial antigens, which are similar to self-antigens, can activate autoreactive T cells, which then cross-react with self-antigens and start attacking other organs in the body (Gough and Simmons, 2007). Many chronic and debilitating autoimmune disorders can't be treated adequately, as no specific therapeutic options exist. Therapy then relies primarily on highly toxic drugs with severe side effects. "When we understand more about the specific immune mechanisms behind the development of autoimmune diseases, we could use immunotherapies more effectively. These support the body's defense mechanisms without severe side effects," explains Hamelmann. Also, scientists suspect that specific HLA alleles make patients more susceptible to severe infections – or, conversely, protect them from such infections (Kenney et al., 2017). To elicit the desired immune response, both HLA/microbial antigens complexes and fitting T cells must exist. When either of these is missing, people tend to develop severe infections. On the other hand, patients with the right combination of HLA and T cells are often more resistant to infections. This is the case, for example, in malaria and HIV/AIDS, when specific HLA alleles protect the person from the infection (Mosaad, 2015).

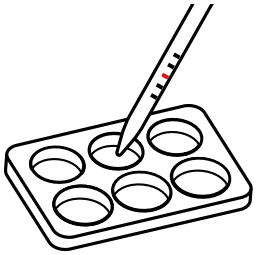
HLA and cancer: how cancer cells escape recognition

The HLA profile can initiate the onset of cancer, or influence the response to chemotherapy, especially in virally-associated cancers (Little et al., 1999). Carcinogenic viruses activate genes that allow cancer cells to escape immune surveillance and proliferate without control. Possible immune escape mechanisms include changes in the structure and function of HLA, loss of expression of tumor antigens, and production of immunosuppressive cytokines. How well cancer patients respond to immunotherapy also depends on their HLA type, as patients with certain HLA class I molecules tend to have better outcomes. In a recent study, scientists observed the response of 369 melanoma patients to immune checkpoint inhibitors. Patients with the HLA-B44 type lived longer than those with the HLA-B62 (Chowell et al., 2017).

New therapeutic options: harnessing HLA for cancer immunotherapy

The body's own peptides that are bound to HLA molecules on healthy cells usually don't generate a T cell response. However, tumor cells contain additional tumor-specific peptides that aren't present on normal cells. These tumor-associated antigens (TAA) can be encoded by mutated genes (neoantigens) or can be derived from proteins that are overexpressed in tumors. When presented by HLA molecules, TAA can activate cytotoxic T cells. These may be able to destroy the tumor cells before they proliferate or metastasize. The immune system's ability to recognize TAA is where immunotherapy begins. Stem-cell transplantation, a life-saving procedure for patients with hematological malignancies, found in bone marrow, for example, was one of the first attempts at immunotherapy. The success of a transplantation strongly depends on the HLA-compatibility between donor and recipient. If they are incompatible, this generates complex immune reactions. The body will reject the graft or cells will lose their function. To avoid rejection, as well as graft-versus-host-disease, HLA typing must be followed by matching of donor and recipient (Howard et al., 2016). In cancer immunotherapy, neoantigens are very attractive targets. They are expressed only in malignant cells, and the T cell repertoire that recognizes them is not affected by central tolerance. Neoantigen-reactive T cells have been found in many tumors, and the number of neoantigens on cancer cells directly correlates to immunotherapy success (Karpanen and Olweus, 2017). However, sometimes autologous antigen-specific T cells don't recognize neoantigens and they fail to stop cancer from spreading. This happens when T cells are inhibited or deleted by tolerance-inducing mechanisms. Alternative approaches, such as using T cells from HLA-matched donors, can cure hematologic malignancies – because these cells recognize that polymorphic peptides in the donor and patient are different (Falkenburg, 2010). Adoptive immunotherapeutic approaches, as the transfer of CAR T cells, use genetically modified T cells from healthy (HLA-matched) individuals. The CAR T cells then respond to neoantigens that were ignored by the patient's tumor-infiltrating T cells (Khalil et al., 2016). Even though neoantigens can be identified quickly, it takes time and resources to assess their immunogenicity. By finding tumor-specific biomarkers, along with greater understanding of each patient's HLA type, researchers can slowly piece together the puzzle of possible therapies for each individual.

HLA-typed cells: an important control system to test new immunotherapies



Scientists are developing a range of strategies to defeat cancer. These strategies include using patients' T cells, HLA-matched T cells, genetically modified T cells, or T cell receptors that recognize HLA-bound TAA and eliminate cancer cells.

Before using T cells in patients, researchers must be sure that these cells will not attack healthy tissues and organs nor have any cytotoxic effects. To do this, they need to test T cells on HLA-matched primary cells from vital organs. "We offer HLA-typed cells from organs such as lung, heart, kidney, or the cardiovascular system, which allow scientists to test their therapies and detect eventual cross-reactions," explains Lisa Hamelmann, Product Manager at PromoCell. "HLA-typed cells provide an optimal control system to test cancer reactive T cells in cell culture. Animal tests don't provide the same relevant evidence, as results cannot be directly transferred to the human system."

Researchers can also use HLA-typed cells to develop new immunotherapies and to look for new antigens or biomarkers. These are convenient and save time because they needn't to be screened. "HLA-typing of primary cells is a long process," says Hamelmann. "Cells have to be first thawed and cultured before extracting the DNA. The genetic material is then sent to a lab for molecular typing. After a couple of weeks, the results are ready and if the HLA type of the tested cells does not match, a new donor has to be found and the process starts again." Speeding up testing can also shorten the development time of new therapies for cancer patients.

Meet the expert

Lisa Hamelmann is our Product Manager at PromoCell. She joined PromoCell in 2018 as a Scientific Support Specialist. In 2020 she became Product Manager for cells and media at PromoCell. As a Product Manager she is responsible for the introduction of custom-tailored solutions, such as the HLA-typed cell service. She knows how important HLA types are in the development of new cell-based therapies.

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12/2022

NEWS

Unlocking the anti-tumor potential of CD4 T cells

How can CD4 T "helper" T cells be leveraged for advanced therapeutics for cancer treatment?

KADEJA JOHNSON, DIGITAL EDITOR, REGMEDNET

In a recent study, researchers from the University of Geneva (UNIGE; Switzerland) have demonstrated that CD4 T lymphocytes, a type of helper T cell, have strong cytotoxic capabilities that can be leveraged for therapeutic applications. By modifying a specific subtype of CD4 T cells, the researchers were able to target an antigen found in many cancer types, including melanoma, lung, ovarian and brain cancer. Their findings may offer an alternative immunotherapy strategy for treating a broad range of tumor types and demonstrate the strong potential for gene transfer of T-cell receptors (TCR) in CD4 T cells for cytotoxic applications.

T cells play a crucial role in the body's immune system, helping to protect the body from infection and disease. Immunotherapies have leveraged the functionality of T cells, primarily CD8 T "killer" cells – cytotoxic cells that target and eliminate diseased and cancerous cells. By modifying these naturally occurring cytotoxic T cells, researchers have harnessed patients' own immune systems to provide an alternative approach in cancer treatment for many patients.

CD8 T cell-based immunotherapy has been a promising strategy in cancer treatment, but growing interest in CD4 T "helper" cells is opening new avenues. As interest in their role grows, researchers are exploring how they could strengthen the fight against cancer.

According to the study's paper, "...emerging evidence suggests that the polyfunctional and cytotoxic subsets of CD4 T cells may be crucial in the immune response against cancer." From being considered as helper cells – supporting the function, migration and proliferation of other immune cells – the researchers, led by Camilla Jandus, have revealed that CD4 T cells can also be cytotoxic.

A key barrier to translating CD4 T cells into effective therapies has been the complexity of their antigen recognition. Unlike CD8 T cells, which recognize peptides presented by HLA class I molecules, CD4 T cells interact with peptides bound to HLA class II molecules – structures that are both polymorphic and expressed in different variations. Moreover, on-target off-tumor toxicity remains a concern, as some target antigens are also expressed in healthy tissues, risking autoimmune responses from CD4 cell-based therapies.

To mitigate these challenges, the researchers focused on NY-ESO-1, an antigen with limited expression in normal tissues (primarily testis and ovary) but abundantly expressed in multiple tumor types such as melanoma, lung and ovarian cancer.

First, they isolated CD4 T cells from the blood, tumor tissue and lymph nodes of melanoma

patients with the HLA-DRB3*02:02 allele and from healthy donors and studied their molecular characteristics. The researchers selected this HLA type due to is prevalence in 50% of the Caucasian population. This enabled them to assess the anti-tumor potential of CD4 T cells in a broader population group, avoiding the challenges associated with the polymorphic nature of CD4 T cells. From this, they identified and isolated a subset if these CD4 T cells (dominant alpha and beta chains) that possessed a TCR that was able to recognize the NY-ESO-1 antigen. Next, these specific TCRs were cloned into lentiviral vectors and transduced into human CD4 T cells and expanded in vitro. Analysis against positive and negative tumor cell-lines revealed that these modified CD4 T cells were able to eliminate NY-ESO-1–positive tumor cells and produced cytotoxic molecules like granzyme B (a protease that induces apoptosis). Additionally, the researchers assessed the modified CD4 T cells in human samples of lung, ovarian and neuroblastoma tumors, and the analysis revealed that the modified T cells could be applied to other tumor types.

Following this, the team evaluated these CD4 T cells modified with the relevant TCRs in both *in vitro* and *in vivo* systems using immunodeficient mice with NY-ESO-1 tumors. Encouragingly, analysis revealed significant tumor regression, with no off-target cytotoxicity observed.

The findings from this study suggest that modified CD4 T cells can potentially efficiently target cancer cells in an addition to their "helper" role. "This dramatically expands the pool of patients who could benefit, especially since the targeted antigen is expressed in many types of cancer," according Jandus.

Looking ahead, the team is preparing a clinical trial involving patients with confirmed HLA-DRB3*02:02 expression and NY-ESO-1–positive tumors. A personalized workflow will involve isolating CD4 T cells from patients, modifying to express the NY-ESO-1 TCR, expanding them ex vivo and reintroducing them as a therapeutic product.

Additionally, the team is exploring the development of allogeneic TCR-modified CD4 T cell banks from healthy donors, matched by HLA typing. They hope this off-the-shelf approach could accelerate treatment initiation, particularly for patients with rapidly progressing or pediatric cancers.

Sources

Source: Saillard M, Cenerenti M, Reichenbach P et al. Engineered CD4 TCR T cells with conserved high-affinity TCRs targeting NY-ESO-1 for advanced cellular therapies in cancer. Sci. Adv. 11(26), eadu5754 (2025).

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REVIEW

Advances in tumor immunotherapy targeting macrophages

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ABSTRACT

Introduction: In recent years, immunotherapy has shown significant therapeutic potential in patients with advanced tumors. However, only a small number of individuals benefit, mainly due to the tumor microenvironment (TME), which provides conditions for the development of tumors. Macrophages in TME, known as tumor-associated macrophages (TAM), are mainly divided into M1 anti-tumor and M2 pro-tumor phenotypes, which play a regulatory role in various stages of tumorigenesis, promote tumorigenesis and metastasis, and cause immunotherapy resistance.

Areas covered: This review focuses on research strategies and preclinical/clinical research progress in translating TAM into antitumor phenotype by referring to the PubMed database for five years. These include small molecule chemotherapy drug development, metabolic regulation, gene editing, physical stimulation, nanotechnology-mediated combination therapy strategies, and chimeric antigen receptor-based immunotherapy.

Expert opinion: It is necessary to explore the surface-specific receptors and cell signaling pathways of TAM further to improve the specificity and targeting of drugs and to strengthen research in the field of probes that can monitor changes in TAM in real time. In addition, the physical stimulation polarization strategy has the advantages of being noninvasive, economical, and stable and will have excellent clinical transformation value in the future.

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Immunotherapy; tumor-associated macrophage; macrophage polarization; tumor microenvironment; tumor therapy

1. Introduction

Up to now, the incidence rate of tumors is still rising, which is one of the leading causes of mortality in the world. As standard treatment methods, traditional resection, radiotherapy, and chemotherapy have limited efficacy for tumors with high recurrence or metastasis rates [1]. In recent years, immunotherapy has become an effective treatment for advanced tumors, providing patients with options [2]. As a treatment with lasting effects, immunotherapy aims to block critical pathways in the immune system and activate and promote autoimmune function to suppress tumor cells. There is an immune memory phenomenon that can effectively limit tumor recurrence and metastasis [1]. The main strategies include immune checkpoint inhibitors, CAR-T cells, and therapeutic cancer vaccines. These mainly rely on T cells that directly attack tumor cells, providing advantages over traditional radiotherapy and chemotherapy and demonstrating strong prospects for anti-cancer- treatment [3]. Despite achieving promising results in several clinical trials, immunotherapy based on immune checkpoint inhibitors (ICI) still faces some obstacles: limited response rates for monotherapy, significant differences in response among different tumors and individuals, and some patients experiencing immune-related adverse reactions [4].

