RSC Advances

REVIEW

View Article Online
View Journal | View Issue

Cite this: *RSC Adv.*, 2025, **15**, 29937

Phenotypic and targeted drug discovery in immune therapeutics: challenges, opportunities, and future directions

Nelson García Vázquez, Hossam Nada, Saurabh Upadhyay and Moustafa T. Gabr *

The development of immune therapeutics has revolutionized modern medicine, particularly in the treatment of cancer and autoimmune diseases. Historically, drug discovery has been guided by two main strategies: phenotypic and target-based approaches. While phenotypic screening has led to the identification of first-in-class therapies, targeted drug discovery has enabled rational drug design based on molecular mechanisms, enhancing precision and therapeutic efficacy. The integration of phenotypic and targeted approaches has been accelerated by advancements in computational modeling, artificial intelligence, and multi-omics technologies, and is reshaping drug discovery pipelines. Herein, key examples of immunomodulatory drugs, including immune checkpoint inhibitors, bispecific antibodies, and small-molecule modulators, are employed to highlight their discovery pathways and mechanisms of action. We also examine emerging hybrid approaches that connect functional and mechanistic insights to accelerate therapeutic development. Leveraging both paradigms, future immune drug discovery will depend on adaptive, integrated workflows that enhance efficacy and overcome resistance.

Received 3rd June 2025
Accepted 14th August 2025

DOI: 10.1039/d5ra03914b

rsc.li/rsc-advances

1. Introduction

Immune therapeutics have emerged as one of the most transformative innovations in contemporary medicine, particularly in the field of immuno-oncology and autoimmune disorders, by harnessing and modulating the body's intrinsic immune defenses.[1] Among the most impactful advances are immune checkpoint inhibitors targeting programmed cell death protein 1 (PD-1), its ligand PD-L1, and cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), which restore antitumor immunity by disrupting key immunosuppressive pathways exploited by cancer cells.[2] These agents have achieved unprecedented and durable clinical responses across multiple tumor types.[1,2] Despite these successes, significant challenges remain, including primary and acquired resistance, variable response rates, and immune-related toxicities.[2] Moreover, the relatively narrow spectrum of validated immune checkpoint targets continues to constrain broader therapeutic applicability, underscoring the urgent need for novel targets and more adaptable drug discovery strategies.[2] Given the clinical impact of immunotherapy across cancer and autoimmune indications, this review specifically focuses on drug discovery strategies for immune-modulating agents. While this review emphasizes clinically approved and advanced stage immunotherapeutics, it also examines emerging discovery strategies, including small molecules, peptides, and hybrid phenotypic and targeted approaches, that aim to expand and refine the current immunotherapy landscape.

The development of immune therapeutics has historically relied on two principal drug discovery strategies: phenotypic and target-based approaches. Phenotypic drug discovery entails the identification of active compounds based on measurable biological responses, often in the absence of prior knowledge regarding their molecular targets or mechanisms of action (Fig. 1).[3] This strategy has been pivotal in discovering first-in-class agents and uncovering novel therapeutic mechanisms.[3] By emphasizing functional outcomes, phenotypic screening captures the complexity of cellular systems and is particularly effective in uncovering unanticipated biological interactions. This approach has been instrumental in identifying immunomodulatory compounds that affect T cell activation, cytokine secretion, and other immune functions.[4] Despite its advantages, phenotypic screening poses challenges in downstream development, especially in target deconvolution.[3,4] These efforts often require advanced follow-up studies using biochemical, proteomic, or genomic methods, potentially prolonging discovery timelines and complicating validation.[3,4]

Target-based drug discovery begins with identifying a well-characterized molecular target, often grounded in established biological insights (Fig. 1).[5,6] This approach uses advances in structural biology, genomics, and computational modeling to guide rational therapeutic design. High-resolution methods like X-ray crystallography and cryo-EM enable detailed views of

Department of Radiology, Molecular Imaging Innovations Institute (MI3), Weill Cornell Medicine, New York, NY 10065, USA. E-mail: mog4005@med.cornell.edu

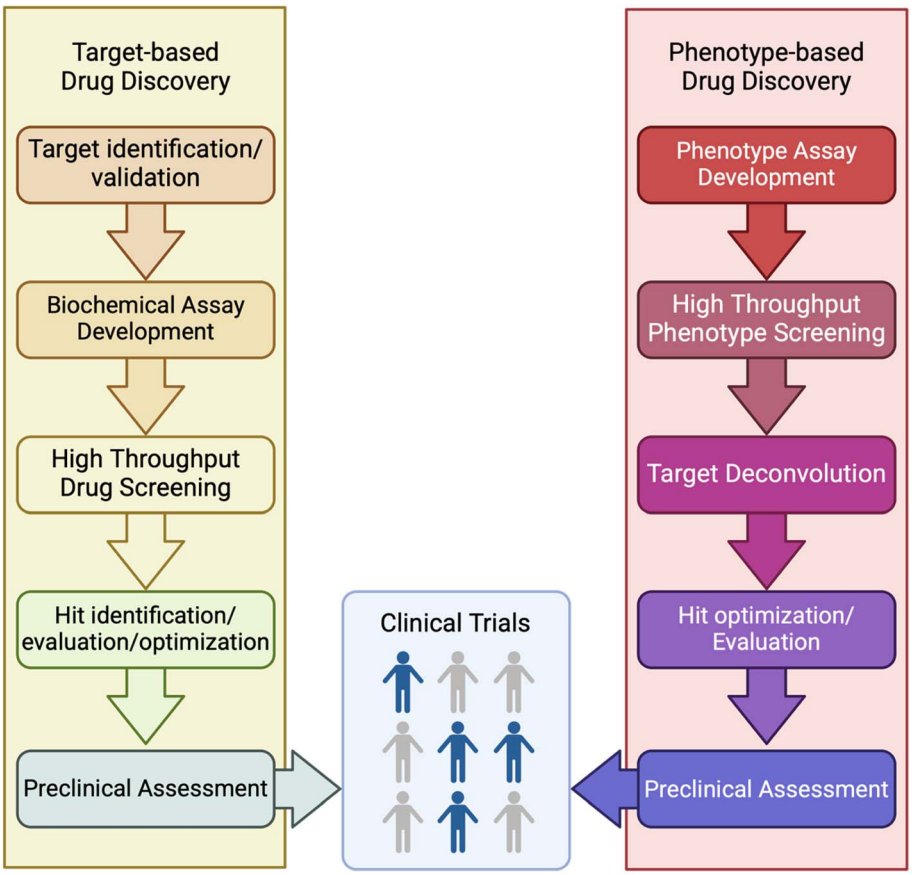

Fig. 1 Target-based and phenotypic drug discovery pipelines (created in https://BioRender.com).

target–ligand interactions, aiding the development of highly specific small molecules, antibodies, and peptide drugs.[5,6] While targeted discovery is highly effective for optimizing compounds against known pathways, it is fundamentally limited by its reliance on validated targets.[7] This dependence limits its applicability to poorly characterized or emerging disease mechanisms.[7] Nonetheless, recent progress in computational structural biology—particularly in predictive modeling and protein structure refinement—has broadened the scope of this approach, enabling the exploration of previously intractable targets and enhancing its utility across diverse therapeutic areas.[8]

Recent technological and methodological advances support the integration of phenotypic and targeted approaches as a means to overcome limitations inherent to each strategy.[9] Target-based workflows increasingly use phenotypic assays to validate candidate molecules, creating a feedback loop between mechanistic precision and biological complexity.[9] For example, a compound identified through structure-guided design can be evaluated in phenotypic systems to assess its impact on cellular behavior and pathway modulation. Conversely, phenotypic screening, when coupled with high-content imaging,[10–12] single-cell transcriptomics,[13,14] and other advanced analytical platforms, can reveal nuanced biological responses that inform target identification and hypothesis refinement. Artificial intelligence (AI)[15,16] and machine learning (ML)[17] are playing a central role in parsing these complex, high-dimensional datasets, enabling the identification of predictive patterns and emergent mechanisms. Moreover, the integration of multi-omics approaches, including genomics, transcriptomics, proteomics, and metabolomics, provides a comprehensive framework for linking observed phenotypic outcomes to discrete molecular pathways, thus facilitating more informed therapeutic discovery and development.[18]

Combining these strengths, researchers are developing hybrid discovery workflows that integrate high-throughput screening, structural biology, and computational modeling into pipelines suited for complex biological challenges. This review critically examines the evolving landscape of immune therapeutics, focusing on how integrated phenotypic and targeted drug discovery strategies are accelerating the development of checkpoint inhibitors, bispecific antibodies, and small molecule modulators to enhance antitumor immunity and address therapeutic resistance.

2. Phenotypic-screening based drugs

Though fewer drugs have been approved *via* phenotypic screening, this approach can overcome limitations inherent to target-based discovery.[19] For example, targeted approaches often experience remarkable attrition due to lack of efficacy

which may stem from flawed target hypotheses or incomplete understanding of compensatory mechanisms. These factors increase false positives and reduce drug approval rates.[19]

Despite the rational design of drugs inhibiting "well-validated" targets, many candidates ultimately fail in clinical trials due to the limitations of single-target approaches.[19] Such strategies often fail to address the complexity of cellular signaling networks and adaptive resistance mechanisms seen in clinical settings.[5,6] In contrast, phenotypic screening provides a powerful and unbiased alternative that circumvents the need for prior knowledge of the molecular target.[3] This approach has been pivotal in discovering successful therapeutics such as thalidomide, underscoring its value in drug discovery.[4] Phenotypic screening is especially beneficial when the underlying biological pathways are poorly characterized or when the therapeutic objective involves modulating multifaceted, system-level immune responses.[3,4] This section presents key therapeutics discovered and developed exclusively through phenotypic screening.

2.1. Immunomodulatory drugs

Among the immunotherapeutics discovered through phenotypic screening, thalidomide and its subsequent 2nd generation analogs stand out as rare examples where both the identification of the parent compound and the subsequent optimization of second-generation analogs were exclusively guided by phenotypic assays.[19] Thalidomide was originally marketed as an anti-emetic for morning sickness in pregnant women.[20] However, the increasing evidence of its association with neurologic toxicities and teratogenicity led to its discontinuation.[21] It was later reintroduced and approved for multiple myeloma, marking its therapeutic resurgence. Moreover, the initial catastrophic introduction of thalidomide has led to stricter regulatory oversight of drug manufacturing and approvals, as well as the establishment of key ethical principles, including informed consent for patients.[22]

Phenotypic screening of thalidomide analogs led to the discovery of two approved derivatives: lenalidomide and pomalidomide.[23] Both lenalidomide and pomalidomide exhibited a significant increase in the potency for down-regulating tumor necrosis factor (TNF) production and reduction in the sedative and neuropathic side effects with few changes in the scaffold of thalidomide.[24] Two key conclusions can be drawn from these findings. First, the shared pharmacological effects suggest a common mechanism of action and target profile among these compounds. Second, the thalidomide scaffold demonstrates strong potential for structural optimization. This is exemplified by the development of lenalidomide and pomalidomide, where minimal chemical modifications to the parent compound resulted in substantial enhancements in therapeutic activity and reductions in toxicity.