In the immunoediting hypothesis, the interaction between the immune system and tumor cells is expected to undergo three phases: tumor elimination, equilibrium, and tumor escape [5]. TME provides conditions for tumor evasion, providing a 'breeding ground' for the occurrence and development of tumors [6]. In addition to cytotoxic lymphocytes (CTL) and natural killer cells (NK) with anti-tumor effects, TME also includes tumor-associated macrophages (TAMs), cancer-associated fibroblasts (CAFs), endothelial cells (EC), regulatory T cells (Tregs), and myeloid suppressor cells [7]. These cell populations affect tumor immune evasion, immune therapy response, and patient survival [8]. Especially TAMs, accounting for 30% −50% of TME cells [9,10], control multiple aspects of tumor growth, including through TGF-β Immune suppression and evasion mediated by mechanisms such as IL-10 By secreting vascular endothelial growth factor (VEGF) to promote angiogenesis and protecting tumors from oxidative stress-mediated resistance to chemotherapy; and promoting tumor growth after radiation [11]. As the relationship between tumors and TAMs becomes increasingly apparent, macrophages, as essential targets in TME, interact with various immune cells through innate and cellular immunity to regulate the immune response in tumor TME. Therefore, targeted therapy of TAMs has become a promising tumor treatment strategy [6,12]. At present, TAM targeting strategies can be roughly divided into three types: (a) eliminating macrophages that already exist in tumor tissue; (b) Inhibition of monocyte/macrophage recruitment;

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Article highlights

- The polarizing TAM from M2 to M1-like phenotype will gradually become an alternative to TAM elimination and blocking recruitment strategies.
- TAM repolarization strategies based on drug preparation and delivery, gene editing, metabolic regulation, physical stimulation, and combination therapy can significantly improve tumor treatment outcomes.
- Tumor therapies based on CAR-M regulation of macrophage M1 polarization have entered clinical evaluation and have shown potential in B-cell leukemia/lymphoma and other hematological malignancies.
- The strategy of regulating macrophage polarization and changing tumor microenvironment based on physical stimulation has the advantages of safety, economy, and convenience and has excellent clinical conversion potential.

(c) The repolarization of TAMs toward an 'immune support' phenotype characterized by the restoration of phagocytosis and antigen presentation ability [13].

This review summarized the heterogeneity and plasticity of TAMs and the effects of different phenotypes of TAMs on tumor progression. In contrast, targeted therapeutic strategies for depleting TAMs and blocking recruitment of TAMs have been introduced. Although these strategies have made outstanding achievements in inhibiting tumor progression, due to the widespread distribution of TAMs, the method of 'universal killing' will destroy the immune balance of the body and lead to serious side effects. Recently, therapies aimed at polarizing TAMs from M2 to M1-like phenotypes have emerged as a highly effective alternative. This article categorizes and focuses on these strategies, including innovative preparation of chemotherapy and small molecule therapeutic drugs, therapeutic strategies that regulate TAMS-related genes, pathways, and metabolism, and noninvasive and economical physical stimulation strategies. Other promising combination treatment options are also described. In addition, this paper briefly introduces cutting-edge clinical and clinical research in this field. Finally, we discuss the development prospects, challenges, and possible future research directions of TAM-based cancer treatment strategies. We aim to understand the role of TAMs in tumor immune regulation more fully, summarize the recent progress of tumor therapy based on TAMs, and provide some references for future research on tumor therapy.

2. Heterogeneity and plasticity of TAMs

Macrophages are widely distributed in various tissues and have functions such as immune monitoring, homeostasis maintenance, tissue repair, and regeneration [6]. Macrophages can exhibit different phenotypes and functions in response to changes in the local microenvironment or the effects of different stimuli, known as polarization. TAMs exhibit significant phenotypic plasticity in their impact on tumor progression, mainly divided into two subtypes with opposite functions: classically activated M1-like TAMs and alternatively activated M2-like TAMs [8] (Figure 1). M1-like TAMs are mainly induced by interferon-gamma (IFN-γ), lipopolysaccharide (LPS), and granulocyte-macrophage colony-stimulating factor (GM-CSF) [10]; this macrophage subpopulation expresses toll-like receptor 2 (TLR2) and TLR4, CD80, CD86, inducible nitric oxide synthase (iNOS), and major histocompatibility complex II (MHCII), and can produce a large number of cytokines, inducing further polarization of macrophages in the feedback cycle, with high antigen presentation and pro-inflammatory cytokines such as IL-12, IL-23 and TNF-α, and has anti-tumor effects [13–16]. It also exerts direct tumor cytotoxicity or induces anti-tumor immune responses by helping to present tumor-related antigens. In contrast, M2-like TAMs are stimulated by Interleukin-4 (IL-4), IL-6, IL-10, IL-13, transforming growth factor β (TGF-β), vascular endothelial growth factor (VEGF), and other M2-specific cytokines, which can provide nutrients to tumor cells, increase angiogenesis, and improve tumor aggressiveness [17,18].

M2 macrophages can be divided into four subtypes: M2a, M2b, M2c, and M2d, and these different subtypes can exert immune regulatory effects through different mechanisms of action. IL-4/IL-13 activates STAT6 by IL-4 receptor α (IL-4 Rα) to obtain M2a type polarization; immune complex induces macrophage differentiation into M2b type; IL-10/TGF-β induces the M2-C subtype by activating STAT3 through the IL-10 receptor [19]. M2d is induced by IL-6, TLR, and adenosine receptor, which promotes angiogenic tumor progression and immunosuppression and inhibition of anti-tumor T cell function, promoting tumor tolerance [20]. Although recent studies have identified the types of macrophage subtypes, the polarization process of macrophages is very complex. In general, single-cell analysis of TAMs in mouse and human tumors reveals a complex picture of macrophages that goes far beyond the simple classification of M1/M2 [21]. A recent study using single-cell transcriptomic and epigenomic analyses of bone marrow (BM) in 11 subjects found and provided a cell map of Neuroblastoma (NB) across all subgroups, defining the cell state of each NB subgroup, disentangling determinants of intra- and inter-tumoral heterogeneity. The study also found that NB cells can signal to the BM microenvironment by reconnecting monocytes specific for macrophage migration inhibitors and midkine signaling, exhibiting M1 and M2 characteristics, marked by activating pro-inflammatory and anti-inflammatory programs, and expressing tumor-promoting factors. This discovery provides a molecular target that disrupts the TME communication or monocyte polarization in the bone marrow metastasis niche for therapeutic opportunities [22]. Emerging research using single-cell RNA-sequencing (scRNA-seq) and spatial transcriptomics has revealed a wealth of new information about the origin, location, stage, and function of TAMs, as well as their unique expression markers and metabolism, which could improve our understanding of ontogenesis, phenotype, and functional plasticity of macrophages. However, the basic rules that determine the diversity of TAMs in tumors, as well as the role of ontogenesis and tumor programming in regulating the functional diversity of TAMs, are still unclear, and how functional subsets change as tumors develop and spread to distant organs is not fully understood [23].

In the acidic, hypoxic, and relatively low-nutrient tumor microenvironment, macrophages of the M2 phenotype grow and

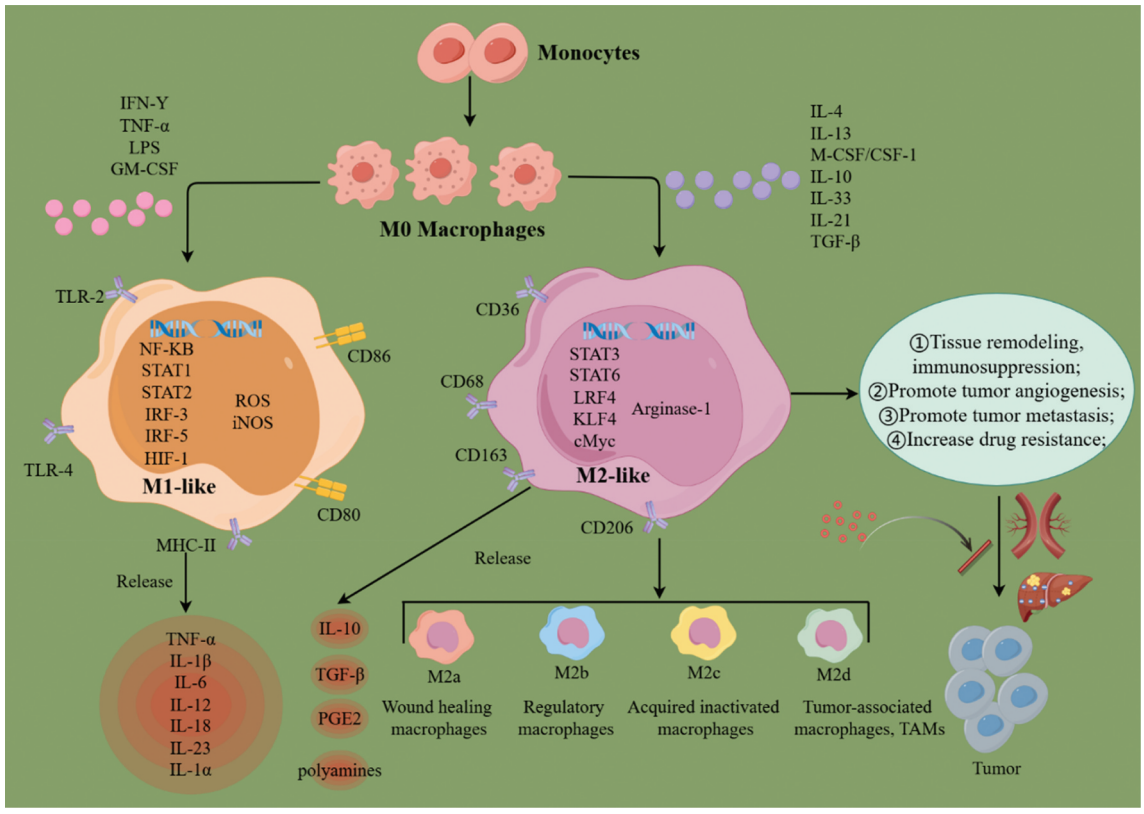

Figure 1. The polarization of macrophages to different phenotypes under the stimulation of different induction factors plays different roles in tumor progression (By Figdraw).

Macrophage polarization refers to the phenotype of macrophages that are activated to express different functions in a specific environment. Macrophages can usually be divided into M1 (pro-inflammatory, classically activated macrophages) and M2 (anti-inflammatory, alternative activated macrophages) types. As M2 macrophages further develop, they are refined into subgroups M2a, M2b, M2c, and M2d.

proliferate slowly. In contrast, macrophages of the M1 phenotype proliferated more strongly. They relied on the aerobic glycolysis pathway to obtain energy for biosynthesis and secretion, which led to the polarization of the M1-like TAMs into the M2-like TAMs [24]. Therefore, in TME, TAMs tend to exhibit M2 polarization and primarily possess M2-like functions, leading to tumor immune suppression of inflammatory responses [25], shielding tumor immune surveillance, promoting tumor occurrence and metastasis, and being closely related to poor tumor prognosis [8,17]. The immune status of TAMs can be consistent with pro-inflammatory M1 or immunosuppressive M2. Previous studies have shown that inducing polarization of M2-like TAMs to M1-like TAMs, reducing the abundance of M2-like TAMs, and increasing the proportion of M1 and M2 macrophages in TAMs can mediate the relief of the immunosuppressive tumor microenvironment (ITM), activation of the host immune system, infiltration of CD8+T cells into TME, and restoration of infiltrating T cell function can improve the therapeutic effect of tumors [26,27]. These studies indicate that reversible M2-M1 polarization has potential therapeutic value in the clinical treatment of tumors.

3. Macrophage depletion and recruitment regulation

One of the strategies of TAMs regulation is to directly eliminate TAMs and reduce the density of TAMs in tumor tissue, thereby reversing the immune-suppressed tumor microenvironment.

Colony-stimulating factor 1, CSF1, is a chemokine and the primary regulator of most macrophage populations. The CSF1 gene is located at the Ip13 breakpoint. It is produced in membrane-bound form, secreted glycoprotein, and proteoglycan, both on the cell surface and in secreted proteoglycan form. It has unique roles in homeostasis and induces proliferation during inflammation [28]. The colony-stimulating factor 1 receptor (CSF-1 R) is a type III tyrosine kinase receptor highly expressed on the surface of macrophages and is the only receptor for CSF-1 [29]. Tumor cells tend to overexpress CSF-1, a monocyte chemoattractant that recruits monocytes from the blood circulation and regulates macrophage proliferation, survival, and differentiation after binding to CSF-1 R. Thus, the CSF-1/CSF-1 R signal has become a significant target for inhibiting macrophage survival and reprogramming M2-like tam to the M1 phenotype. Removal of macrophages by inhibiting CSF1/CSF1R inhibited the differentiation, proliferation, and survival of mouse M2 macrophages. On the other hand, blocking the CSF1/CSF1R axis can repolarize macrophages to M1 macrophage function, enhance the role of macrophages in antigen presentation, and increase anti-tumor T cell response [30].