Subsequent studies identified cereblon, a substrate receptor of the CRL4 E3 ubiquitin ligase complex, as the primary binding target of these compounds. Thalidomide and its analogs bind to cereblon, altering the substrate specificity of the E3 ligase and leading to the ubiquitination and proteasomal degradation of specific neosubstrates, most notably the lymphoid transcription factors IKZF1 (Ikaros) and IKZF3 (Aiolos).[25,26] The degradation of IKZF1/3 is now recognized as the key mechanism underlying the anti-myeloma activity of lenalidomide and pomalidomide. Clinically, patients who respond to these agents exhibit significantly higher cereblon expression levels—approximately threefold higher—compared to non-responders.[27] Moreover, a strong correlation has been observed between elevated cereblon expression and improved treatment outcomes, including partial or complete responses, whereas lower expression levels are associated with stable or progressive disease.[28] Beyond their clinical use in multiple myeloma, thalidomide and its analogs have become foundational components in targeted protein degradation strategies. As cereblon-binding E3 ligase ligands, they are widely used in the design of proteolysis-targeting chimeras (PROTACs), which hijack the ubiquitin-proteasome system to selectively degrade disease-relevant proteins. This approach has catalyzed a new era of drug discovery with significant translational potential across oncology and other therapeutic areas.[29,30]

2.2. Bispecific antibodies

Bispecific antibodies (bsAbs) are another class of therapeutics which has benefited from phenotypic screening. Antibodies (Abs), otherwise known as immunoglobulins (IgG), are key proteins of the immune system which are responsible for targeting foreign substances and infectious agents.[31] Abs are composed of two light chains and two heavy chains which are linked by disulfide bonds, forming a monomer with a molecular weight of 146–160 kDa.[31] The hypervariable regions within the heavy and light chains makes up the antigen-binding sites of Abs.[31]

Traditionally, an antibody is considered to have two identical antigen-binding sites (two HL fragments) that make it bivalent and monospecific. Immunoglobulins are expressed both as membrane-bound receptors on B lymphocytes and as soluble molecules secreted by plasma cells. Soluble Abs exhibit high affinity and specificity in binding a wide range of natural and artificial molecules (antigens). The ability of Abs to recognize and bind diverse antigens stems from their diversity which encompass 10^8–10^{10} unique antigen-binding variants.[31–33] Conversely, bsAbs feature two distinct antigen-binding sites which enables them to interact with two different targets which increases their functionality and therapeutic range.[34,35] However, bsAbs present a challenge in selecting the optimal target combination for enhanced functional activity.[34,36]

2.3. Cytokine-based therapies

The discovery of interleukin-2 (IL-2) serves as a classic example of a therapeutic agent identified through phenotypic screening.[38] It originated from the observation that T cells exhibited robust proliferation when cultured under specific conditions. This finding led to the hypothesis that a soluble factor, secreted by the T cells themselves, was responsible for driving their growth.[38] Subsequent biochemical purification

and characterization of this factor ultimately led to the identification and naming of IL-2.[38]

IL-2 is a prototypical four-α helix cytokine whose expression is controlled at the mRNA level by signals from CD28 and the T cell receptor.[38] IL-2 exerts its biological effects by establishing a receptor complex composed of three distinct subunits IL-2Rα (CD25), IL-2Rβ (CD122) and the common γ chain (γc). Structural studies of IL-2 bound to the extracellular domains of these receptor chains in a quaternary complex revealed that the interaction sites on IL-2 for each receptor subunit are distinct and non-overlapping. Notably, the α chain (CD25) does not interact directly with the β (CD122) or γc chains, maintaining a modular receptor assembly.[39,40]

IL-2 is essential for immune homeostasis, regulating T (Treg) cell function and fine-tuning effector lymphocyte responses.[41] Paradoxically, the diverse roles of IL-2 have been exploited therapeutically: low doses of recombinant IL-2 are used to promote Treg-mediated immunosuppression in autoimmune and inflammatory diseases while high doses stimulate anti-tumor immune responses.[42,43] Recent advances in understanding the functional, biophysical, and structural properties of IL-2 have paved the way toward the development of novel IL-2 formulations.

The discovery of IL-2 exemplifies the fundamental principles of phenotypic screening, wherein a distinct cellular phenotype—in this case, T cell proliferation—prompted investigation into the underlying molecular driver. This strategy underscores the value of observing functional cellular outcomes without prior assumptions about specific molecular targets. Rather than initiating drug discovery with a defined target, phenotypic screening prioritizes the identification of bioactive compounds based on their observable effects.[42,43] Building on this framework, it is essential to explore how structural insights can complement phenotypic approaches in the context of immune checkpoint targeting. By elucidating key structural domains of immune checkpoint molecules, researchers have been able to rationally design more selective and effective therapeutic interventions. Clinically, high-dose IL-2 has been used to boost effector T cell responses in metastatic melanoma and renal cell carcinoma, while low-dose IL-2 promotes Treg expansion to treat autoimmune diseases such as lupus and graft-*versus*-host disease.[42,43]

Beyond oncology, immunotherapeutics have demonstrated remarkable success in autoimmune and inflammatory diseases. In neuroinflammation, natalizumab (anti-α4 integrin) and ocrelizumab (anti-CD20) have significantly improved outcomes for patients with multiple sclerosis.[44] Belimumab, an anti-BLyS monoclonal antibody, is approved for systemic lupus erythematosus (SLE).[45] Additionally, biologics such as dupilumab (IL-4Rα antagonist) for atopic dermatitis and asthma,[46] secukinumab (IL-17A inhibitor) for psoriasis and psoriatic arthritis,[47] and canakinumab (IL-1β inhibitor) for autoinflammatory syndromes[48] illustrate the expanding therapeutic reach of immune modulation. These examples highlight how immune-based strategies are being successfully adapted across diverse disease states beyond cancer.

2.4. Phenotypically guided antibodies for rare diseases

Several therapeutic antibodies approved for rare diseases have been developed through phenotypically guided screening strategies that rely on functional endpoints rather than predefined targets. Eculizumab, a monoclonal antibody targeting complement protein C5, was originally identified based on its ability to inhibit complement-mediated hemolysis in cellular assays.[49] It became the first approved treatment for paroxysmal nocturnal hemoglobinuria (PNH) and has since been approved for atypical hemolytic uremic syndrome (aHUS).[49] Its long-acting successor, ravulizumab, was developed through iterative optimization of the eculizumab scaffold using pharmacokinetically guided functional screens.[50]

Another example is emapalumab, a monoclonal antibody against interferon-gamma (IFN-γ), developed through cytokine-response assays for patients with primary hemophagocytic lymphohistiocytosis (HLH), a rare and life-threatening immune disorder.[51] These examples highlight how phenotypic screening can play a central role in identifying and optimizing therapeutic antibodies, particularly in contexts where disease mechanisms are complex or not fully elucidated.

3. Targeted drug discovery

Targeted drug discovery in immune checkpoint modulation relies on structural insights into key binding domains of PD-1, CTLA-4, and LAG-3.[2] PD-1's FG and BC loops facilitate ligand interactions, while CTLA-4's FG loop and β-sheet strands form a stable ligand interface.[2] LAG-3's D1 and D2 domains are essential for MHC class II binding and dimerization, crucial for therapeutic targeting. While immune checkpoint inhibitors such as PD-1, PD-L1, CTLA-4, and LAG-3 have demonstrated profound clinical success, the landscape of immune therapeutics extends far beyond these targets. Stimulatory immune checkpoints, including CD40, OX40, and 4-1BB (CD137), play a crucial role in promoting T-cell activation and enhancing anti-tumor immunity.[2] Agonistic antibodies targeting these co-stimulatory molecules are being actively developed to amplify immune responses, often in combination with checkpoint blockade.[2] In this section, we primarily focus on PD-1, PD-L1, CTLA-4, and LAG-3 as representative immune checkpoints due to their well-characterized structures, extensive clinical validation, and central roles in the evolution of cancer immunotherapy. This section explores how structural studies reveal binding dynamics that block immune evasion and enhance therapeutic strategies. Cryo-EM and X-ray crystallography further refine small-molecule and peptide-based inhibitors, paving the way for precision immunotherapy.

3.1. Key structural domains of immune checkpoints for drug discovery

Understanding the structural features of immune checkpoints is crucial for developing targeted therapeutics that enhance or inhibit immune responses. In PD-1, the IgV region is responsible for the flexible ligand and therapeutic binding interactions that are characteristic of PD-1, with the FG loop (residues

Pro130 and Lys131) and BC loop being the most important.[52] The FG loop, a key binding hotspot, is a strong binding region and it is essential for the binding of PD-1 ligands like PD-L1. The N terminal loop, although distant from the PD-1/PD-L1 interface, additionally contributes to the binding of PD-1/PD-L1 through hydrogen binding.[53] This type of interaction at the two sites shows how flexible the PD-1 molecule is and illustrates the possibility of designing inhibitors that target the range of structure and dynamic changes.[54]

Likewise, regions of CTLA-4 that are also vital for its functional regulation are present within its IgV-like domain is focused here.[55] The FG loop (residues Tyr104 and Tyr105) and strands A and G of the front β-sheet form an interface for ligand binding.[56] These regions are identified as targets because they stabilize interactions mediated by hydrophobic residues like Tyr102, Tyr107, Tyr109, and Tyr110, as well as polar residues such as Asn106. The FG loop's structural flexibility boosts CTLA-4's potential as a target for selective modulation, forming specific and stable complexes.[57]

LAG-3 has a distinct structure for both D1 and D2 domains, which are required for its function.[58] The D1 domain binds MHC class II molecules with high affinity, and the D2 domain is key for dimerization.[59] Met171 residue in the D2 domain and bulky N-linked glycans stabilize the dimerization interface, with glycosylation crucial for structural flexibility.[60] This flexibility enables LAG-3 to adopt various conformations, affecting its cell surface interactions and functions. The features of LAG-3 described above are crucial for expanding its therapeutical range, especially when targeting interfaces that are glycosylation dependent or when needing to change the protein's conformation.

The structures of PD-1, CTLA-4, and LAG-3 show that these proteins are dynamic with adjustable domains and interfaces. This makes them suitable for targeting strategies to interfere with immune checkpoint pathways. The structures of PD-1, CTLA-4, and LAG-3 reveal dynamic interfaces that can be exploited for immune checkpoint modulation, forming the foundation for structure-guided therapeutic design.

3.2. Structure-guided targeting of immune checkpoints

Understanding of the structural basis of the PD-1/PD-L1 interaction has provided insight into immune checkpoint regulation and therapeutic intervention. Structure of PD-1 complexed with anti-PD-1 drugs, pembrolizumab and nivolumab, explain the mechanisms behind their functioning. Both the antibodies have the similar biding site on PD-1 and prevents binding of PD-1 and PD-L1.[61,62] Pembrolizumab induces structural changes at BC loop as well as FG loop of PD-1, while nivolumab stabilizes FG loop leading to disruption of its binding with PD-L1 thus increasing T cell activation. Furthermore, pembrolizumab optimizes its binding through flexible C'D loop indicating that structural dynamics are vital in providing therapeutic targets.[63] PD-L1 studies also provide insights which are complementary. The crystal structure of PD-L1 with small molecules like BMS-202 and BMS-8 reveals a unique mechanism where dimerization is induced by these molecules.[64] At the dimer–dimer interface, these compounds attach to PD-L1 thus stabilizing its dimeric structure and preventing PD-1 from interacting. In case of BMS-202 and BMS-8, size exclusion chromatography (SEC) and NMR experiments have confirmed that they cause PD-L1 to dimerize, hence blocking interaction between PD-1/PD-L1.[65] These findings underscore the potential of small molecules in modulating immune checkpoints and highlight the possibilities opened by structural studies.