Currently, different antibodies and small molecules targeting CSF1R signaling have been extensively studied. A variety of CSF-1 R inhibitors (such as PLX3397, BLZ-945, DCC-3014, and JNJ-40346527) have entered clinical studies for the treatment of solid tumors, where they are used alone or in combination to deplete TAMs, reshape TME, and redevelop TME. And then exert an anti-

tumor effect. PLX3397 was approved by the FDA in 2019 to treat tenosynovial giant cell tumors (TCGT) [31,32]. Preclinical trials have shown that PLX3397 has anti-tumor effects in mice with lung, breast, prostate, melanoma, and gastrointestinal stromal tumors (GIST). PLX3397 is also being studied in clinical trials, both alone and in combination with other drugs, and has shown some therapeutic potential in glioblastoma, melanoma, and metastatic breast cancer. In addition, PLX3397 can improve the efficacy of chemotherapy or radiation therapy. A previous study established a preclinical mouse model of sarcoma, and systemic administration of PLX3397 significantly inhibited primary tumor growth and spontaneous lung metastasis and improved metastasis-free survival. In addition, the experimental results showed that the effect of PLX3397 was not limited to the consumption of TAMs but also reduced FOXP3+ regulatory T cells and increased the migration and invasion of CD8 T cells to the tumor [33]. Another study developed an intelligent glutathione (GSH)-responsive targeted carrier system, Co-deliver the interleukin-12 (IL-12) gene and colony-stimulating factor-1 receptor (CSF-1 R) inhibitor PLX3397 (PLX) to activate host immunity through local expression of IL-12. Meanwhile, the function of tumor-associated macrophages (TAM) is regulated by blocking CSF-1/CSF-1 R signaling to enhance anti-tumor immunity and reverse immune escape [34]. Based on current evidence, PLX3397 would provide a promising immunotherapy approach for many cancer types, including cancer, melanoma, and sarcoma. It would encourage its use in combination with immune checkpoint-blocking therapy and traditional chemotherapy and radiation [33]. A recent study found a novel and highly effective CSF-1 R inhibitor C19, which selectively promoted the secretion of chemokine CXCL9 by macrophages, induced the recruitment of CD8+ T cells to tumors, reduced the infiltration of immunosuppressive Tregs/MDSCs, and effectively reprogrammed M2-like tam to M1 phenotype. Moreover, remodeling TME significantly improves the efficiency of anti-PD-1 Colorectal cancer (CRC) [32].

Another TAMs depletion strategy is based on the application of Bisphosphonates. BP drugs (clodronate, zoledronate, etc.) can affect the proliferation of TAMs and induce their apoptosis, reduce new angiogenesis, improve the outcome of anti-tumor therapy, and high affinity and toxicity of BP to tam have been observed in preclinical study models [35,36]. However, its antitumor activity is often limited by short plasma half-life and off-target effects. Therefore, to improve the pharmacokinetics of bisphosphonates and reduce adverse reactions, researchers usually use bisphosphonate liposome preparations or nanoparticles to target the consumption of TAMs [37].

In melanoma cells, Clodronate Liposomes deplete TAMs through apoptosis [38]. The FDA has approved zoledronic acid (ZA) as the third-generation BP, which can block the mevalonate pathway (a critical metabolic pathway in eukaryotes) and cause macrophage apoptosis [36]. It has been used to reduce the abundance of TAMs and induce M1 polarization of TAMs to inhibit bone metastasis in cancer patients [39]. To improve the ability to target macrophages and eliminate TAMs in tumors, a recent study constructed M2-like TAMs targeting nanoliposomes and using M2 macrophage-binding peptide (M2pep) peptide to modify the surface, explicitly selecting and preferentially binding to M2-like TAMs. The results indicate that the liposome can reshape TME, including the normalization of tumor blood vessels, enhancement of tumor perfusion, relief of tumor hypoxia, an increase of immune promoting cytokines, and decrease of immune-suppressing cytokines, resulting in various anti-tumor immune responses [36]. In addition, chemotherapy drugs are also designed to induce TAMs and tumor cell death.

Another way to consume TAM is to reduce replenishment by blocking the recruitment of circulating inflammatory monocytes to the tumor site. The mobilization and recruitment of monocytes from the bone marrow to the tumor site depend highly on chemokine signaling. There are 28 chemokines in the CC chemokine subfamily (CCL1-CCL28), among which CCL2, CCL3, CCL5, CCL15, CCL18, and CCL26 have specific regulatory effects on pathological processes such as tam invasion and polarization in tumors [40]. On the one hand, chemokines lead to the recruitment of protumorigenic immune cells, such as MDSCs, Tams, tumor-associated neutrophils (TAN), and regulatory T cells (Tregs), thereby inducing tumor immune escape and promoting tumor development. Conversely, chemokines inhibit tumor growth by mediating anti-tumor immune responses, such as recruiting CD4+T cells, CD8+T cells, and natural killer cells (NK cells) [41].

The role of chemokines in the crosstalk between tumor and TAM has been described in detail [42].

In recent years, many chemokines and their receptors have been found to regulate TAM recruitment, infiltration, and polarization in TME. A small Phase I clinical trial has shown that a CCR5 antagonist combined with chemotherapy can prolong overall survival in patients with metastatic colorectal cancer [43]. Chordoma is a sporadic and locally aggressive interstitial malignant bone tumor with an incidence of only 0.8% and a high local recurrence rate (43%-85%). Chordomas originate from undifferentiated embryonic nodal remnants and occur in the axial bone, of which the sacrum accounts for about 50%, followed by the base of the skull and the spine at 35% and 15%, respectively. This tumor is insensitive to radiotherapy and targeted therapy and is ineffective to chemotherapy. Endoscopic transnasal and oral approach to skull base surgery is still the primary treatment method for this disease [44]. A recent study showed that chordoma regulates macrophage recruitment and M2 polarization through the action of autocrine CCL5 on tumor cells and paracrine CCL5 to promote tumor immune escape. At the same time, polarized M2 macrophages can significantly enhance the proliferation and metastasis of chordoma, forming a vicious cycle. The study further found that both CCL5 knockout and blocking of the CCL5-CCR5 axis can dramatically inhibit the malignant progression of chordoma and the polarization of M2 macrophages, providing a research basis for improving the prognosis of chordoma patients [44]. Macrophage recruitment and blocking strategies have been developed from using one chemokine antagonist to using double antagonists. In addition, some studies have used chemokine antagonists, chemotherapeutic drugs, and immunotherapy drugs to improve anti-tumor effects [42]. For example, CCR2 antagonists, CCR5 antagonists, and CXCR4 antagonists have been used to

eliminate macrophages, thereby enhancing the anti-tumor efficacy of anti-PD-1/PD-L1 [45–48]. However, chemokines also play a role in the recruitment of cytotoxic lymphocytes, so selecting chemokine receptors should be careful.

A recent study has developed a TME adaptive complex (Gel/(REG+NG/LY)) that simultaneously carries regorafenib (REG) and selective TGF-β Inhibitors (LY), increases tumor infiltration of CD8+T cells, reduces recruitment of TAMs and myeloid-derived suppressor cell (MDSCs), effectively inhibit tumor growth and liver metastasis, and have significant potential in improving the prognosis of advanced cancer patients [49] (Figure 2(a–c)). Another study demonstrated that NADPH oxidase 4(NOX4)within the tumor-induced reactive oxygen species (ROS) stimulates the production of various cytokines (including CCL7, IL8, CSF-1, and VEGF-C) through PI3K/Akt signaling dependence to recruit M2-TAMs and induce polarization. JNK/HB-EGF axis leads to activation of JNK and release of HBEGF, thereby promoting the growth of non-small cell lung cancer (NSCLC) cells. Animal experiments have shown that the use of ROS scaffold acetylcysteine to eliminate ROS or the NOX4 inhibitor to inhibit NOX4 activity can inhibit tumor growth while reducing the percentage of total (F4/80 +) and M2 (CD206+) macrophages in tumor tissue [50] (Figure 2(d,e)). In addition, the S100 protein family, as a regulatory protein, plays a vital role in the development of tumors. The overexpression of S100P is closely related to the occurrence and invasion of many tumor cells (such as pancreatic cancer and colorectal cancer). Recently, Gao et al.'s study confirmed for the first time that the expression of S100P protein is significantly upregulated in lung adenocarcinoma (LUAD) tissue, affecting the chemotaxis of TAMs by activating the PKA/c-Jun pathway to promote the release of chemokines such as CCL2 and CCL5 and promoting TAMs polarization toward an M2-like phenotype through phosphorylation of transcription factors such as STAT3 and NF-κB. This study demonstrates that inhibiting the activation of the PKA/c-Jun signaling pathway and reducing the production of CCL5 can weaken macrophage recruitment and polarization ability. This study suggests that S100P is expected to become a target for TME treatment and a diagnostic marker for LUAD, providing another strategy for treating LUAD patients. However, The expression levels of S100P protein may vary in different tumor tissues, so further exploration of the role and mechanism of S100P protein in TAMs is needed in other tumor models [51].

Tumor cells overexpress CD47, transmitting a 'do not eat me' signal that can connect with the signal regulatory protein alpha (SIRPα) on macrophages, evading attacks from phagocytic cells. RS17 peptide is an anti-tumor peptide that can specifically bind to CD47 on tumor cells and block CD47-SIRP α Signal transduction can actively target tumor cells and reshape TAMs phenotype. Tang et al. fused extracellular vesicles (M1 EVs) derived from M1 macrophages with liposomes and designed a mixed nanocarrier (hEL-RS17) using RS17 peptide for surface modification. They packaged the chemotherapeutic agent, photosensitizer IR820, and immunomodulator in hEL-RS17. After NIR laser irradiation, the combined treatment mode mediated by phototherapy, chemotherapy, and immunotherapy reshapes the immunosuppressive TME, allowing more M1-like TAMs to penetrate the tumor site and

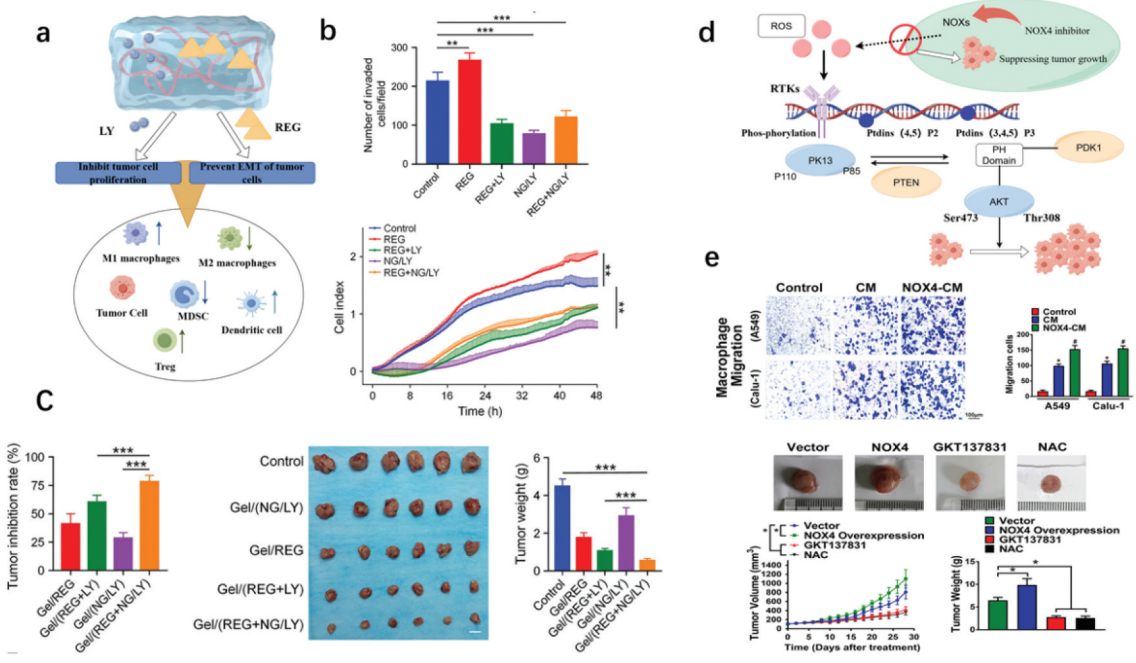

Figure 2. Blocking macrophage recruitment strategies mediates tumor therapy (By Figdraw).

(a) Gel/(REG+NG/LY) co-carrying REG and selective TGF-β inhibitors increased tumor invasion of CD8+T cells and decreased recruitment of tam and MDSCs; (b) Real-time monitoring of the effects of NG/LY and REG on the migration ability of CT26 cells, as well as the number of CT26 cells infected after 48 h incubation with different drugs, showed the inhibitory effect of REG +NG/LY on tumor cell migration; (c) Tumor inhibition rate of subcutaneous CT26 tumor mice in different groups after treatment, CT26 tumor images and tumor weight of each treatment group showed significant anti-tumor effects of Gel/(REG+NG/LY). Cited: Advanced materials. 2022;34:e2200449. doi: 10.1002/adma.202200449. (d-e) NOX4 promotes macrophage migration and M2 polarization and the therapeutic effect of NOX4 inhibitors on tumors. Cited: Redox biology. 2019;22:101116. doi: 10.1016/j.redox.2019.101116.

improve its phagocytic ability, achieving enhanced anti-tumor effects [52].

In conclusion, eliminating macrophages in TME or preventing the recruitment of macrophages can significantly inhibit tumor growth. However, in addition to being distributed in tumors, macrophages are also distributed in various organs and in maintaining tissue homeostasis, regeneration, and immune defense mechanisms against pathogens. Chemical drugs indiscriminately clearing macrophages can disrupt the immune balance, leading to serious side effects [53]. The maturity of this technology still requires much clinical exploration to accurately grasp the degree and duration of TAM consumption. Recently, strategies aimed at polarizing TAMs from M2 phenotype to M1-like phenotype have become an alternative to chemotherapy [53] (Figure 3).

4. Macrophage polarization strategy

4.1. TAM polarization strategy based on chemotherapy drugs and small molecule drug regulation

The FDA has approved some drugs to promote macrophage polarization toward the M1 phenotype and inhibit the transition to the M2 phenotype to improve the survival rate of advanced cancer patients [20]. Table 1.

Cabazitaxel enhances the anti-tumor ability of programmed cell clearance by activating and inducing macrophages to transition to an M1-like phenotype, which can effectively treat triple-negative breast cancer (TNBC) [54]. A recent study demonstrated that vitamin C can prevent bladder tumors by promoting M1-like TAM polarization through in vitro studies of MB49 cancer cells and in vivo models of subcutaneous MB49 bladder cancer in C57BL/6 mice [55]. In recent years, studies have shown that Bufalin can inhibit the overexpression of NF-κB resulting in p65-p50 heterodimer being superior to p50 homodimer in the nucleus, thereby activating the NF-κB signaling pathway, increasing the production of immune-stimulating cytokines, transforming most macrophages into the M1 phenotype. At the same time, Bufalin promotes the production of pro-inflammatory cytokines in tumors, facilitates the recruitment and activation of cytotoxic CD8+T and CD4 +Th1 cells, and these activated immune T cells increase the expression of surface PD-1 and then enhance the activity of anti-PD-1 antibody [56]. In addition, Wnt secreted by tumor cells can activate β-catenin signaling, induce M2-like TAM differentiation, and lead to HCC progression. Recent studies

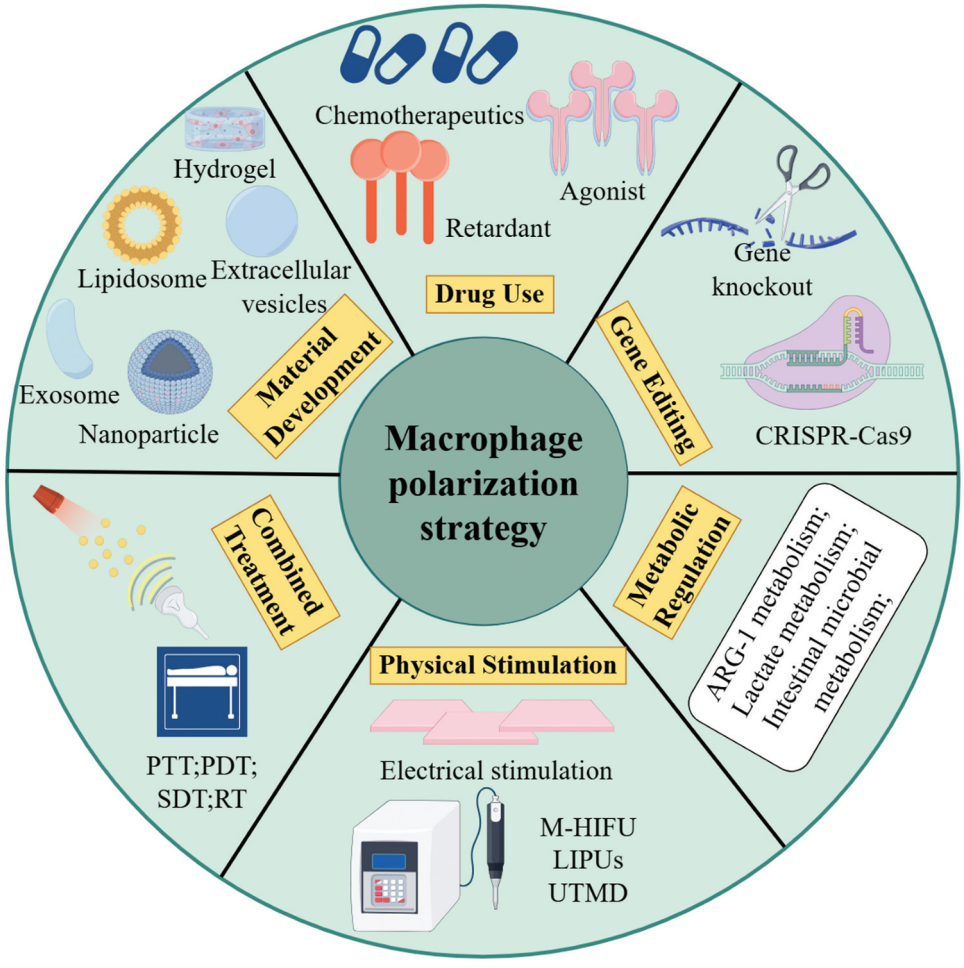

Figure 3. Regulatory strategies and nanoagents mediating macrophage repolarization (By Figdraw).

Strategies have been used to regulate the transformation of macrophages from the M2 phenotype to the M1 phenotype, reverse the tumor immunosuppressive microenvironment, and improve the body's anti-tumor immune response.

Table 1. Small molecule drugs for tumor-associated macrophage polarization.

Drug or adjuvants	Function	Tumor type	References
VBL	Activation of the NF-κB signaling pathway, Upregulating cyba which encodes p22phox protein expression	Lewis Lung Carcinoma (LLC)	[9]
Cabazitaxel	Activation of the NF-κB signaling pathway	Triple-Negative Breast Cancer (TNBC)	[54]
Vitamin C	Produces H_2O_2, activates NK cells,T cells	Bladder cancer	[55]
Bufalin	Activation of the NF-κB signaling pathway inhibited the release of Wnt1	Hepatocellular carcinoma (HCC)	[56,57]

have shown that Bufalin can block the transcription and secretion of Wnt1 in M2 macrophages, thereby blocking communication between Wnt1 and tumor cells, inactivating the β-catenin signaling cascade in HCC cells, thereby inhibiting HCC cell proliferation and malignant transformation, further expanding the application of Bufalin [57].

The different polarization states of macrophages can be identified and distinguished by their different cell shapes. Under the microscope, M1 macrophages appear large and round, while M2 macrophages are stretched and elongated cells. It has been proven that manipulating changes in cell morphology can regulate the polarization state of macrophages, thereby expressing different functions [9]. Recent studies have shown that vinblastine (VBL) targets microtubules in the cytoskeleton and induces the activation of NF-κB and the production of Cyba-dependent reactive oxygen species polarize TAMs into M1 phenotype, and through the NF-κB-Cyba-ROS axis activates CD8+T cells and promotes tumor immune response. In addition, VBL can promote the nuclear translocation of transcription factor EB, induce lysosomal biogenesis, enhance macrophage phagocytic function, and thus enhance its inhibitory effect on colorectal and pulmonary tumor [9]. There are many microtubule-targeted drugs similar to VBL used for anti-tumor therapy. Still, their mechanism of regulating tumor immune response is not yet precise, and further research is needed in this direction.

Monoamine oxidase A (MAO-A) is an outer mitochondrial membrane-bound enzyme encoded by the X-linked MAOA gene. Usually, it maintains the normal functioning of the brain. However, recent studies by Wang et al. demonstrated that in solid tumors, MAO-A can control ROS levels in TAMs via the MAO-A-ROS axis, thereby regulating the immunosuppressive polarization of TAMs through IL-4/IL-13-induced JAK-Stat6 signaling pathway sensitivity. It further demonstrated that MAOI, previously used to treat depression, can effectively induce TAM reprogramming and inhibit tumor growth in preclinical mouse and human xenograft tumor models. The study offers the possibility of future use of MAOI for combined oncology therapy [58].

4.1.1. Toll-like receptor (TLR) agonists

Regulating macrophage polarization to M1 type is essential for tumor treatment. At present, the regulation of macrophage polarization is mainly focused on CD40 agonists, Toll-like receptor (TLR) agonists, and phosphatidylinositol-3-kinase-γ (PI3Kγ) inhibitors [6]. Toll-like receptors (TLRs) are pattern recognition receptors widely expressed in immune cells, including macrophages [5], and are involved in activating immune stimulus responses. They are crucial in triggering innate and adaptive immunity [3]. Once TLR recognizes its ligand, the TLR signaling pathway will be activated, triggering the secretion of pro-inflammatory cytokines, which is beneficial for producing M1 macrophages [59]. Targeting TLR with immune regulatory molecules is a promising approach. Previous studies have shown that using Toll-like receptor (TLR) agonists to activate the NF-κB signaling pathway not only promotes the repolarization of anti-inflammatory M2 macrophages into pro-inflammatory M1 phenotypes but also enhances macrophage phagocytosis and destroys tumor cells [3] Table 2.

Several TLR agonists with immunostimulatory activity have been studied, and reprogramming of TAMs in different tumor models has shown increased cytotoxic activity against tumors and the production of immune-stimulated cytoplasmic division [21]. A recent study proposed a new anti-cancer immune regulatory molecule, Streptococcus pneumonia endopeptidase O (PepO) virus protein, as a TLR2/4 ligand agonist. By activating PI3K/AKT/mTOR and inhibiting the JAK2-STAT3 pathway, M2-like TAMs were successfully converted into M1-like TAMs in TNBC immunotherapy, enhancing the anti-tumor properties of the chemotherapy drug doxorubicin [60].

Table 2. Toll like receptor (TLR) agonists.

Material type	Drug or adjuvants	Function	Tumor type	References
Streptococcus PepO virulence protein	TLR2/4 ligand agonist	Activating PI3K/AKT/mTOR inhibiting the JAK2-STAT3 pathway	TNBC	[60]
GY101	selective TLR7 agonist	Induced the secretion of IL-6, IL-12, TNF-α and IFN-γ in mouse splenic lymphocytes	Colon cancer Melanoma 4T1	[61]
Chip-RS	Loaded Toll like receptor agonist, SHP-2 inhibitor	Downregulate SHP-2, weakening its ability to initiate 'don't eat me' signal	Breast Cancer	[62]
ChiP-CeR	matrix tumortargeting peptide (ChiP), photosensitizer, immune adjuvant	In situ killing of tumor cells by PDT effect The ICD effect activates antigen release Effectively stimulate the polarization of M1 macrophages	Breast Cancer	[63]
MOLP	Targeting the TLR4 receptor	Induces the expression of CXCL9 and CXCL10 and increases the infiltration of T cells in the tumor	LLC	[65]
LPS	TLR4 agonist	TLR4 activation by systemic administration of LPS suppressed the progression of OsA via stimulation of CD8+ T-cells	Osteosarcoma (OsA)	[66]

TLR7, as a transmembrane signal transduction receptor expressed on the surface of endosomes, has become an attractive target for tumor immunotherapy. Two of its derivatives have shown promising immune-stimulating activity in various preclinical models. Recently, Ren et al. prepared a new selective TLR7 agonist, GY101. The results showed that injection of GY101 can activate CD8+T cell infiltration and polarization of M2-like macrophages toward M1-like macrophages, improve the immune response in vivo, and significantly inhibit tumor growth in different animal models [61]. However, although Toll-like receptor agonists can repolarize TAMs to the M1 phenotype, their immunotherapeutic effects still need to be improved due to the poor phagocytic ability of macrophages [8]. Moreover, TLR agonists exhibit low targeting specificity and poor pharmacokinetic behavior, which may bring some severe side effects [62]. To address these limitations, agonists rely heavily on nanoparticle systems for delivery. Due to the increased expression of CD47 on tumor cells, it can recognize the signal regulatory protein alpha (SIRP alpha) on macrophages, leading to immune escape and phagocytic inhibition. Recently, Liu et al. constructed a chimeric peptide engineering biomarker (Chip-RS) using a macrophage-targeted chimeric peptide (ChiP) loaded Toll-like receptor agonist and a protein tyrosine phosphatase 2 (SHP-2) inhibitor containing the Src homology 2 (SH2) domain (SHP099) to repolarize M2 macrophages into M1 macrophages, reverse the immunosuppressive TME, and downregulate SHP-2, weakening its ability to initiate 'do not eat me' signal mediated evasion of macrophage phagocytosis and improving the phagocytic ability of M1 macrophages [63]. Based on the above work experience, the research team has provided a combination therapy strategy: photodynamic-triggered tumor immunotherapy. They prepared a chimeric peptide-engineered self-delivery nanomedicine (Chip-CeR) encapsulated with photosensitizers and TLR7/8 agonists. In vivo studies have shown that this PDT-initiated immunotherapy has a significant synergistic therapeutic effect in inhibiting primary and metastatic tumors [62].