Structural investigations have also given deep insights into CTLA-4/tremelimumab complex.[66] Unlike its normal ligand B7-1/2, tremelimumab interacts with an interface of CTLA-4 that includes the essential motif 97MYPPPY102 present in FG loop. Such binding outcompetes B7-1/2 and thus restores T-cell functioning and potentiates anti-tumor effects. Protein interface analysis has identified important amino acid residues in F and G strands of CTLA-4 β-sheets whose mutation are critical for antibody binding. Notably, Bio-Layer Interferometry (BLI) assays show that antibodies like HL32 and Ipilimumab block B7-1 binding through overlapping epitopes.[67] However, structure of HL32 shows a pH-dependent affinity that dissociates at the acidic endosomal pH to maintain recycling of CTLA-4 while ipilimumab maintains its bound state causing it to be degraded within lysosomes. This detailed understanding of pH-dependent binding dynamics demonstrates how structural data can inform the design of antibodies with reduced toxicity.

X-ray crystallography and cryo-EM have been used in the structural analysis of LAG-3, revealing that its D1 and D2 domains play a critical role in ligand binding and thus causing immune inhibition.[59] The major site for MHC class II interaction has been marked to be the D1 domain with a 25-residue loop stabilizing its interaction. Such knowledge can then be applied in designing therapeutic antibodies such as Favezelimab that inhibits D1-MHC class II interactions and reinstates T-cell functionalities (Fig. 2).[68] Moreover, *cis*-dimerization of LAG-3 mediated through D2 domain is crucial for its inhibitory activity.[59] To target dimerization interface of D2, C9B7W type antibodies were introduced that interrupted dimerization and thus blocked the binding of both MHC class II and FGL1 ligands by LAG-3 protein.[59] These findings highlight the synergistic roles of D1 and D2 domains in LAG-3 biology and provide complementary pathways for therapeutic intervention.

These structural insights highlight the pivotal role of high-resolution studies in immune checkpoint drug discovery. By revealing key interaction sites and guiding the design of therapeutics, structure-based approaches have advanced the development of more precise and less toxic inhibitors. This foundation paves the way for exploring antibody-based therapies, which leverage structural understanding to enhance immune checkpoint modulation. These structural insights underscore the role of high-resolution studies in guiding immune checkpoint drug discovery, paving the way for the development of novel therapeutic agents with improved specificity and reduced toxicity.

3.3. Antibody-based therapies: PD-1/PD-L1 and CTLA-4 inhibitors

Structural knowledge has directly contributed to the development of therapeutic antibodies that block immune checkpoint

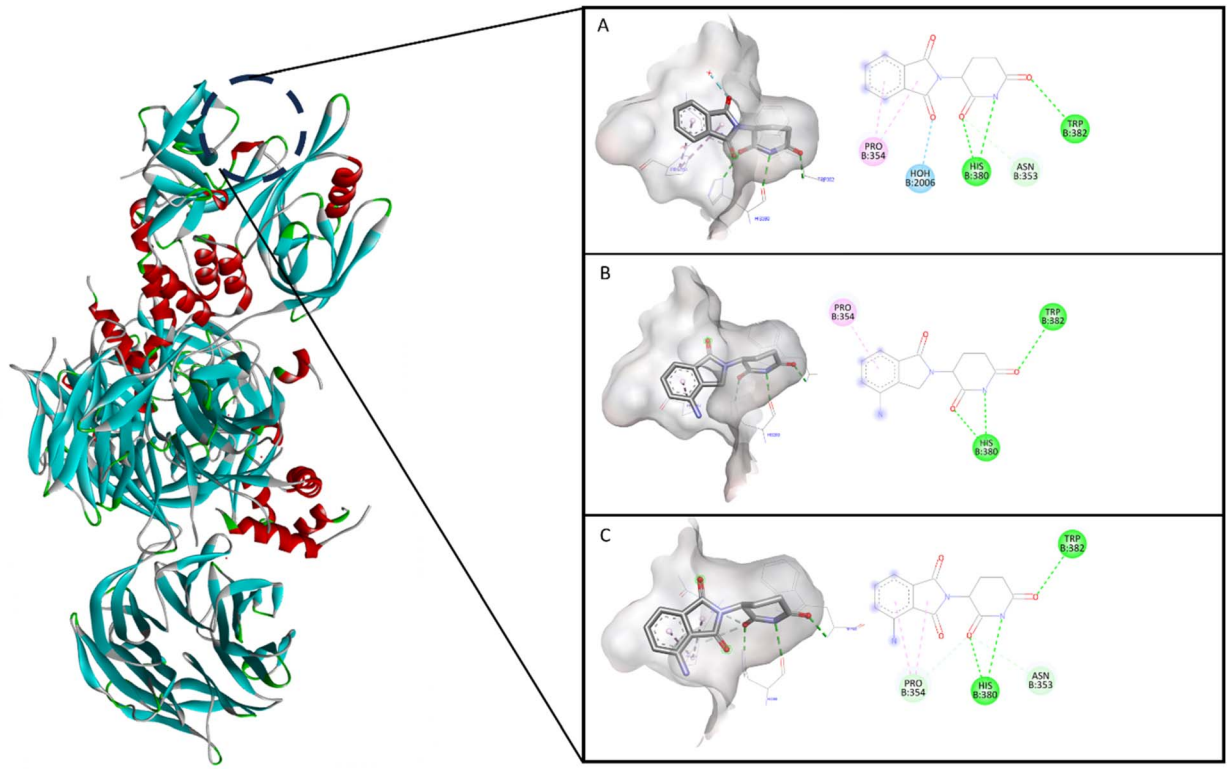

Fig. 2 3D structure of the DDB1-CRBN E3 ubiquitin ligase bound to (A) thalidomide, (B) lenalidomide and (C) pomalidomide obtained from PDB ID: 4CI1, 4CI2 and 4CI3, respectively.

signaling and restore immune function in cancer treatment. Monoclonal antibodies against PD-1/PD-L1 axis have shown remarkable activity in therapeutic trials for melanoma, non-small cell lung cancer (NSCLC) and other malignancies.[69] These IgG4 antibodies like, pembrolizumab and nivolumab, bind to PD-1 thereby blocking its interaction with PD-L1 and resulting in restoration of T-cell function.[37] Pembrolizumab possesses hinge region mutation at S288P that makes it more stable while binding to the CC′-loop of PD-1 and nivolumab selectively binds to N-terminal loops on PD-1 thus making it more effective at reviving immune functionality. These PD-1 inhibitors restore T cell function by blocking PD-1 interaction with PD-L1, preventing inhibitory signaling. They are approved for various cancers including melanoma, non-small cell lung cancer, and Hodgkin lymphoma.[69]

PD-L1 specific antibodies like atezolizumab, avelumab and durvalumab binds with great affinity to the forward β-sheet of PD-L1.[70] For example, atezolizumab has dissociation constant of 0.4 nM which blocks NSCLC-associated immunosuppression mediated by PD-LI (Fig. 3).[71] Similarly, avelumab and durvalumab manifest great potency with dissociation constants amounting to 42.1 pM and 667 pM respectively.[72,73] The impact has been most apparent in improving outcomes in Merkel cell carcinoma and bladder cancer. Another nanobody, KN035, has a remarkable feature of targeting PD-L1 CC′FG strands, providing a new therapeutic strategy.[74] This suggests the potential of producing small molecule inhibitors that can simulate nanobody interactions thereby broadening the therapeutic arsenal. These anti-PD-L1 antibodies inhibit ligand-receptor engagement, restoring antitumor immunity. Atezolizumab is approved for NSCLC and triple-negative breast cancer; avelumab for Merkel cell carcinoma; and durvalumab for bladder and lung cancers.[74]

CTLA-4 inhibitors help treat cancer by blocking the interaction between CTLA-4 and B7-1/2 hence restoring T-cell activation. The first checkpoint inhibitor approved for metastatic melanoma was ipilimumab which is an IgG1 monoclonal antibody that binds to CTLA-4.[75] Ipilimumab binds to the large, buried surface area of CTLA-4 with a high binding affinity (K_D = 5.25 nM). It interacts with the front β-sheet of CTLA-4 and overlaps with the CTLA-4:B7 recognition surface, creating direct steric hindrance that prevents B7 ligand binding (Fig. 3). This blockade inhibits T-cell downregulation and enhances the antitumor activity of T-cell lymphocytes, making it a potent immune checkpoint inhibitor. Ipilimumab blocks CTLA-4:B7 interactions, enhancing T cell activation. It was the first immune checkpoint inhibitor approved for metastatic melanoma.[75]

Tremelimumab, an IgG2 antibody has been designated as orphan drug for mesothelioma and is undergoing trials in combination therapies.[76] It binds to CTLA-4 at a site overlapping with the CTLA-4:B7 interaction interface (Fig. 3). Its IgG2 subclass minimizes antibody-dependent cellular cytotoxicity (ADCC), making it preferable due to its reduced systemic side

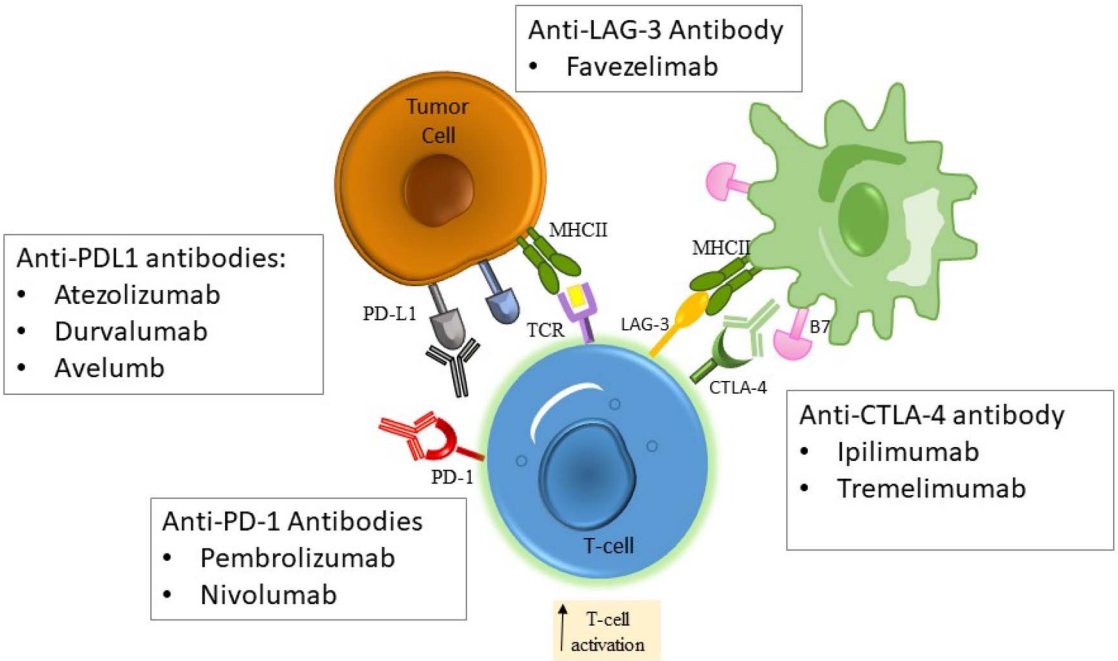

Fig. 3 Antibody based therapy for immune checkpoint inhibition. PL-1 expressed on the T-cells interacts with the PDL-1 expressed on the tumor cells. This interaction causes inactivation of T-cells. By blocking PD-1/PD-L1 interactions by utilizing anti-PD-1 and anti-PD-L1 antibodies help restore anti-tumor function of T-cells. Similarly, CTLA-4 expressed on the T-cells interacts with B7 on the antigen presenting cells and causes immunosuppression. Anti-CTLA-4 antibodies mediate activation, proliferation, and tumor-antigen responsiveness of T-cells.