During tumor progression, macrophages transition to the M2 phenotype, which promotes tumor invasion, metastasis, and angiogenesis. M2-like TAMs have also been proven to be one of the most radiation-resistant cells. Its mediated ITM significantly impairs the killing effect of radiation therapy on tumors, and the number of M2-like TAMs increases after high-dose radiation (>10 Gy), further leading to the failure of radiation therapy. Zhang et al. coupled the toll-like receptor agonist TLR7/8a with radiosensitive peptide hydrogel (Smac-TLR7/8 hydrogel). Upon γ-ray radiation, Smac-TLR7/8 hydrogel can polarize macrophages into M1 phenotype by activating the NF-κB pathways, successfully alleviate ITM, and enhance cytotoxicity, phagocytosis, and DNA damage during RT in vitro, and overcome radiation resistance [64]. TLR agonists are also used as adjuvants for anti-cancer vaccines and combined with checkpoint inhibitors or adoptive T cell therapy for 'warm' immune cold tumors [21].

In addition, studies have shown that some natural polysaccharides can also serve as biological response modulators (BRMs), producing excellent immune enhancement effects by activating the host's immune system (innate and acquired immunity), with minimal cytotoxicity to human cells and great potential for combination therapy. Dendrobium officinale polysaccharides promote the polarization of TAMs toward the M1 phenotype by targeting TLR2 receptors of TAMs; Moringa oleifera leaf polysaccharides (MOLP) promote polarization of M2-TAMs toward the M1 phenotype by targeting TLR4 receptors, induces expression of CXCL9 and CXCL10, and increases T cell infiltration in tumors [65]. Natural lipopolysaccharide (LPS), as a Toll-like receptor 4 (TLR4) agonist, is a potent innate immune stimulator that can directly stimulate and polarize immune cells such as macrophages, as well as indirectly activate, recruit, and maintain effective T cell responses. Based on this, researchers utilized MP-LPS (an innovative chemically detoxified monophosphorylated LPS) as an effective treatment for Osteosarcoma (OSA). After intravenous administration of animal tumor models, 50% of the tumors regressed, and the remaining 50% progressed. This study provides a good reference for immunomodulatory treatment strategies for highly drug-resistant tumors such as OsA [66].

4.1.2. STING agonists

In recent years, the cyclic GMP AMP synthase (cGAS) – interferon gene stimulatory factor (STING) signaling pathway has gradually become an effective target in cancer immunotherapy [67]. cGAMP binds to STING, and then activates TANK-binding kinase 1 (TBK1) and inhibitor of κB kinases (IKKs), and activates the transcription factor interferon regulatory factor 3 (IRF3) and NF-κB, respectively. In turn, it induces the production of type I interferons (IFNs) (especially IFN-β) and other immunostimulatory molecules, activates dendritic cells (DC), promotes infiltration of natural killer cells (NK), and repolarizes TAMs into M1 phenotypes, promoting anti-tumor immune effects [68]. Based on the importance of this pathway, many attempts have been made in the preparation of drugs targeting this pathway.

Wu et al. designed and synthesized a STING agonist with EGFR antibodies (tumor cells overexpressing epidermal growth factor receptors) to produce antibody-drug conjugates (ADCs). ADC can couple therapeutic compounds (payloads) with monoclonal antibodies through appropriate ligands, utilizing the antibody's ability to specifically recognize overexpressed cell surface molecules in malignant cells to deliver payloads to tumors, reducing unnecessary exposure to normal tissue [69]. Experiments have shown that this ADC can activate the STING pathway, strongly induce the expression of type-I interferences and co-stimulatory molecules (such as CD80 and CD86), and exhibit good tolerance and potent anti-tumor effects in a mouse melanoma tumor model. STING ADC and anti-PD-L1 antibodies can synergistically promote the activation of DC, T cells, and natural killer cells, induce polarization of TAMs from M2 to M1, and activate multiple aspects of the anti-tumor immune response [68]. Currently, 14 types of ADCs have been approved for clinical use, and more ADCs are under clinical development [70]. Studies use STING agonists to effectively reprogram M2-like TAMs in TME and activate other myeloid cells, overcoming TME-mediated resistance to PARP inhibitors and providing new treatment options for patients with PARP-acquired resistance [71]. Poly ADP-ribose polymerase (PARP) inhibition can trigger anti-tumor solid immune

responses and have promising therapeutic effects in clinical practice. Many PARP inhibitors have been approved by the FDA [72].

However, these STING agonists face some limitations in clinical use, which greatly limit their anti-tumor effects and clinical applications. For example, it is difficult to reach the local tumor area, Insufficient retention time within the tumor, Higher drug doses are required to achieve the desired drug concentration in the tumor area, and unavoidable systemic side effects [68]. Therefore, recent research has focused on developing effective drug delivery strategies to overcome these limitations, activate the STING pathway, and improve ITM [73].

Exosomes are extracellular vesicles secreted by cells, with a 50–200 nm diameter. Due to its ideal safety, stability, and immunogenicity, it is used as a perfect carrier for delivering chemotherapy drugs, proteins, small interfering RNA (siRNA), and microRNA (miRNA), playing an essential role in anti-tumor immune responses [74]. Cheng and his colleagues synthesized a genetically engineered hybrid exosome by fusing M1 exosomes from M1 macrophages and GT exosomes from genetically engineered CD47 overexpressing tumor cells. Further, encapsulate the DNA targeting agent (SN38) and STING agonist (MnO2). They exploited CD47's tumor-targeting ability to extend the blood circulation time of extracellular vesicles. Animal experiments have shown that mixed exosomes can combine SN38-induced DNA damage with Mn2+ mediated cGAS/STING activation in the acidic intracellular microenvironment, thereby inducing TAMs to polarize into M1 phenotype at the tumor site and inducing immunogenic cell death (ICD). The delivery system also promotes DC maturation, enhances the infiltration of CTL and NK cells in the tumor area, produces significant anti-tumor and anti-metastatic effects, and realizes a new strategy to enhance cancer immunotherapy by activating the STING pathway and improving ITM [73].

4.2. TAM polarization strategy based on gene editing

MiR-182, as one of the most upregulated microRNAs in tumor tissues, has been proven to be an essential regulatory factor in promoting metastasis in various tumors (adenocarcinoma, lung cancer, glioma, melanoma, and ovarian cancer). Previous studies by Ma and his colleagues showed that TGF-β can regulate tumor cells. The signal induces miR-182 expression in macrophages, directly inhibiting TLR4, leading to NF-κB inactivation and M2 polarization of TAMs. The TGF-β/miR-182/TLR4 axis represents this pathway. Moreover, they targeted miR-182 inhibitors to macrophages in tumors through cationized mannan-modified extracellular vesicles, effectively inhibited the expression of miR-182, inhibited M2 phenotype polarization, and finally slowed down the development and deterioration of breast cancer. This study has laid the theoretical foundation for RNA-based macrophage-targeted therapy [27].

Although some studies in recent years have explored the possibility of achieving repolarization of TAMs from M2-like to M1-like and demonstrating impressive antitumor effects in animal models, tumor cells continue to produce macrophage colony-stimulating factor (M-CSF) and bind to the colony-stimulating factor 1 receptor (CSF1-R) on macrophages, inducing activation of downstream signaling pathways, leading to the possibility of delayed return of repolarized M1 macrophages to M2 macrophages. On the other hand, the overexpressed transmembrane protein CD47 in tumor cells can bind to the signal regulatory protein alpha (SIRP alpha) on macrophages and activate the phosphatase SHP-1 and SHP-2 in macrophages, further mediating the transition from M2 to M1. These factors can lead to the loss of sustained immune response. Therefore, how to repolarize TAMs from pro-tumor M2 phenotype to antitumor M1 and maintain macrophages in the M1 state for a long time to obtain long-term antitumor immunity has become a challenge that continues to be overcome [75].

Using the sgRNA and Cas9 endonuclease guide, the CRISPR-Cas9 system can precisely manipulate critical genes involved in tumor development and immune response, with advantages such as solid specificity, high efficiency, and simplicity. Recently, it has been recognized as an ideal genome editing tool in biomedical research. CRISPR-Cas9 can knock out several related genes, permanently reshaping TAMs into antitumor M1-like phenotypes while maintaining adaptability [76]. Zhao et al. reported a CRISPR-Cas9 genome editing strategy that selectively and accurately enhances the repolarization of M2-TAMs to M1 in TME without retransformation, disruption of immune homeostasis, or systemic toxicity, significantly improving antitumor efficacy with a robust immune response in animal models of breast cancer [75]. CRISPR-Cas9 must be accurately transferred to the nucleus of target cells to avoid side effects. However, the high molecular weight and easy degradation of Cas9 sgRNA RNP hinder its in vivo application through traditional drug delivery systems. Therefore, an effective delivery carrier is crucial for successfully applying the CRISPR-Cas9 genome editing system in vivo. A recent study found that functionalized nanovesicles (NVs) from Escherichia coli protoplasts can effectively encapsulate Cas9 sgRNA RNP and modify it with galactosamine-conjugated phospholipid derivatives to enhance targeted macrophage characteristics. This method is easy to prepare, has high encapsulation efficiency, good safety, and efficient targeted delivery, and can accurately reprogram TAMs, demonstrating good efficacy in vivo tumor therapy [76].

In addition, as one of the STAT transcription factor families, STAT1 can be phosphorylated and activated by phosphokinases such as JAK1 and enter the nucleus to regulate target gene expression. STAT1 has been proven to be a central transcription factor regulating macrophage polarization, and activation of STAT1 can enhance macrophage transformation to the M1 phenotype [77]. Recently, Jiang et al.'s research has shown that knocking out the Tripartite motif-containing (TRIM) gene can promote macrophage M1 polarization by activating the JAK1/STAT1 signaling pathway, thereby inhibiting the development of HCC. In addition, it has been proven that TRIM65 is an oncogene of various tumors, upregulated in 16 tumor types represented by HCC, and related to tumor grading, staging, survival rate, and tumor cell proliferation. This study provides new therapeutic targets for various tumors [78] (Table 3).

Table 3. TAM polarization strategy based on gene editing.

Material type	Drug or adjuvants	Function	Tumor type	References
AntagomiR-182-Loaded M-EVs	AntagomiR-182	Leading to miR-182 inhibition,mediates TGF-β/miR-182/TLR4 axis blocking, macrophage reprogramming, and tumor suppression	Breast cancer	[27]
X-ray guided and triggered remote control of a CRISPR-Cas9 genome editing system (X-CC9)	CRISPR-Cas9	Blocking the expression of CSF1-R and SIRRP α	Breast Cancer	[75]
An in vivo CRISPR-Cas9 system targeting TAMs	A Cas9-sgRNA ribonucleoprotein targeting Pik3cg, A pivotal molecular switch of macrophage polarization, Bacterial CpG-rich DNA fragments, acting as potent TLR9 ligands	Opening the way for cancer immunotherapy, overcoming challenges related to cell viability and safe, precise delivery in vivo	Breast Cancer	[76]
—	TRIM gene knockout	Activating the JAK1/STAT1 signaling pathway	HCC	[78]

4.2.1. Trained immunity

Innate immune cells undergo long-term functional reprogramming after encountering primary immune stimuli, and this change increases the effectiveness of the response to subsequent stimuli. This memory-like ability of innate immune cells is called 'trained immunity.' It is driven by epigenetic changes in hematopoietic progenitor cells in the bone marrow: from bone marrow progenitor cells to peripheral monocytes (centrally trained immunity) and from peripheral monocytes to local innate immune cells, such as macrophages (peripheral trained immunity) [79]. Thus, continuously differentiating and producing peripheral innate immune cells with a memory-like phenotype elevated to a high-potency state over time, resulting in an enhanced response to subsequent immune stimulation.