effects while maintaining anti-tumor efficacy. These advances demonstrate how antibody engineering, guided by structural insights, continues to refine immune checkpoint therapies for enhanced efficacy and safety. Tremelimumab is under investigation for use in mesothelioma and hepatocellular carcinoma, and acts similarly to ipilimumab by blocking CTLA-4.[76]

3.4. Innovative antibody engineering for enhanced immune checkpoint therapy

Antibodies have been genetically engineered in the laboratory to improve their specificity to precisely target their receptor, minimize the immune response against the antibody and enhance their therapeutic potential. Technologies such as:

Non-fucosylated antibodies generated by removal of fucose, an oligosaccharide, from the Fc region exhibited enhanced binding to the FcγRIIIa receptor expressed by immune cells and significantly increases antibody dependent cellular cytotoxicity (ADCC).[77] Preclinical studies with non-fucosylated PD-L1 monoclonal antibodies have shown enhanced peripheral T-cell activation and reduced regulatory T-cell function leading to more potent anti-tumor activity. Such engineered antibodies demonstrate a way forward in improving therapeutic outcomes.

3.4.1. Pro formulations. The prodrug antibodies called Probody™ therapeutics, are designed to avoid off-target effects by using masking peptides that hinder antigen-binding areas.[78] These masks are cleaved by tumor-specific proteases, allowing the antibody to bind only at the tumor site. CX-072 is a PD-L1 targeting Probody™ that has been shown to exhibit less systemic toxicity in preclinical studies, and it is currently undergoing clinical trials for solid tumors and lymphomas.[79]

3.4.2. Bispecific antibodies. Bispecific antibodies combine two different molecules to enhance the anti-tumor defense.[80] M7824 which is a fusion protein of PD-L1 and TGF-βRII has demonstrated some hopes in early clinical trials in biliary tract and colon cancers. In a similar vein, MGD013 targets both PD-L1 and LAG-3 which have shown promising results in treatment of solid tumors as well as hematologic malignancies.[81,82]

Despite their successes, there are various setbacks encountered by antibody-based therapies including resistance, immune-related adverse events (irAEs) among patients with varying rates of response.[83] Current research efforts concentrate on understanding mechanisms of resistance, optimization of dosing strategies together with exploring combination approaches so as to achieve efficacy while minimizing side effects. Future developments in antibody engineering such as antibody–drug conjugates (ADCs) or multi-specific antibodies may help overcome these problems thereby advancing cancer immunotherapy further (Fig. 4).[84]

3.5. Peptide-based and small-molecule inhibitors

An antibody-based approach to treat cancer is seen as too costly, administratively burdensome and having stability problems, thereby leading to the emergence of peptide-based therapies and small molecule drugs which are considerably considered as cost-effective, administratively less complex, and more stable alternatives or additions for cancer treatment. One such alternative approach that modulates immune checkpoints is

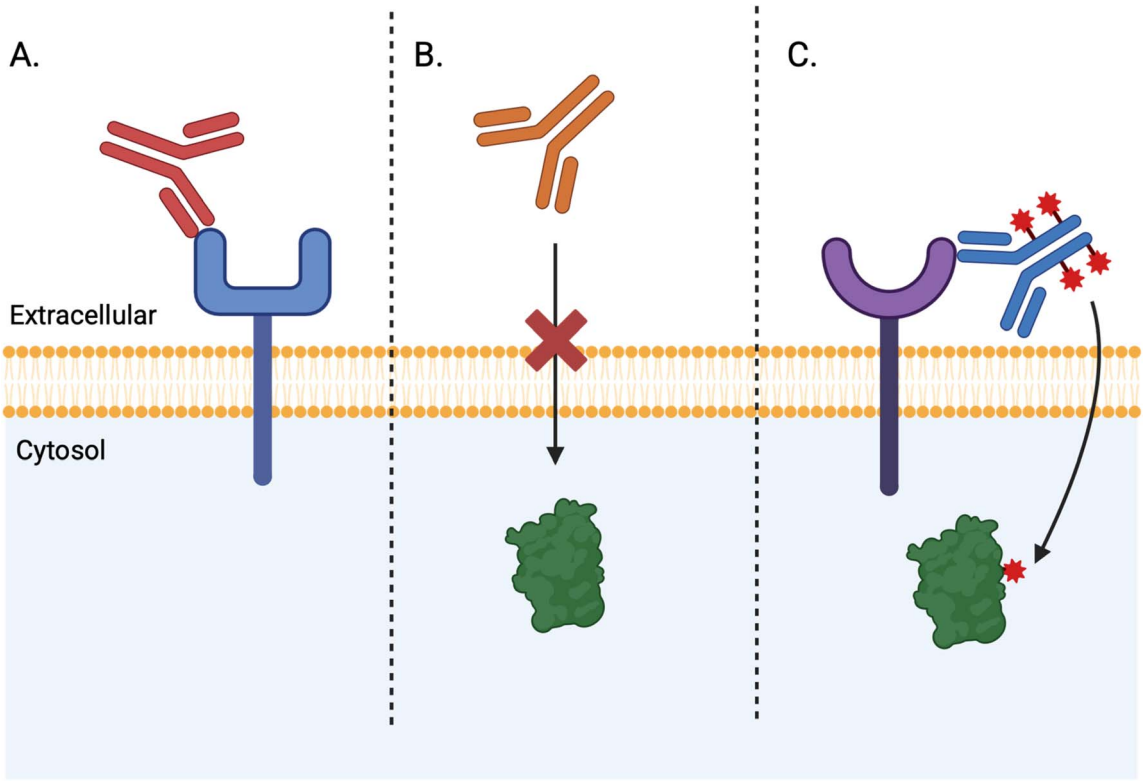

Fig. 4 Challenges of antibody therapies and innovations. (A) Antibodies bind surface, membrane, proteins on the extracellular space. (B) Targeting of cytosolic proteins with antibodies remains a challenge due to inability to cross cell membrane. (C) Antibody–drug conjugates as a targeted drug delivery system (created in https://BioRender.com).

targeting key regulatory pathways like ubiquitination and degradation (Fig. 5). For instance, IL-2 promotes FBXO38-mediated ubiquitination and degradation of PD-1 thus increasing its anti-cancer efficacy while curcumin destabilizes PD-L1 through inhibition of deubiquitination hence improving its anti-CTLA-4 therapy.[85] Only a limited number of small molecule inhibitors targeting immune checkpoints, particularly PD-1/PD-L1, have emerged from rational medicinal chemistry efforts. Among the most studied are biphenyl-based compounds developed by Bristol Myers Squibb (BMS), which demonstrated submicromolar potency in disrupting PD-1/PD-L1 interactions.[86] Despite their strong *in vitro* activity, these hydrophobic compounds (*e.g.*, BMS-1001, BMS-202, BMS-200, and BMS-1166, Fig. 5) have not progressed into clinical development. Several companies, including Incyte, Arising International, Chemocentryx, Polaris Pharmaceuticals, and Maxinovel, have developed related scaffolds centered on the biphenyl core.[86] Notably, INCB086550 (Fig. 5) from Incyte has shown promising antitumor activity in humanized mouse models and is currently being evaluated in a phase I clinical trial. Early

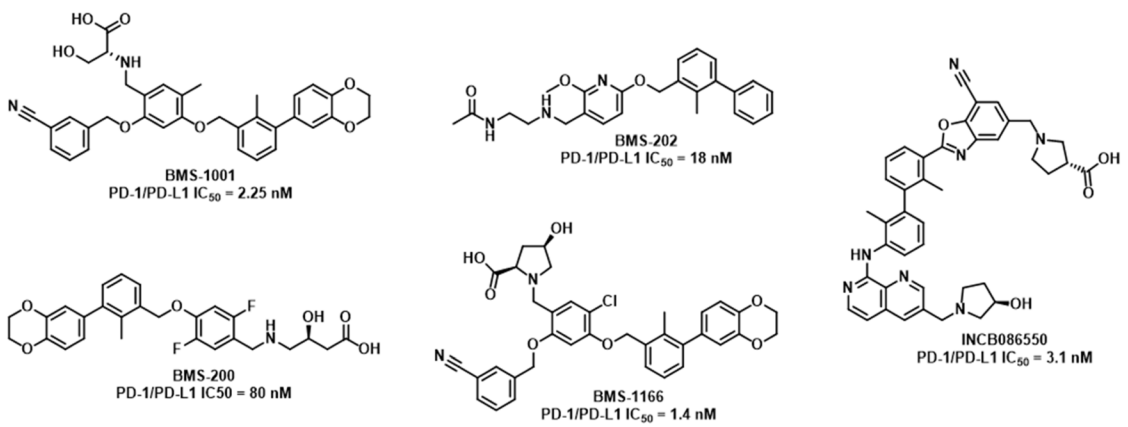

Fig. 5 Chemical structures of small molecule inhibitors of PD-1/PD-L1 interaction.

results indicate enhanced T-cell activation and immune modulation consistent with PD-1/PD-L1 blockade.[86] In parallel, academic efforts have focused on optimizing BMS-derived leads, while others have explored structure-based virtual screening to expand the repertoire of small molecule inhibitors targeting PD-1 and related immune checkpoints. Inhibitors like 2-bromopalmitate disrupt palmitoylation on PD-L1 destabilizing it thereby enhancing anti-tumor immunity.[87] Small molecules like YPD-29B have been reported to function as PD-L1 modulators. They work by inducing PD-L1 dimerization, which can destabilize the protein and promote its internalization. Once internalized, PD-L1 undergoes lysosomal degradation, effectively reducing its presence on the cell surface (Fig. 6).[88]

3.5.1. PD-1/PD-L1 peptide-based inhibitors. Peptide based inhibitors are designed based on the structural insights into their binding interfaces to block the PD-1/PD-L1 interaction. The first peptide antagonist known as (D)PPA-1 was made using mirror-image phage display so as to constrain tumor growth *in vivo* by inhibiting the interaction between PD-1 and PD-L1.[89] The optimized peptides like PL120131 target specific residues on PD-1 thus relieving cells from apoptotic signaling induced by PD-L1.[90] Other molecules, TPP-1 and UNP-12, also have been reported to considerably decrease the size of tumors in preclinical experiments.[91] Furthermore, NP-12 in combination with tumor vaccines or cyclophosphamide has shown increased activity against melanoma as well as colon cancers.[92] Another peptide called PD-LYSO, is based on an interesting approach which enhances lysosomal degradation of PD-L1 improving therapeutic outcomes (Fig. 6).[93] Peptides such as PL120131 and PD-LYSO inhibit PD-1/PD-L1 binding or promote PD-L1 degradation, leading to enhanced T cell-mediated cytotoxicity in preclinical models.[93]

3.5.2. CTLA-4 peptide-based inhibitors. In the context of CTLA-4, peptide-based therapies have shown the potential to disrupt CTLA-4 binding to B7 ligands. For example, a synthetic peptide called p344 specifically binds the 99MYPPPY104 loop of CTLA-4 to prevent CTLA-4 from interacting with B7-1.[94] Computational tools like Rosetta were used to design cyclic peptides such as peptide cyc[EIDTVLTPTGWVAKRYS] which has been tested experimentally for its ability to increase CD8+ T-cell cytotoxicity and decrease tumour growth in lung cancer models.[95] Another promising approach utilizes helix-loop-helix (HLH) peptides, such as ERY2-4, a modified version of a CTLA-4 binding peptide inhibits CTLA-4/B7-1 interaction while boosting lymphocyte responses without cross-reactivity with CD28 thereby making it a valuable candidate for immunotherapy.[96] Furthermore, monobody proteins derived from the MYPPPY motif of CTLA-4 such as CFN13 and its Fc-fusion variant CFN13-Fc are potent inhibitors of CTLA-4/CD80 interactions further supporting the therapeutic potential of peptide-based CTLA-4 inhibitors.[97]

3.5.3. LAG-3 peptide-based inhibitors. Lymphocyte activation gene-3 (LAG-3) expressed on immune cells is another important immune checkpoint. A peptide, LFP-6, was also shown to inhibit the binding of FGL1 to LAG-3 thus enhancing T cell function *in vivo*.[98] A proteolysis-resistant version of this peptide, LFP-D1, as well as the bispecific peptide LFOP targeting PD-1/PD-L1 and LAG-3/FGL1 have also been found to synergize with radiotherapy thereby improving anti-tumor immunity (Qian *et al.* 2024). Additionally, cyclic peptides such as Cyclo (CVPMTYRAC) disturb LAG-3/HLA-DR interactions, activating CD8+ T cells while reducing regulatory T cells.[99] Cyclo has been useful for imaging LAG-3 expression and analysis of immunotherapy responses in murine melanoma models after labeling this peptide with gallium-68 to produce 68Ga-NOTA-XH05.[100]

These advancements in peptide- and small-molecule-based immune checkpoint inhibitors highlight their potential as promising alternatives to traditional antibody therapies. With

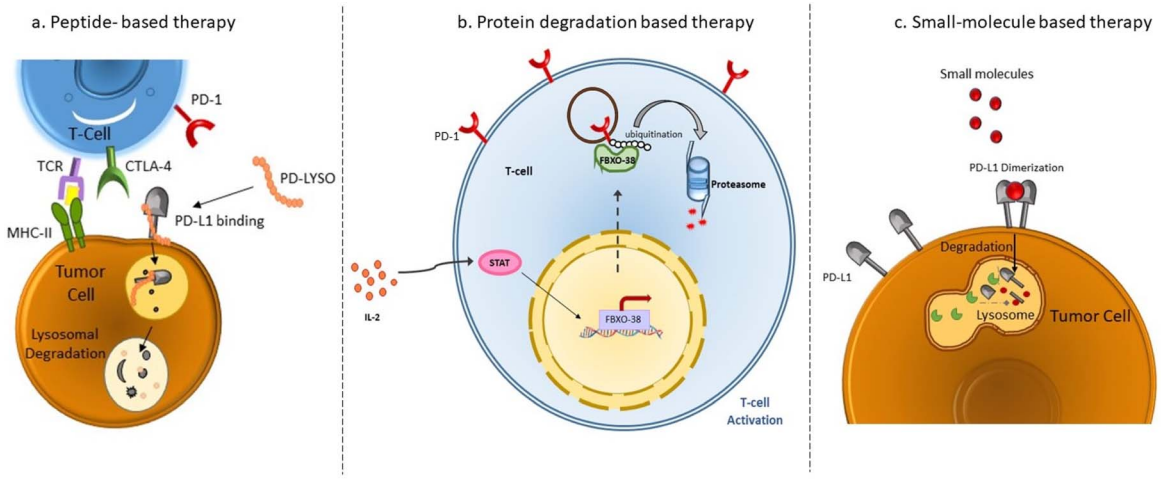

Fig. 6 Mechanisms of immune checkpoint inhibitors. (A) Peptides such as PD-LYSO are specially designed with structural insights to bind PD-L1 expressed on tumor cells and subsequently causes internalization and lysosomal degradation of PD-L1. (B) IL-2 causes upregulated expression of FBXO-38 within T-cells that results in ubiquitination of PD-1 and causes proteasomal degradation of PD-1, hence inhibiting PD-1/PD-L1 axis. (C) Small molecules like YPD-29B causes dimerization and destabilization of PD-L1, subsequently resulting in internalization and lysosomal degradation of PD-L1.

improved tumor penetration, favorable pharmacokinetics, and reduced production costs, these innovative approaches are paving the way for more effective and accessible cancer immunotherapies. Further optimization and clinical validation of these therapies could revolutionize the field of immuno-oncology.

3.5.4. Small molecules from antibody pharmacophores (SMAbPs). Over the past few years, our lab has pioneered the discovery of first-in-class small molecule inhibitors of immune checkpoints using various approaches.[101–109] This work has involved fluorescence resonance energy transfer (FRET) assays in screening chemical libraries, which resulted in the discovery of small molecules targeting V-domain immunoglobulin suppressor of T cell activation (VISTA),[101] ICOS (inducible costimulator of T cells),[102] and LAG-3.[103,104] Additionally, we employed pharmacophore-based virtual screening using PyRod, a software that enables for visualizing pharmacophoric binding pocket characteristics and identifying hot spots for hit discovery, in the identification of small molecules targeting various immune checkpoints.[105–107] Notably, our group was the first to successfully utilize affinity selection mass spectrometry (ASMS) for identifying immune checkpoint inhibitors, leading to the discovery of ICOS-targeted small molecules.[108]

Our most remarkable advancement in this field has been the introduction of small molecules from antibody pharmacophores (SMAbPs) as a new workflow for the discovery of small molecules targeting immune checkpoints.[109] SMAbPs leverages co-crystal structures of checkpoints with monoclonal antibodies to build pharmacophore maps for virtual screening. The application of SMAPs to five immune checkpoints resulted in hits with submicromolar potency in both cell-free and cellular assays.[109] Notably, SMAPs identified the most potent inhibitors targeting T-cell immunoglobulin and mucin domain 3 (TIM-3) (MG-T-19, Fig. 7) and VISTA (MG-V-53, Fig. 7) reported to date, as well as first-in-class small molecule modulators of BTLA (B- and T-lymphocyte attenuator) (MG-B-28, Fig. 7), 4-1BB (MG-I-62, Fig. 7), and CD27 (MG-C-30, Fig. 7).[109]

3.6. Targeting immunosuppressive cell populations: TAMs and Tregs

In addition to immune checkpoint blockade, targeting immunosuppressive cell populations within the tumor microenvironment—namely tumor-associated macrophages (TAMs) and regulatory T cells (Tregs)—has emerged as a complementary strategy to enhance antitumor immunity.[1] These cell types play critical roles in establishing immune tolerance, suppressing cytotoxic T lymphocyte (CTL) activity, and promoting tumor progression.[1]

TAMs are typically skewed toward an M2-like, anti-inflammatory phenotype that supports tumor growth, angiogenesis, and metastasis. Therapeutically, reprogramming or depleting TAMs has been pursued through inhibition of colony-stimulating factor 1 receptor (CSF1R), a key regulator of macrophage survival and differentiation.[1] Small-molecule CSF1R inhibitors such as pexidartinib (approved for tenosynovial giant cell tumor) and monoclonal antibodies like emactuzumab have demonstrated the ability to reduce M2 macrophage infiltration and enhance the efficacy of immune checkpoint therapies in preclinical and early clinical settings.[1]

Tregs, characterized by high expression of CD25, CTLA-4, and the chemokine receptor CCR4, suppress effector T-cell activity and contribute to immune evasion.[1] Targeting Tregs has focused on selectively disrupting their recruitment and function in tumors without inducing systemic autoimmunity. CCR4-targeted monoclonal antibodies such as mogamulizumab have been shown to deplete intratumoral Tregs and are currently under investigation in combination with PD-1 inhibitors for refractory solid tumors and T-cell lymphomas.[1] Additional strategies involve low-dose cyclophosphamide or anti-CD25 approaches, though these carry the risk of depleting activated effector T cells.

Collectively, TAM and Treg targeting strategies are gaining momentum as critical components of combination immunotherapy regimens. These approaches aim to reshape the tumor immune landscape, alleviate immunosuppression, and amplify

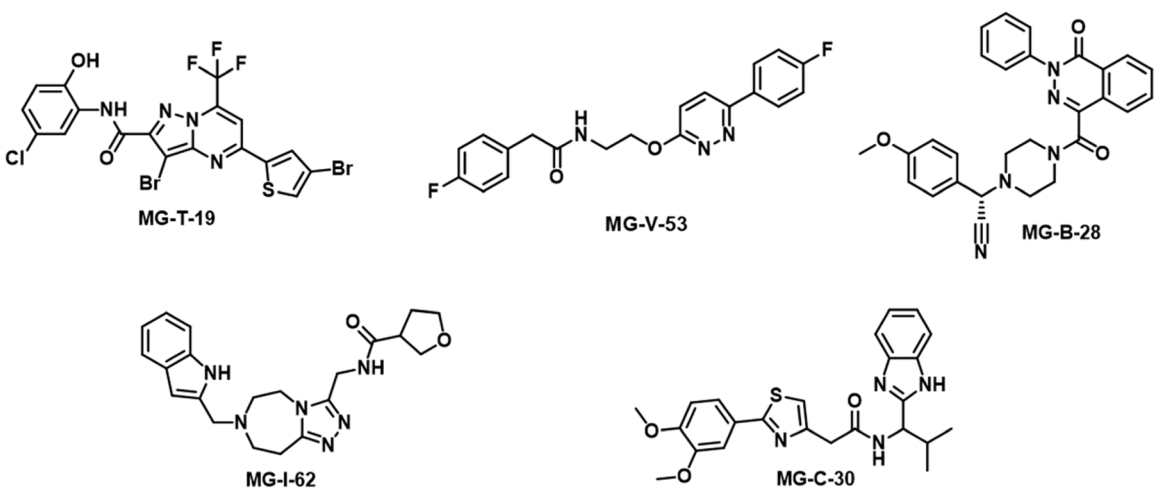

Fig. 7 Chemical structures of small molecules identified using SMAbPs targeting TIM-3, VISTA, BTLA, 4-IBB, and CD27.