A recent study constructed a nanohybrid vaccine based on Omv (Omv: Bacterial outer membranes rich in pathogen-associated molecular patterns with the potential to promote Trained immunity) as Trained immunity-related vaccines (TIrV). TIrV showed significant antitumor effects in both MC38 colorectal cancer and B16-F10 melanoma models by targeting TAMs. This study suggests that induced TAM training represents a potential link between innate and adaptive immunity and that trained immunity can fight cancer independently of adaptive immunity [79]. Another study reported that administering bacterial Omv 1 week before tumor vaccination significantly enhanced mouse antitumor immunity. Based on the mechanism, the natural nanostructure Omv is endocytosed by innate immune cells, accompanied by pathogen-associated molecular patterns (PAMPs) entering the cytoplasm, activating the inflammasome signaling pathway, and inducing IL-1β secretion. The entry of IL-1β into the bone marrow induces lineage metastasis and epigenetic remodeling of hematopoietic progenitor cells. It ultimately enhances the reactivity of differentiated peripheral blood APCs to subsequent tumor vaccination. In addition, the trained immune inducer Omv is potent and versatile and can be artificially modified by genetic engineering to enhance its biological function. Omv is now widely used in the clinic, and several Omv-based group B meningococcal vaccines are being used [80].

Trained immunity is rapidly becoming a hot topic in tumor escape and immunotherapy. Certain pro-inflammatory stimuli, such as beta-glucan, Bacillus Calmette-Guerin (BCG), oxidized low-density lipoprotein, and uric acid, have been identified as trained immune inducers. BCG is the only TIrV approved for the treatment of bladder cancer.

4.3. TAM polarization strategy based on metabolic regulation

The metabolic regulation of TME is a promising strategy for improving immunotherapy and has received widespread attention in recent years [81] Table 4. The balance of arginine metabolism plays a vital role in the polarization of TAMs. Different arginine metabolism pathways drive the transformation of TAMs into two different phenotypes, M1 and M2, resulting in distinct immune effects. Arginase-1 (Arg-1) catalyzed arginine metabolism leads to TAMs tending to polarize into M2 subtypes. The overexpression of Arg-1 in M2 can decompose L-Arg (L-arginine), limit the availability of L-Arg to T cells, create a vicious cycle, inhibit anti-tumor immune response, hinder the expansion and activation of CTL, mediate tumor evasion of T lymphocyte clearance, and ultimately promote tumor metastasis. Based on this, a recent study restored the balance of arginine metabolism in the TME through targeted delivery of L-arginine (L-Arg), combined with the PDT-induced ICD effect, significantly promoted the M1 polarization of TAMs and increased the proportion of CD8+T cells. Animal experiments have shown that reshaping ITM by regulating arginine metabolism can enhance the immune response after PDT and significantly improve the survival rate of mice [82].

Lactic acid participates in various critical metabolisms in TME, and its excessive secretion promotes the polarization of TAMs toward an immunosuppressive phenotype while hindering tumor infiltration of cytotoxic T lymphocytes and exacerbating tumor immune escape. The regulation of lactate metabolism may be a promising target in cancer immunotherapy. Most solid tumors rely on aerobic glycolysis to generate energy, known as the 'Warburg effect,' which produces large amounts of lactate in the tumor cytoplasm and TME, promoting tumor development. Tian et al.'s study synergistically regulated the pH of TME by using two drugs, lonidamine and syrosingopine. The former inhibited hexokinase in mitochondria to block glycolysis pathways and lactate production, while the latter inhibited the expression of lactate transporter MCT-4 on tumor cell membranes and reduced lactate efflux. In vivo studies have shown that this strategy-mediated reduction of extracellular lactate promotes polarization of TAMs into M1 phenotype while increasing the proportion of NK cells and reducing the number of Treg cells [81].

Table 4. TAM polarization strategy based on metabolic regulation.

Material type	Drug or adjuvants	Function	Tumor type	References
Lonidamine and syrosingopine incorporated liposomes (L@S/L)	Lonidamine, Syrosingopine	Inhibited hexokinase in mitochondria to block glycolysis pathways and lactate production, Inhibited the expression of lactate transporter MCT-4 on tumor cell membranes and reduced lactate efflux	TNBC	[81]
Multifunctional nanodrug (called HNHFPA)	Photosensitizer TCPP and Fe3+, L-Arg Hyaluronic acid -L-norvaline (L-Nor, an Arg-1 inhibitor)	Generate nitric oxide NO, Produced ROS, Induced tumor ICD, L-Nor suppressed the Arg-1 overexpressed in M2, Reversed the ITM with increased ratios of M1 and CD8+ T cells	TNBC	[82]
DL@NP-M-M2pep	DL, HCC membrane, M2pep	Inhibiting the PI3K/Akt pathway, Activating the NF- κB pathway	HCC	[84]

In addition, research has shown that microorganisms can play essential roles in human health and diseases by regulating key processes of metabolism, inflammation, and immunity [83]. Moreover, the gut microbiota and its metabolites can connect with the liver through the hepatic portal vein (known as the 'Gut-Liver Axis'), thereby regulating the development of HCC, which has good application prospects. D-lactate (DL), as an endogenous immune modulator derived from small molecules in the gut microbiome, has been shown to enhance the phagocytic function of Kupffer cells (a key feature of M1 macrophages) [84]. Han et al.'s study further suggests that DL can interact with TLR2 and TLR9 on macrophages by inhibiting the PI3K/Akt pathway and activating the NF-κB pathway-mediated response to promote macrophage polarization from M2 to M1, successfully reshaping immunosuppressive TME in allogeneic transplantation and situ HCC mouse models, and improving tumor immune efficacy [85].

4.4. TAM polarization strategy based on physical stimulation

In addition to regulating macrophage polarization through biological or chemical stimuli, physical stimuli's regulatory effects and mechanisms on macrophage polarization have also been widely studied in recent years (Figure 4(a)). Among them, electrical signals, an essential type of physical stimulation, can directly program cellular behavior through electrogenic mediation and have received significant attention in biomedical research. Kong et al., driven by ultrasonic radiation β-PVDF film (a superior piezoelectric material) induces spontaneous polarization of the crystal phase of the film, releases local charges, promotes Ca2+ to flow in through voltage-gated channels, and establishes Ca2±CAMK2A-NF-κB axis, it can encourage the release of TNF-α, IL-1β and other pro-inflammatory factors, and induce M1 macrophage polarization

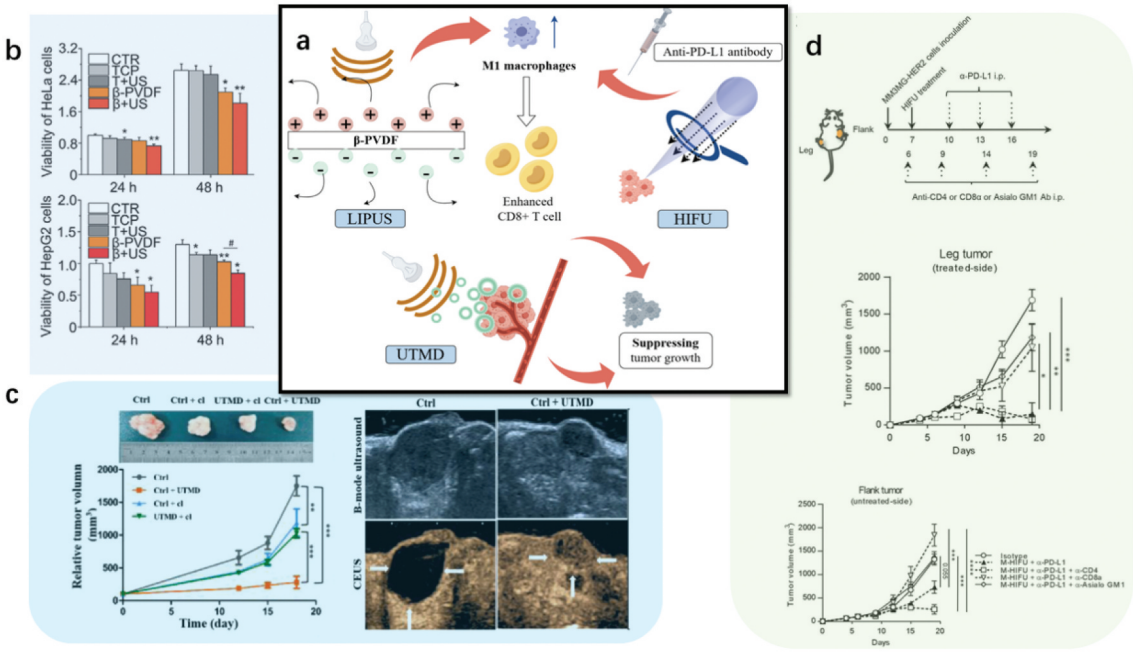

Figure 4. Ultrasound stimulation strategy induced the remission of tumor immunosuppressive microenvironment and improved the efficiency of tumor immunotherapy (By Figdraw).

(a) (Physical stimulation) Ultrasound-mediated macrophage repolarization improves the efficiency of tumor therapy; (b) In wireless mode, M1 polarization of macrophages was enhanced by ultrasonic radiation β-PVDF film. Cited: Advanced science. 2021;8:2100962. (c) After UTMD mediation, in situ, tumor vascular perfusion was enhanced, and tumor growth slowed down. Cited: Biomedicine & pharmacotherapy. 2023;160:114322. (d) Physical disruption of the TME by M-HIFU repolarizes TAM, enhances T-cell infiltration, and, when combined with anti-PD-L1 antibody, mediates superior systemic antitumor immune responses and distant tumor growth suppression. Cited: Journal for Immunotherapy of Cancer. 2022;10.

(Figure 4(b)). This treatment strategy, which enhances the pro-inflammatory response of macrophages through ultrasound-assisted piezoelectric material-mediated local electrical signals, significantly improves the immunotherapeutic effect of tumors. Moreover, radio stimulation is a noninvasive treatment method that is cheap, efficient, easy to use, and has great potential in clinical translation [86].

Ultrasound has been widely used in the biomedical field due to its adequate energy focusing, deep penetration, ease of operation, and cost-effectiveness. Ultrasound mainly exerts its therapeutic effects through cavitation effects, mechanical effects, and local thermal effects [87]. These effects can cause cell damage and recruit specific immune cells, including NK cells, macrophages, CD4+ and CD8+T cells [88]. Abe et al.'s previous reports indicated that mechanical high-intensity focused ultrasound (M-HIFU) could increase the infiltration of DC and T cells in tumors, enhance anti-tumor immunity, and reduce the risk of tumor metastasis after treatment with M-HIFU [89]. Their subsequent research further demonstrated that the physical damage of M-HIFU to TME can induce the transformation of macrophage subtypes in tumors. Moreover, the synergistic effect of M-HIFU and anti-PD-L1 antibodies in treating tumors can upregulate the gene expression of CD8+T cells, exhibiting a more significant systemic anti-tumor effect than single therapy [90]. (Figure 4(d)).

Ultrasound-targeted microbubble destruction (UTMD) has been previously used in tumor imaging to evaluate prognosis. Promoting efficient drug delivery through the interaction between microbubbles and vascular endothelium is also possible. In clinical treatment, UTMD has also been proven to enhance the chemotherapy effect of gemcitabine for advanced pancreatic cancer [91]. Research by Lin and his colleagues has shown that UTMD can induce the repolarization of TAMs from M2 type to M1 type, induce anti-tumor immunity, and achieve tumor vascular normalization, thereby inhibiting their growth and metastasis; it has shown significant therapeutic effect in the tumor model of pancreatic cancer (PaCa) (Figure 4(c)). This study provides new regulatory targets for tumor vascular normalization treatment strategies and expands the application scope of UTMD in clinical practice [26].

4.5. Other TAM polarization strategies (PTT/PDT/SDT)

With the growing understanding of tumor-related immune responses, it is believed that activating the innate immune system to attack tumor cells systematically may suppress the primary tumor and metastasis and that after ICD [51], tumor cells can release Danger-associated molecular patterns (DAMPs), which are essential for the transformation of immune 'hot' tumors [92]. Liu et al. prepared a tumor artificial vaccine, multi-responsive Advanced Nanoparticles (RMmAGL), with pH, enzyme, and near-infrared (NIR) multi-responsive properties. These nanoparticles can exert photothermal therapy (PPT) effects after near-infrared radiation to eliminate primary tumors. Then, PPT-mediated ICD induces dendritic cell (DC) maturation, activates the immune system, and releases TLR7 agonists, directly mediating TAMs M1 polarization, further enhancing immunotherapy, and inhibiting distant tumor metastasis [53]. Recently, Wang and his colleagues used an M1-type macrophage membrane camouflaged ferrous-supply-regeneration nano platform (M1mDDTF) to kill tumor cells in situ through photothermal conversion and chemotherapy drug release and synergistically enhance the ICD effect in situ, inducing TAMs to polarize toward M1 phenotype and achieve immune suppression of tumors [93]. A recent study combined negatively charged IONs with adeno-associated viral type 2 (AAV2) and expressed the phototherapeutic protein KillerRed after intravenous injection into cells. The results showed that after ION-AAV2 treatment, the levels of tumor-associated M1 macrophages increased by about 1.80 times, while the number of M2 macrophages decreased by 0.88 times, significantly stimulating CD8+T cell activation, achieving a tumor treatment strategy combining photodynamic and viral therapy [94].

Compared with PTT and PDT, sonodynamic therapy (SDT) has advantages such as noninvasive, high tissue penetration ability, and high spatiotemporal selectivity for drug release. SDT based on conventional focused ultrasound (FUS) has shown positive effects on tumor treatment. However, the focal area of FUS is small, the amount of DAMP released is small, the efficiency of immunotherapy is minimal, and the ICD induced by SDT alone cannot activate effective systemic anti-tumor immunity. A recent study used focused acoustic vortex (FAV) mediated SDT combined with Dox chemotherapy drugs (FAV-mediated ultrasound chemotherapy) to prepare nanoliposomes as ICD inducers. The lateral size of FAV action is several times that of FUS, increasing the interaction area between ultrasound and tumor and enhancing mechanical effects. Further combination with immune checkpoint blockade (ICB) can increase the infiltration of cytotoxic T lymphocytes and NK cells, polarize M2 macrophages into M1 macrophages, and realize the combined application of various therapeutic strategies mediated by ultrasound stimulation [92].

These nano platform-based therapeutic strategies can promote the release of chemotherapy drugs and the production of ROS to kill tumors directly. The resulting ICD effect induces pro-inflammatory macrophage polarization by initiating the NF-κB pathway. However, low cellular uptake efficiency and various chemical modifications may affect the therapeutic effect on tumors. In addition, the complex chemical processes involved in the preparation of nanoparticles may also cause some safety issues [5].

4.6. Clinical study of macrophage polarization regulation strategy

As end-differentiated mononuclear phagocytes, macrophages play an essential role in the adaptive and innate immune systems. They have become a potential target of tumor immunotherapy and are currently the focus of research [95]. Because TAMs contribute to chemotherapy resistance, treatments that target either monotherapy or combination chemotherapy with TAMs are being tested in preclinical and clinical trials. The tyrosine kinase inhibitor PLX3397, which targets CSF-1 R, c-Kit, and Flt3, blocks tumor progression by depolarizing the M2 phenotype of TAMs, and the drug is already in clinical trials in patients with melanoma, prostate cancer, and glioblastoma

[96]. Several inhibitors and monoclonal antibodies targeting the IL-8-CXCR1/2 pathway are in different stages of clinical trials, most of which are well tolerated and have specific anti-tumor activity [97]. In addition to monotherapy, inhibitors targeting CSF-1 or CSF-1 R are also tested with chemotherapy. For example, PLX3397 is used in combination with paclitaxel in patients with advanced solid tumors. PLX3397 combined with eribulin was used in breast cancer patients. PLX3397 and vemurafenib in patients with BRAF mutant melanoma; PLX3397 combined with sirolimus is used in patients with advanced sarcoma [21,98].

Current strategies targeting macrophages, including mononuclear phagocytes for cell therapy, such as Chimeric antigen receptor macrophages (CAR-M), have entered clinical evaluation [99]. It has also shown potential in B-cell leukemia/lymphoma and other hematological malignancies. The availability, stability, and standardization of genetically engineered macrophages make it possible to use macrophages more efficiently as adoptive transfer therapy cells, and this technology has been recognized as a valuable alternative solution, with many preclinical and clinical studies underway [87]. Previous studies by Zhang and his colleagues have developed induced pluripotent stem cells (iPSCs)-derived, CAR-expressing iPSC-derived Macrophage (CAR-iMac). Experiments have shown that CAR expression endows antigen-dependent macrophages with functions such as cytokine expression and secretion, polarization toward anti-tumor phenotype, and enhancement of anti-tumor cell activity in vivo. This study provides for the first time an unlimited source of IPS-derived engineered CAR-macrophage cells to eliminate tumor cells. However, this approach borrows CAR t cells containing the CD3ζ activation domain and cannot induce sustained polarization of macrophages toward an M1-like phenotype [87].

TLRs act as pattern recognition receptors (PRRs) and regulate immune cells by recognizing different PAMPs. TLR4, a typical TLR member widely expressed in bone marrow cells such as macrophages, can interact with adaptor molecules through the toll/IL-1 r (TIR) signaling domain to lead to nuclear translocation of nuclear factor κB (NF-κB)/p65 and promote the expression of pro-inflammatory cytokines. Based on the biological mechanism of TLR4, a recent study by the team designed second-generation M1-polarized CAR macrophages with anti-tumor efficacy: The intracellular TIR domain of TLR4 was introduced into the CAR to stimulate and maintain the M1-like phenotype after iMACs involvement in the antigen, improving the anti-tumor effect. In vivo experiments have shown that designed second-generation CAR macrophages can induce TIR to enhance cytotoxicity to tumor cells in an NF-κB pathway-dependent manner, promote antigen presentation ability, and maintain the polarization of CAR-iMac. The study provides insights into the mechanism of action of bone marrow-based immune cell therapy for solid tumors [95]. However, the process of CAR-M-based tumor treatment strategy is complicated, and the preparation cost is exceptionally high, which is difficult for most patients to afford clinically. In addition, due to the toxicity and other side effects of this treatment method, its clinical application is further limited.

5. Conclusions

As the most abundant population of immune cells in TME, targeted treatment of TAMs has become a promising tumor treatment strategy. Reprogramming TAMs or inhibiting TAMs recruitment can effectively inhibit tumor progression. Although significant progress has been made in halting tumor progression through macrophage depletion and blocking macrophage recruitment, these approaches result in the loss of the innate immune-stimulating role of macrophages as primary phagocytes and specialized antigen-presenting cells (APCs) in solid tumors. Therefore, today's cutting-edge research strategies mainly focus on polarizing TAMs from the M2 phenotype. Small-molecule regulatory drugs targeting macrophage surface receptors and internal signaling pathways remain the most direct and efficient regulatory strategies, and multi-drug combination therapy strategies have been developed. With the deepening of the understanding of macrophages, macrophage polarization strategies based on metabolic pathway regulation and gene editing have become a research hotspot. However, improving the targeted regulation of these methods still needs to be further explored. CAR-M-based tumor therapy has entered clinical evaluation and succeeded in hematological malignancies. Still, the complex preparation process and high cost have hindered the clinical development of this treatment strategy. Recently, the strategy of physical stimulation to regulate TAM polarization has the advantages of safety, simplicity, and high clinical conversion efficiency. However, only some studies are based on this strategy, and further exploration is needed for its mechanism.

6. Expert opinion

Based on receptors on the surface of macrophages and their corresponding signaling pathways within the cell, a series of small molecule regulatory drugs and biological response modulators have been developed, such as the reprogramming of TAMs via CD40 agonists, HDAC inhibitors, PI3K-γ inhibitors, and creatinine, and are currently in active clinical evaluation. However, the targeting specificity of prepared small-molecule drugs is low, and inevitable systemic side effects may also accompany the pharmacokinetic behavior during administration. In addition, a gene-editing strategy based on the CRISPR-Cas9 system can reshape macrophages into an anti-tumor M1-like phenotype [75]. However, the CRISPR-Cas9 system only works best when accurately transferred into the target cell's nucleus. Due to the limited efficacy of single therapy, combination therapy has been considered a more effective way to treat tumors in recent years. For example, immunotherapy combined with PDT, SDT, RT, and chemotherapy selectively targets a variety of signal pathway molecules related to polarization to produce more specific and compelling anti-tumor effects. This multi-pronged approach is expected to become the primary cancer treatment method in the future, but it requires a drug-loading platform as a tool for drug delivery. Fortunately, with the rapid development of nanomedicine, many nanomaterials with excellent physical and chemical properties have been developed as universal drug delivery platforms. These nanoparticles can achieve targeted drug delivery

and precise TAM reprogramming through surface modification and can also provide suitable carriers for combination therapy strategies. It positively regulates immunity and induces a robust anti-tumor immune response [62,73,76]. In addition, noninvasive labeling and observation of TAM may help to understand prognostic changes in TME. However, in clinical practice, the identification of TME can only be confirmed by immunohistochemistry, flow cytometry, or gene expression analysis, which are all performed in vitro. A recent study utilized the characteristics of macrophage mannose receptor CD206 as a marker of m2 macrophages to develop a nanoimaging probe: azido-sugar-encapsulated CD206-targeted liposomes, which then specifically targets tam overexpressing CD206, realize the targeted imaging of TAM in breast cancer, and monitor the complex TAM response [100]. However, this emerging field still faces challenges in terms of efficacy, safety, and manufacturing: (i) The design and preparation of nanoparticles with various biological and chemical modifications on the surface may lead to increased toxicity and side effects; (ii) The unique physical and chemical properties of nanoparticles and the cumbersome preparation process may lead to their aggregation, dissolution, denaturation, and deactivation during long-term storage; (iii) As a high-tech product, the complexity and high cost of nanoparticle preparation process limit the large-scale production of nanoparticles, hindering their promotion and application in clinical practice.

In addition, although current studies have identified certain specific targets, such as miR-182, that regulate macrophage polarization by targeting TLR4 and inhibiting downstream NF-κB signaling, it has been demonstrated that the use of miR-182 inhibitors can inhibit macrophage M2 phenotypic polarization. However, studies have shown that miR-182 can also induce macrophages to promote tumor directional polarization through other ways, such as regulating the accumulation of triglycerides in cancer cells and regulating the expression of HIF-1α signaling [101]. In other words, the current study is not comprehensive enough to explore the mechanism of specific targets mediating macrophage polarization, and further research is needed.

In addition to regulating macrophage polarization through biological or chemical stimuli, the regulatory effects and mechanisms of physical stimuli on macrophage polarization have also been widely studied in recent years. As an essential type of physical stimulation, electrical signals can enhance macrophages' pro-inflammatory response and enhance tumors' immunotherapeutic effect. Ultrasound performs its therapeutic effects primarily through cavitation, mechanical, and local thermal effects, causing cell damage and recruiting specific types of immune cells, including natural killer (NK) cells, macrophages, CD4+ and CD8+T cells, and mast cells [88]. Low-intensity focused ultrasound (LIUS) is a safe, noninvasive, and cost-effective mechanical stimulation that has emerged in recent years and has positively affected neuro regulation, fracture healing, inflammation improvement, and metabolic regulation [102,103]. It can enhance the anti-tumor effect of chemotherapy drugs and inhibit cell invasion and clonal formation, promoting differentiation of CSCs (cancer stem cells) and reducing their drug resistance and migration [104]. In addition, the mechanical effects of LIPUS also play a crucial role in regulating various cellular functions, such as controlling stem cell differentiation and proliferation, tumor cell apoptosis, cell migration, delivery of genes and molecules into cells for personalized immunotherapy [105], has shown great potential in cancer treatment. However, the vast majority of current studies only use physical stimulation as a means of drug delivery, and there are still few studies on the regulation of macrophage polarization-mediated disease therapy based on physical stimulation, mainly in inflammation-related lesions. Physical stimulation, as a noninvasive treatment method, also has the characteristics of simple operation, high efficiency, good stability, and high-cost performance, and it has great potential in clinical transformation. It is hoped that we can strengthen exploration in this research field in the future.

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Declaration of interest

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Isolation of Tumor Associated Macrophages (TAM) from Fresh Tumor Tissue

Application Note

Background

Tumor-associated macrophages (TAM) represent an important constituent of the tumor microenvironment (TME). After being educated by cancer cells, TAM adopt an anti-inflammatory, pro-tumor and pro-metastatic M2-like phenotype promoting progression of the disease [1]. So far, circulating precursor monocytes were thought to represent the main cellular origin of TAM, however there is increasing evidence that tissue-resident macrophages may also be recruited, especially with increasing malignancy of the tumor [2].

MMP2/9, B7-H4, STAT-3, CD163, and CD206 have been used as putative markers for classification of TAM within different macrophage subsets. Indeed, due to the high plasticity and heterogeneity of these cells, a consensus marker profile for TAM has not been established so far [3].

A high local TAM infiltration within the tumor generally represents an indicator of a poor prognosis. However, the high plasticity of the macrophage as a cell type also poses new opportunities by means of the targeted reprogramming of TAM to a pro-inflammatory / anti-tumor phenotype in the context of novel therapeutic interventions [2]. Therefore, TAM have now become another important target in anti-cancer research.

Using the PromoCell Primary Cancer Culture System (C-28081), TAM can be isolated from primary tissue samples of solid tumors as non-proliferating adherent cells and can be cultured for at least two weeks.