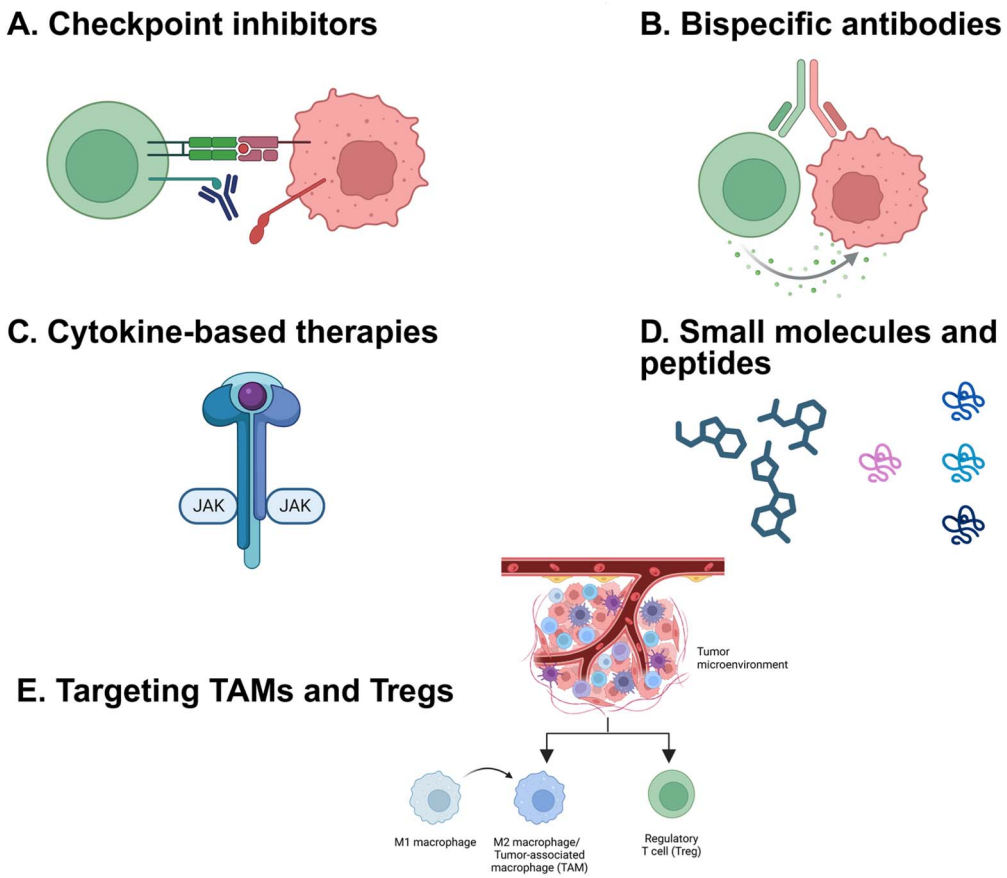

Fig. 8 Summary of molecular mechanisms of representative immune-modulating therapeutic classes. (A) Checkpoint inhibitors restore T cell function by blocking the interactions between negative immune checkpoints and their binding partners. (B) Bispecific antibodies engage CD3 on T cells and tumor-associated antigens (*e.g.*, CD19) to promote T cell-mediated killing. (C) Cytokine-based therapies (*e.g.*, IL-2) activate immune cells through JAK/STAT signaling in a dose-dependent manner. (D) Small molecules and peptides disrupt checkpoint signaling through dimerization, degradation, or direct binding. (E) Agents targeting TAMs and Tregs reprogram or deplete immunosuppressive cells in the tumor microenvironment to enhance antitumor immunity (created in https://BioRender.com).

responses to checkpoint inhibitors, particularly in immunologically "cold" tumors. As understanding of the cellular and molecular regulators of these suppressive populations deepens, future efforts are likely to focus on precision targeting, biomarkers of response, and rational combination designs. A consolidated overview of the molecular mechanisms associated with these immunomodulatory strategies is illustrated in Fig. 8.

4. Case studies of hybrid discovery workflows

Hybrid technologies in drug discovery combine the use of phenotypic screening with molecular profiling and genetic to obtain and enhance therapeutic agents. This approach helps bridge functional and mechanistic insights which can lead to finding of predictive biomarkers as well as novel therapeutic candidates. Hybrid methodologies give a comprehensive framework for enhancing tumor immunotherapy and targeted therapies by addressing highly complex mechanisms such as synthetic lethality, modulation of tumor microenvironment as well as resistance to drugs.

One persuasive illustration is the application of ENMD-2076 and TAK-901 aurora kinase inhibitors in triple-negative breast cancer (TNBC).[110] 15-Gene tumor immunologic phenotype (TIP) signature coupled with high-throughput sequencing-based screening (HTS2) was used by researchers to identify compounds which could reprogram expression of TIP genes.[111] These inhibitors upregulated the Th1 chemokines CXCL10 and CXCL11, enhancing T-cell infiltration into the tumor microenvironment and greatly enhancing the effectiveness of anti-PD-1 therapy in preclinical TNBC models. This method highlights how combination therapies modulating the immune environment within tumors that overcome resistance can be identified through hybrid workflows.

Similarly, this applies to the treatment of EGFR wild-type (EGFR-WT) tumors like head and neck squamous cell carcinoma (HNSCC) and lung adenocarcinoma, where hybrid methods employed clinical data in combination with genetic profiling to find out new therapeutic targets. Clinical trials such as Impower 150 proved that combining atezolizumab, bevacizumab, paclitaxel and carboplatin could improve patients' progression-free survival significantly.[112,113] Moreover, new

mutations were analyzed, including FCGR2B, IGF1R, and KIT, and this also led to the development of innovative therapies like BI-1206 and imatinib mesylate.[114] This finding demonstrates how phenotypic data, together with molecular information, can inform multi-drug regimens aimed to treat specific patient groups.[115]

The growing integration of AI and ML in immune drug discovery is revolutionizing both phenotypic and targeted approaches. In phenotypic screening, AI enables high-throughput analysis of complex cellular imaging data, facilitating the extraction of subtle morphological and functional changes that would be difficult to detect manually. Deep learning models are being trained on high-content imaging datasets to recognize immune cell activation patterns, cytokine release profiles, and tumor cell killing responses with increasing precision. Importantly, AI is not expected to render traditional phenotypic screening obsolete, but rather to complement and extend its capabilities. By enhancing feature extraction, hit prioritization, and predictive modeling, AI can bridge phenotypic observations to mechanistic insights more efficiently. In addition, AI is playing a growing role in structure-based drug design, virtual screening, and *de novo* molecule generation—particularly when integrated with multi-omics and real-world clinical data. The convergence of AI and phenotypic assays represents a synergistic evolution that will continue to enhance the resolution and translational relevance of immune drug discovery pipelines.

5. Challenges and resistance in immune checkpoint therapy

Despite the transformative success of immune checkpoint inhibitors (ICIs), a significant proportion of patients fail to respond, and many responders eventually develop resistance.[2] These limitations stem from both primary (innate) and acquired mechanisms of resistance. Primary resistance may result from poor immunogenicity, absence of tumor-infiltrating lymphocytes (TILs), or low expression of checkpoint ligands such as PD-L1.[2] Tumors with a "cold" immune phenotype—characterized by limited antigen presentation or suppressed interferon signaling—are particularly unresponsive to ICIs.[2]

Acquired resistance, on the other hand, often emerges after initial response and may involve upregulation of alternative immune checkpoints (*e.g.*, TIM-3, TIGIT, LAG-3), loss of neoantigen expression, or mutations in interferon-γ signaling and antigen processing pathways.[2] For example, mutations in JAK1/JAK2 or B2M can result in insensitivity to T cell-mediated killing and loss of MHC class I presentation, respectively.[2]

To overcome these challenges, several strategies are under investigation. Rational combination therapies, such as dual checkpoint blockade (*e.g.*, PD-1 + LAG-3 inhibitors) or ICIs combined with kinase inhibitors (*e.g.*, VEGFR, MEK), have shown promise in restoring immune responsiveness.[2] Epigenetic modulators such as HDAC or DNMT inhibitors may reprogram the tumor microenvironment to enhance antigenicity and T cell infiltration. Additionally, oncolytic viruses, STING agonists, and cytokine therapies are being developed to stimulate local immune activation in poorly inflamed tumors. The integration of predictive biomarkers and adaptive trial designs will also be critical for optimizing these interventions and tailoring treatment to dynamic tumor–immune interactions.[2]

6. Conclusions and outlook

The future of drug discovery lies not in choosing between phenotypic and targeted approaches but in recognizing how they can complement each other. As technology continues to advance, hybrid discovery strategies will become increasingly refined, leveraging computational modeling, high-content screening, and multi-omics integration to bridge functional outcomes with molecular mechanisms. These evolving workflows offer a path forward in addressing the complexity of immune therapeutics, reducing attrition rates, and accelerating the development of more effective treatments. Ultimately, the challenge is not just discovering new drugs but ensuring they reach patients with greater precision, fewer setbacks, and sustained efficacy. With an expanded toolkit and a growing appreciation for the strengths of both paradigms, the next era of drug discovery will not be defined by rigid categories but by the flexibility to adapt, integrate, and innovate.

Hybrid discovery workflows are poised to shape the future of immune therapeutics by integrating high-throughput screening, computational modeling, and mechanistic validation in a dynamic and iterative manner. These workflows combine the functional relevance of phenotypic assays with the precision of target-based design, enhanced by AI-driven pattern recognition and multi-omics data integration. For instance, early-stage phenotypic hits can be rapidly triaged through transcriptomic or proteomic profiling to infer putative mechanisms of action, followed by structural modeling for rational optimization. Such workflows not only shorten discovery timelines but also allow real-time adaptation to emerging resistance mechanisms or biomarker-defined patient subgroups. Looking ahead, next-generation immunotherapy pipelines will likely rely on closed-loop systems in which *in vitro* functional screening, computational analytics, and medicinal chemistry cycles are tightly interwoven. This adaptive strategy represents a departure from linear discovery models and is particularly well-suited for addressing the complexity and dynamism of the tumor immune microenvironment.

Precision medicine has become increasingly central to the development and clinical implementation of immune therapeutics. By leveraging patient-specific molecular and immunologic features, precision-guided immunotherapy enables the stratification of responders and the rational design of combination strategies. Biomarkers such as PD-L1 expression levels are now routinely used to guide the use of PD-1/PD-L1 checkpoint inhibitors in NSCLC and melanoma. Tumor mutational burden (TMB) has emerged as another important predictor of immune responsiveness, correlating with increased neoantigen presentation and T cell infiltration. In certain malignancies, such as microsatellite instability-high (MSI-H) colorectal

cancer, high TMB has been associated with robust responses to immune checkpoint blockade. HLA typing and mutational analyses are further being explored to identify patient populations most likely to benefit from peptide-based vaccines or neoantigen-specific T cell therapies. Emerging technologies such as single-cell transcriptomics and spatial profiling are also being employed to map immune cell states within tumors and to tailor immunotherapy regimens accordingly. These advances highlight how precision medicine is reshaping immune drug discovery, enabling patient-centric approaches that increase therapeutic efficacy while minimizing off-target effects.

Conflicts of interest

There are no conflicts to declare.

Data availability

No primary research results, software or code have been included and no new data were generated or analyzed as part of this review.

Acknowledgements

We acknowledge funding from the National Institutes of Aging (NIA) RF1AG084635 (PI: Gabr).