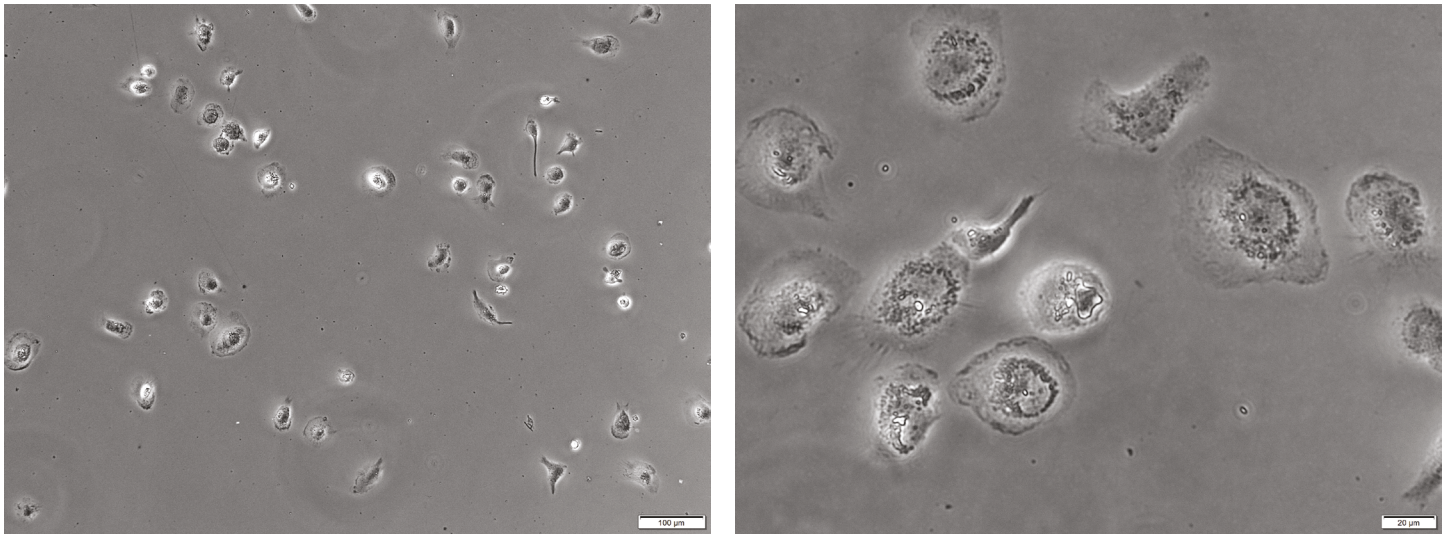

Figure 1. Phase contrast images of Tumor Associated Macrophages (TAM) isolated from primary human tumor tissue. *The TAM were isolated according to the given isolation protocol from human tumor tissue, a lung metastasis of an unknown primary tumor. The cells show the characteristic "fried-egg"-phenotype of M2-like macrophages. Images were taken 4 days after isolation (magnification left 100x, right 400x).*

TAM Isolation Protocol

I. Materials

- Fresh tumor tissue (0.2–3 grams; ≥ 1 gram is optimal)
- Hanks Balanced Salt Solution (HBSS) with Ca^{2+}/Mg^{2+} without Phenol Red (C-40370)
- Primary Cancer Culture System (C-28081)
- M-CSF from E.coli or HEK cells
- Gentamicin (50 mg/ml stock)
- Phosphate buffered saline (PBS) without Ca^{2+}/Mg^{2+} (C-40232)
- Accumax (e.g. Sigma #A7098) for tissue digestion
- Scalpel / forceps / scissors
- Cell strainers of descending size down to 70 µm (e.g. 400 / 100 / 70 µm)
- Tilt-roll-shaker, rotary mixer or comparable
- Tissue culture treated 24 well-plates (also refer to protocol step II.9)

II. TAM isolation procedure

The Primary Cancer Cell Medium D-ACF allows for the isolation and culture of tumor associated macrophages (TAM) while reliably preventing stromal overgrowth.

Keep in mind that the cells do not proliferate and that the yield of isolated TAM from primary tumor tissue may be low depending on the specific sample and type of tumor.

Note: The following isolation protocol is a basic method suitable for a wide range of solid tumors. However, alternative methods for isolation of tumor-derived single cells may be adequate and advisable for demanding tissues regarding their successful homogenization.

1

Wash and weigh the tumor tissue (day 0)

Remove visible residues of healthy tissue from the tumor. Place the tumor sample in a tube and wash twice with a generous amount of PBS and vigorous shaking. Then weigh the tumor tissue in a pre-tared sterile petri dish.

Note: The tumor tissue should be as fresh as possible and stored in HBSS at 2 to 8 °C immediately after surgical removal. Tissue up to 6 hours old is optimal for isolation purposes. However, successful isolations have been accomplished from tumor samples as old as 24 hours. Keep in mind that recently applied chemical or radiation therapy may affect the isolation results.

2

Homogenize the tumor tissue (day 0)

Place the washed tumor sample on the lid of a petri dish. Add a small volume (1–2 ml) of Primary Cancer Cell Medium D-ACF to the tumor tissue and dissect it into small pieces using a scalpel. Homogenize the tissue to a "slurry" or into small pieces of approx. 1 mm^3 by additionally mincing the tissue chunks using the scalpel. Avoid attrition of the tissue.

3

Wash the homogenized tumor tissue (day 0)

Transfer the homogenized tumor tissue to a 50 ml tube using forceps. Add 10x the volume (w/v) of PBS and vortex or mix vigorously. Let the tissue pieces settle for 2 minutes and then aspirate the supernatant. Repeat if there is still a lot of blood/debris observable. Aspirate as much as possible of the PBS without losing the tissue.

Note: If there is floating homogenized tissue, use a sieve, e.g. 400 µm, for separating the washed, homogenized tissue from the washing buffer.

4

Perform the enzymatic digest of the tumor tissue (day 0)

Resuspend the tissue pellet in Accumax solution at a concentration of 20 ml per gram of tumor tissue. Incubate at room temperature (20-25°C) with gentle but constant mixing, e. g. by a tilt-roll mixer at 50 rpm. Digest until the solution becomes distinctly turbid. Depending on the type of tissue, this is typically the case after approximately 30–60 minutes. A 45 minute incubation is a good starting point.

Note: Do not digest the tissue longer than necessary and never digest for longer than 60 min ince this may significantly compromise cell viability. Always perform the digestion reaction at room temperature and consult the Accumax manual for instructions on proper storage and handling.

5

Remove tissue residues from the sample (day 0)

Let the remaining tissue pieces settle down for 2 minutes. In order to obtain a single cell suspension, progressively filter the turbid supernatant using cell strainers of descending pore size down to 70 µm, e.g. 400 µm > 100 µm > 70 µm.

Note: Discard the remaining tissue pieces.

6

Dilute the sample with medium (day 0)

Dilute the single-cell suspension at least 1:1 with Primary Cancer Cell Medium D-ACF or a suitable buffer, e.g. PBS / Albumin / EDTA. Use a higher dilution ratio if the solution is still viscous.

7

Obtain the isolated single cells (day 0)

Pellet the cell suspension for 15 minutes at 350 x g at room temperature and carefully aspirate the supernatant without disturbing the cell pellet. Leave a small amount (20 – 50 µl) of supernatant left.

Note: Use 15 ml conical tubes and strictly adhere to the given centrifugation speed and time for best results in the centrifugation step!

8

Resuspend the obtained single cells (day 0)

First, resuspend the cell pellet by snipping with your fingers. Then resuspend the cells in Primary Cancer Cell Medium D-ACF supplemented with 250 ng/ml M-CSF per 1ml medium per gram of isolated tumor tissue. Adapt the amount of medium if necessary, e.g. use 500 µl of medium for 0.5 grams of tumor tissue.

Note: In case cell clumps are observed, which cannot be effectively resuspended, filter the cell suspension once more through a 70 µm cell strainer before proceeding to the next step.

9

Let the cells attach (day 0)

Plate the resuspended cells at approx. 500 µl cell suspension per cm^2 of culture surface and add 50 µg/ml Gentamicin. Incubate overnight at 5% CO_2 and 37°C in the incubator.

Note: Do not use the NCCD Reagent for treatment of the culture surface! Just use standard TC-treated plasticware for TAM-isolation.

Note: 24well plates are well-suited for tumor samples of at least 1 gram. For smaller tissue samples 48well or even 96well plates might be more adequate to match the recommended seeding density and number of samples aimed.

10

Wash the adherent cell fraction (day 1)

By vigorously swirling the tissue culture vessel loosen non-adherent cells and aspirate them. Wash the remaining adherent cells three times with warm PBS by swirling the vessel and aspirating the supernatant.

11

Use the isolated TAM for your experiments or continue their culture (day 1)

The isolated adherent TAM may now be used for your experiments. Alternatively, continue the culture at 37°C with 5% CO_2 using Primary Cancer Cell Medium D-ACF and 250 ng/ml M-CSF and perform regular media changes every 3–4 days.

Note: Make sure to apply some water/buffer to the surrounding wells of the sample(s) in order to limit evaporation during prolonged culture.

Note: The use of alternative culture media instead of the Primary Cancer Cell Medium D-ACF may result in rapid stromal overgrowth of the TAM culture.

Product listing

Product	Size	Catalog Number
Primary Cancer Culture System	250 ml	C-28081
Dulbecco's PBS without Ca^{2+} / Mg^{2+}	500 ml	C-40232

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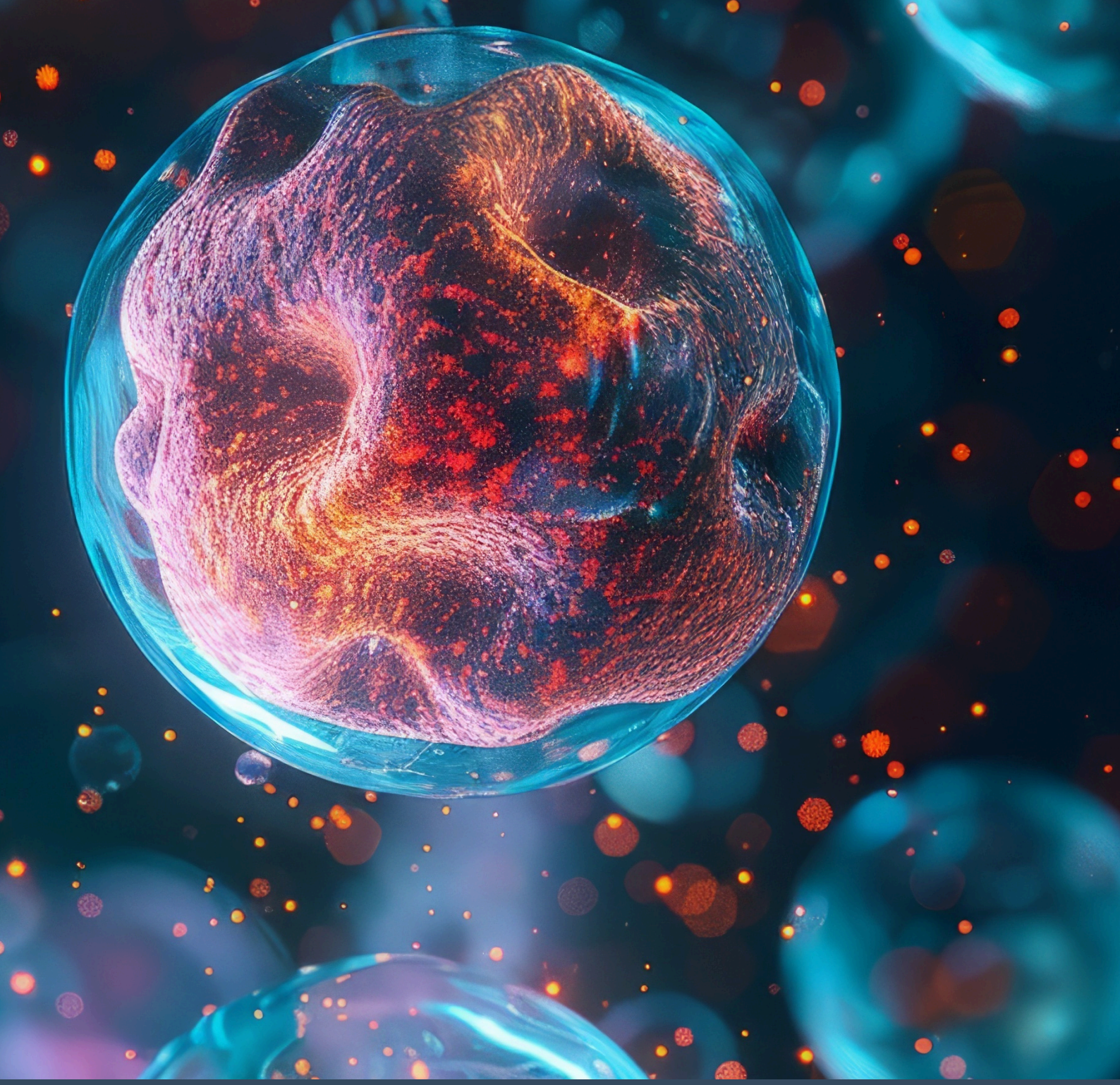

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