References

1 J. H. Cha, L. C. Chan, M. S. Song and M. C. Hung, *Cold Spring Harbor Perspect. Med.*, 2020, **10**, a036863.
2 M. Hossain, *Int. Immunopharmacol.*, 2024, **143**, 113365.
3 E. L. Berg, *Cell Chem. Biol.*, 2021, **28**, 424–430.
4 F. Vincent, A. Nueda, J. Lee, M. Schenone, M. Prunotto and M. Mercola, *Nat. Rev. Drug Discovery*, 2022, **21**, 899–914.
5 A. C. Rufer, *Drug Discovery Today*, 2021, **26**, 875–886.
6 A. S. Hauser, M. M. Attwood, M. Rask-Andersen, H. B. Schiöth and D. E. Gloriam, *Nat. Rev. Drug Discovery*, 2017, **16**, 829–842.
7 M. M. Attwood, D. Fabbro, A. V. Sokolov, S. Knapp and H. B. Schiöth, *Nat. Rev. Drug Discovery*, 2021, **20**, 839–861.
8 J. P. Renaud, A. Chari, C. Ciferri, W.-T. Liu, H.-W. Remigy, H. Stark and C. Wiesmann, *Nat. Rev. Drug Discovery*, 2018, **17**, 471–492.
9 M. Leveridge, C. W. Chung, J. W. Gross, C. B. Phelps and D. Green, *SLAS Discovery*, 2018, **23**, 881–897.
10 S. Lin, K. Schorpp, I. Rothenaigner and K. Hadian, *Drug Discovery Today*, 2020, **25**, 1348–1361.
11 R. Hughes, R. Elliott, J. Dawson and N. Carragher, *Cell Chem. Biol.*, 2021, **28**, 338–355.
12 F. Bellomo, D. Medina, E. Leo, A. Panarella and F. Emma, *J. Inherited Metab. Dis.*, 2017, **40**, 601–607.
13 V. Marx, *Nat. Methods*, 2021, **18**, 9–14.
14 J. Lee, D. Hyeon and D. Hwang, *Exp. Mol. Med.*, 2020, **52**, 1428–1442.
15 A. Gangwal and A. Lavecchia, *Drug Discovery Today*, 2024, **29**, 103992.
16 D. Paul, G. Sanap, S. Shenoy, D. Kalyane, K. Kalia and R. Tekade, *Drug Discovery Today*, 2021, **26**, 80–93.
17 D. Catacutan, J. Alexander, A. Arnold and J. Stokes, *Nat. Chem. Biol.*, 2024, **20**, 960–973.
18 C. Chen, J. Wang, D. Pan, X. Wang, Y. Xu, J. Yan, L. Wang, X. Yang, M. Yang and G.-P. Liu, *MedComm*, 2023, **4**, e315.
19 J. G. Moffat, J. Rudolph and D. Bailey, *Nat. Rev. Drug Discovery*, 2014, **13**, 588–602.
20 W. Rehman, L. M. Arfons and H. M. Lazarus, *Ther. Adv. Hematol.*, 2011, **2**, 291–308.
21 A. Ornoy, *Reprod. Toxicol.*, 2006, **22**, 214–226.
22 T. Lemmens, *J. Law Med. Ethics*, 2013, **41**, 163–184.
23 M. J. Nutt and S. G. Stewart, *Drug Discovery Today*, 2024, **29**, 104010.
24 A. Barbarossa, D. Iacopetta, M. S. Sinicropi, C. Franchini and A. Carocci, *Curr. Med. Chem.*, 2022, **29**, 19–40.
25 Y. Liu, X. Huang, X. He, Y. Zhou, X. Jiang, S. Chen-Kiang, S. R. Jaffrey and G. A. Xu, *FASEB J.*, 2015, **29**, 4829–4839.
26 X. B. Chang and A. K. Stewart, *Int. J. Biochem. Mol. Biol.*, 2011, **2**, 287–294.
27 T. Ito and H. Handa, *Congenital Anomalies*, 2012, **52**, 1–7.
28 R. Martiniani, V. Di Loreto, C. Di Sano, A. Lombardo and A. M. Liberati, *Adv. Hematol.*, 2012, **2012**, 842945.
29 X. Li and Y. Song, *J. Hematol. Oncol.*, 2020, **13**, 50.
30 X. Sun, H. Gao, Y. Yang, M. He, Y. Wu, Y. Song, Y. Tong and Y. Rao, *Signal Transduction Targeted Ther.*, 2019, **4**, 64.
31 S. E. Sedykh, V. V. Prinz, V. N. Buneva and G. A. Nevinsky, *Drug Des., Dev. Ther.*, 2018, **12**, 195–208.
32 L. A. Rabia, A. A. Desai, H. S. Jhajj and P. M. Tessier, *Biochem. Eng. J.*, 2018, **137**, 365–374.
33 S. S. Sidhu and F. A. Fellouse, *Nat. Chem. Biol.*, 2006, **2**, 682–688.
34 A. F. Labrijn, M. L. Janmaat, J. M. Reichert and P. W. H. I. Parren, *Nat. Rev. Drug Discovery*, 2019, **18**, 585–608.
35 Z. Wu and N. V. Cheung, *Pharmacol. Ther.*, 2018, **182**, 161–175.
36 H. Shim, *Biomolecules*, 2020, **10**, 360.
37 A. Thakur, M. Huang and L. Lum, *Blood Rev.*, 2018, **32**, 339–347.
38 T. Dreier, G. Lorenczewski, C. Brandl, P. Hoffmann, U. Syring, F. Hanakam, P. Kufer, G. Riethmuller, R. Bargou and P. A. Baeuerle, *Int. J. Cancer*, 2002, **100**, 690–697.
39 A. K. Abbas, E. Trotta, D. R. Simeonov, A. Marson and J. A. Bluestone, *Sci. Immunol.*, 2018, **3**, eaat1482, DOI: **10.1126/sciimmunol.aat1482**.
40 H. P. Kim, J. Imbert and W. J. Leonard, *Cytokine Growth Factor Rev.*, 2006, **17**, 349–366.
41 J. J. O'Shea, M. Gadina and R. M. Siegel, *Clin. Immunol.*, 2019, **127**, 127–155.
42 J.-X. Lin and W. J. Leonard, *Annu. Rev. Immunol.*, 2019, **37**, 295–324.
43 J. Damoiseaux, *Clin. Immunol.*, 2020, **218**, 108515.
44 M. Piccioni, Z. Chen, A. Tsun and B. Li, *T Helper Cell Differentiation and Their Function*, 2014, vol. 67, p. 97.

45 K. Sladowska, P. Kawalec, P. Holko and O. Osiecka, *Neurol. Sci.*, 2022, **43**, 5479–5500.
46 J. Singh, N. Shah and A. Mudano, *Cochrane Database Syst. Rev.*, 2021, **2**, CD010668.
47 H. Harb and T. Chatila, *Clin. Exp. Allergy*, 2020, **50**, 5–14.
48 H. Blair, *Drugs*, 2021, **81**, 483–494.
49 H. Gram, *Pharmacol. Res.*, 2020, **154**, 104139.
50 K. Wijnsma, C. Duineveld, J. Wetzels and N. van de Kar, *Pediatr. Nephrol.*, 2019, **34**, 2261–2277.
51 K. Shahid and S. Qayyum, *Cureus*, 2023, **15**, e46185.
52 A. Cheloff and H. Al-Samkari, *Drugs Today*, 2020, **56**, 439–446.
53 D. Chen, S. Tan, H. Zhang, H. Wang, W. He, R. Shi, Z. Tong, J. Zhu, H. Cheng, S. Gao, Y. Chai, J. Qi, M. Xiao, J. Yan and G. F. Gao, *iScience*, 2019, **14**, 113–124.
54 W. Liu, H. Jin, T. Chen, G. Zhang, S. Lai and G. Liu, *Front. Mol. Biosci.*, 2020, **7**, 574759.
55 K. M. Zak, P. Grudnik, K. Magiera, A. Domling, G. Dubin and T. A. Holak, *Structure*, 2017, **25**, 1163–1174.
56 K. Harper, C. Balzano, E. Rouvier, M. G. Mattei, M. F. Luciani and P. Golstein, *J. Immunol.*, 1991, **147**, 1037–1044.
57 H. Gao, H. Cai, J. Liu, X. Wang, P. Zheng, M. Devenport, T. Xu, F. Dou, Y. Liu and A. Zhou, *Cell Discovery*, 2020, **6**, 79.
58 C. Yu, A. F. Sonnen, R. George, B. H. Dessailly, L. J. Stagg, E. J. Evans, C. A. Orengo, D. I. Stuart, J. E. Ladbury, S. Ikemizu, R. J. Gilbert and S. J. Davis, *J. Biol. Chem.*, 2011, **286**, 6685–6696.
59 B. Huard, R. Mastrangeli, P. Prigent, D. Bruniquel, S. Donini, N. El-Tayar, B. Maigret, M. Dreano and F. Triebel, *Proc. Natl. Acad. Sci. U. S. A.*, 1997, **94**, 5744–5749.
60 Q. Ming, D. Antfolk, D. A. Price, A. Manturova, E. Medina, S. Singh, C. Mason, T. H. Tran, K. S. M. Smalley, D. W. Leung and V. C. Luca, *Nat. Commun.*, 2024, **15**, 7513.
61 E. Baixeras, B. Huard, C. Miossec, S. Jitsukawa, M. Martin, T. Hercend, C. Auffray, F. Triebel and D. Piatier-Tonneau, *J. Exp. Med.*, 1992, **176**, 327–337.
62 S. Horita, Y. Nomura, Y. Sato, T. Shimamura, S. Iwata and N. Nomura, *Sci. Rep.*, 2016, **6**, 35297.
63 S. Tan, H. Zhang, Y. Chai, H. Song, Z. Tong, Q. Wang, J. Qi, G. Wong, X. Zhu, W. J. Liu, S. Gao, Z. Wang, Y. Shi, F. Yang, G. F. Gao and J. Yan, *Nat. Commun.*, 2017, **8**, 14369.
64 B. Roither, C. Oostenbrink, G. Pfeiler, H. Koelbl and W. Schreiner, *Cancers*, 2020, **13**, 35297.
65 K. M. Zak, P. Grudnik, K. Guzik, B. J. Zieba, B. Musielak, A. Domling, G. Dubin and T. A. Holak, *Oncotarget*, 2016, **7**, 30323–30335.
66 A. Ganesan, M. Ahmed, I. Okoye, E. Arutyunova, D. Babu, W. L. Turnbull, J. K. Kundu, J. Shields, K. C. Agopsowicz, L. Xu, Y. Tabana, N. Srivastava, G. Zhang, T. C. Moon, A. Belovodskiy, M. Hena, A. S. Kandadai, S. N. Hosseini, M. Hitt, J. Walker, M. Smylie, F. G. West, A. G. Siraki, M. J. Lemieux, S. Elahi, J. A. Nieman, D. L. Tyrrell, M. Houghton and K. Barakat, *Sci. Rep.*, 2019, **9**, 12392.
67 J. Y. Lee, H. T. Lee, W. Shin, J. Chae, J. Choi, S. H. Kim, H. Lim, T. W. Heo, K. Y. Park, Y. J. Lee, S. E. Ryu, J. Y. Son, J. U. Lee and Y. S. Heo, *Nat. Commun.*, 2016, **7**, 13354.
68 U. A. Ramagopal, W. Liu, S. C. Garrett-Thomson, J. B. Bonanno, Q. Yan, M. Srinivasan, S. C. Wong, A. Bell, S. Mankikar, V. S. Rangan, S. Deshpande, A. J. Korman and S. C. Almo, *Proc. Natl. Acad. Sci. U. S. A.*, 2017, **114**, E4223–E4232.
69 A. K. Mishra, S. Shahid, S. S. Karade, P. Agnihotri, A. Kolesnikov, S. S. Hasan and R. A. Mariuzza, *Structure*, 2023, **31**, 1149–1157.
70 A. Rajan, C. Kim, C. R. Heery, U. Guha and J. L. Gulley, *Hum. Vaccines Immunother.*, 2016, **12**, 2219–2231.
71 S. Tan, K. Liu, Y. Chai, C. W. Zhang, S. Gao, G. F. Gao and J. Qi, *Protein Cell*, 2018, **9**, 135–139.
72 F. C. Santini and C. M. Rudin, *Expert Rev. Clin. Pharmacol.*, 2017, **10**, 935–945.
73 J. M. Collins and J. L. Gulley, *Hum. Vaccines Immunother.*, 2019, **15**, 891–908.
74 H. T. Lee, S. H. Lee and Y. S. Heo, *Molecules*, 2019, **24**, 1190.
75 F. Zhang, X. Qi, X. Wang, D. Wei, J. Wu, L. Feng, H. Cai, Y. Wang, N. Zeng, T. Xu, A. Zhou and Y. Zheng, *Oncotarget*, 2017, **8**, 90215–90224.
76 E. J. Lipson and C. G. Drake, *Clin. Cancer Res.*, 2011, **17**, 6958–6962.
77 L. Calabro, A. Morra, E. Fonsatti, O. Cutaia, C. Fazio, D. Annesi, M. Lenoci, G. Amato, R. Danielli, M. Altomonte, D. Giannarelli, A. M. Di Giacomo and M. Maio, *Lancet Respir. Med.*, 2015, **3**, 301–309.
78 K. Mori, S. Iida, N. Yamane-Ohnuki, Y. Kanda, R. Kuni-Kamochi, R. Nakano, H. Imai-Nishiya, A. Okazaki, T. Shinkawa, A. Natsume, R. Niwa, K. Shitara and M. Satoh, *Cytotechnology*, 2007, **55**, 109–114.
79 H. H. Assi, C. Wong, K. A. Tipton, L. Mei, K. Wong, J. Razo, C. Chan, B. Howng, J. Sagert, M. Krimm, L. Diep, A. Jang, M. T. Nguyen, N. Lapuyade, V. Singson, R. Villanueva, M. Paidhungat, S. Liu, V. Rangan, O. Vasiljeva, J. W. West, J. H. Richardson, B. Irving, D. Daniel, M. Belvin and W. M. Kavanaugh, *Cancer Immunol. Res.*, 2021, **9**, 1451–1464.
80 A. Naing, F. Thistlethwaite, E. G. E. De Vries, F. Eskens, N. Uboha, P. A. Ott, P. LoRusso, J. Garcia-Corbacho, V. Boni, J. Bendell, K. A. Autio, M. Randhawa, G. Durm, M. Gil-Martin, M. Stroh, A. L. Hannah, H. T. Arkenau and A. Spira, *J. Immunother. Cancer*, 2021, **9**, 842945.
81 Y. Sun, X. Yu, X. Wang, K. Yuan, G. Wang, L. Hu, G. Zhang, W. Pei, L. Wang, C. Sun and P. Yang, *Acta Pharm. Sin. B*, 2023, **13**, 3583–3597.
82 K. M. Knudson, K. C. Hicks, X. Luo, J. Q. Chen, J. Schlom and S. R. Gameiro, *Oncoimmunology*, 2018, **7**, e1426519.
83 J. J. Luke, M. R. Patel, G. R. Blumenschein, E. Hamilton, B. Chmielowski, S. V. Ulahannan, R. M. Connolly, C. A. Santa-Maria, J. Wang, S. W. Bahadur, A. S. Weickhardt, A. S. Asch, G. Mallesara, P. Clingan, M. Dlugosz-Danecka, M. Tomaszewska-Kiecana, H. Pylypenko, N. Hamad, H. L. Kindler, B. J. Sumrow, P. Kaminker, F. Z. Chen, X. Zhang, K. Shah, D. H. Smith,

A. De Costa, J. Li, H. Li, J. Sun and P. A. Moore, *Nat. Med.*, 2023, **29**, 2814–2824.
84 Q. Yin, L. Wu, L. Han, X. Zheng, R. Tong, L. Li, L. Bai and Y. Bian, *Front. Immunol.*, 2023, **14**, 1167975.
85 M. E. Goebeler, G. Stuhler and R. Bargou, *Nat. Rev. Clin. Oncol.*, 2024, **21**, 539–560.
86 B. Hou, T. Chen, H. Zhang, J. Li, P. Wang and G. Shang, *Front. Immunol.*, 2023, **14**, 1123244.
87 N. Fuchs, L. Zhang, L. Calvo-Barreiro, K. Kuncewicz and M. Gabr, *J. Pers. Med.*, 2024, **14**, 68.
88 H. Yao, C. Li, F. He, T. Song, J. P. Brosseau, H. Wang, H. Lu, C. Fang, H. Shi, J. Lan, J. Y. Fang and J. Xu, *RSC Chem. Biol.*, 2021, **2**, 192–205.
89 F. Lai, M. Ji, L. Huang, Y. Wang, N. Xue, T. Du, K. Dong, X. Yao, J. Jin, Z. Feng and X. Chen, *Acta Pharm. Sin. B*, 2022, **12**, 2845–2858.
90 H. N. Chang, B. Y. Liu, Y. K. Qi, Y. Zhou, Y. P. Chen, K. M. Pan, W. W. Li, X. M. Zhou, W. W. Ma, C. Y. Fu, Y. M. Qi, L. Liu and Y. F. Gao, *Angew. Chem., Int. Ed.*, 2015, **54**, 11760–11764.
91 R. J. Boohaker, V. Sambandam, I. Segura, J. Miller, M. Suto and B. Xu, *Cancer Lett.*, 2018, **434**, 11–21.
92 H. Liu, Z. Zhao, L. Zhang, Y. Li, A. Jain, A. Barve, W. Jin, Y. Liu, J. Fetse and K. Cheng, *Cancer*, 2019, **7**, 270.
93 P. G. Sasikumar, R. K. Ramachandra, S. Adurthi, A. A. Dhudashiya, S. Vadlamani, K. Vemula, S. Vunnum, L. K. Satyam, D. S. Samiulla, K. Subbarao, R. Nair, R. Shrimali, N. Gowda and M. Ramachandra, *Mol. Cancer Ther.*, 2019, **18**, 1081–1091.
94 H. Wang, H. Yao, C. Li, H. Shi, J. Lan, Z. Li, Y. Zhang, L. Liang, J. Y. Fang and J. Xu, *Nat. Chem. Biol.*, 2019, **15**, 42–50.
95 S. V. Podlesnykh, K. E. Abramova, A. Gordeeva, A. I. Khlebnikov and A. I. Chapoval, *Molecules*, 2021, **26**, 1123.
96 R. Thakkar, D. Upreti, S. Ishiguro, M. Tamura and J. Comer, *RSC Med. Chem.*, 2023, **14**, 658–670.
97 T. M. R. Ramanayake Mudiyanselage, M. Michigami, Z. Ye, A. Uyeda, N. Inoue, K. Sugiura, I. Fujii and D. Fujiwara, *ACS Chem. Biol.*, 2020, **15**, 360–368.
98 F. Liu, L. Su, Z. Chen, D. Feng, J. Wei and J. Sun, *Biochem. Biophys. Res. Commun.*, 2019, **513**, 694–700.
99 Y. Qian, Y. Sun, P. Shi, X. Zhou, Q. Zhang, Q. Dong, S. Jin, L. Qiu, X. Niu, X. Zhou, W. Zhao, Y. Wu, W. Zhai and Y. Gao, *Acta Pharm. Sin. B*, 2024, **14**, 1150–1165.
100 W. Zhai, X. Zhou, H. Wang, W. Li, G. Chen, X. Sui, G. Li, Y. Qi and Y. Gao, *Acta Pharm. Sin. B*, 2020, **10**, 1047–1060.
101 P. Yuan, Y. Long, N. Wei, Y. Wang, Z. Zhu, J. Han, D. Jiang, X. Lan and Y. Gai, *J. Immunother. Cancer*, 2024, **12**, 328.
102 M. T. Gabr and S. S. Gambhir, *J. Am. Chem. Soc.*, 2020, **142**, 16194–16198.
103 S. A. Abdel-Rahman, K. Świderek and M. T. Gabr, *RSC Med. Chem.*, 2023, **14**, 1767–1777.
104 S. A. Abdel-Rahman, A. U. Rehman and M. T. Gabr, *ACS Med. Chem. Lett.*, 2023, **14**, 629–635.
105 S. A. Abdel-Rahman, L. Zhang and M. T. Gabr, *SLAS Discovery*, 2023, **28**, 188–192.
106 S. A. Abdel-Rahman, V. Talagayev, S. Pach, G. Wolber and M. T. Gabr, *J. Med. Chem.*, 2023, **66**, 11464–11475.
107 L. Calvo-Barreiro, V. Talagayev, S. Pach, S. A. Abdel-Rahman, G. Wolber and M. T. Gabr, *ChemMedChem*, 2023, **18**, e202300305.
108 N. Fuchs, L. Calvo-Barreiro, V. Talagayev, S. Pach, G. Wolber and M. T. Gabr, *ACS Med. Chem. Lett.*, 2024, **15**, 1884–1890.
109 L. Zhang, L. Calvo-Barreiro, V. de Sousa Batista, K. Świderek and M. T. Gabr, *ChemMedChem*, 2024, **19**, e202400545.
110 S. A. Abdel-Rahman and M. T. Gabr, *Sci. Adv.*, 2024, **10**, eadq5540.
111 J. R. Diamond, S. G. Eckhardt, T. M. Pitts, A. van Bokhoven, D. Aisner, D. L. Gustafson, A. Capasso, S. Sams, P. Kabos, K. Zolman, T. Colvin, A. D. Elias, A. M. Storniolo, B. P. Schneider, D. Gao, J. J. Tentler, V. F. Borges and K. D. Miller, *Breast Cancer Res.*, 2018, **20**, 82.
112 H. Wang, S. Li, Q. Wang, Z. Jin, W. Shao, Y. Gao, L. Li, K. Lin, L. Zhu, H. Wang, X. Liao and D. Wang, *Sci. Adv.*, 2021, **7**, eabc4074.
113 M. Reck, T. S. K. Mok, M. Nishio, R. M. Jotte, F. Cappuzzo, F. Orlandi, D. Stroyakovskiy, N. Nogami, D. Rodriguez-Abreu, D. Moro-Sibilot, C. A. Thomas, F. Barlesi, G. Finley, A. Lee, S. Coleman, Y. Deng, M. Kowanetz, G. Shankar, W. Lin and M. A. Socinski, *Lancet Respir. Med.*, 2019, **7**, 387–401.
114 M. A. Socinski, M. Nishio, R. M. Jotte, F. Cappuzzo, F. Orlandi, D. Stroyakovskiy, N. Nogami, D. Rodriguez-Abreu, D. Moro-Sibilot, C. A. Thomas, F. Barlesi, G. Finley, S. Kong, A. Lee, S. Coleman, W. Zou, M. McCleland, G. Shankar and M. Reck, *J. Thorac. Oncol.*, 2021, **16**, 1909–1924.
115 O. Kholod, W. Basket, D. Liu, J. Mitchem, J. Kaifi, L. Dooley and C. R. Shyu, *Cancers*, 2022, **14**, 1542.