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Research Paper

New approach methodologies to assess wanted and unwanted drugs-induced immunostimulation☆

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A B S T R A C T

This review examines various classes of drugs, focusing on their therapeutic and adverse effects, particularly in relation to immunostimulation. We emphasize the potential of new approach methodologies (NAMs) to study both expected and unexpected immunostimulatory effects. By evaluating the modes of action of different immunostimulatory drugs, we aim to provide insights into effectively assessing unwanted immunostimulatory responses. The review begins by exploring drugs that stimulate the immune system—including immunostimulants, monoclonal antibodies, chemotherapeutics, and nucleic acid-based drugs—to outline NAMs that could be employed to evaluate immunostimulation.

1. Introduction

Xenobiotics, including drugs, can stimulate the immune system by acting on different targets and pathways. The immunostimulatory drugs aim to stimulate immune cell subtypes and block or activate the endogenous regulation of the immune response. When not controlled, immunostimulation may lead to immune-mediated pathologies (e.g., allergies or autoimmune diseases). Humans are continuously exposed to a range of immunotoxic substances, a part of them are pharmaceuticals, and their immunotoxicity may be associated with high morbidity and mortality (Volger, 2014).

The use of immunostimulatory drugs is suggested to treat several

Abbreviations: ADCC, Antibody-Dependent Cell-Mediated Cytotoxicity; AMO, Anti-miRNA Oligonucleotide; APC, Antigen-Presenting Cell; ASO, Antisense Oligonucleotides; CAR-T, Chimeric Antigen Receptor T; CAR, Chimeric Antigen Receptor; CCL, Chemokine Ligand; CCR, C-C Chemokine Receptor; CLR, C-type Lectin-like Receptor; COX2, Cyclooxygenase-2; CRS, Cytokine Release Syndrome; CTLA-4, T Cell Lymphocyte Costimulation Inhibitor; CTL, Cytotoxic T Lymphocyte; DC, Dendritic Cell; EBV, Epstein-Barr virus; ELISA, Enzyme-Linked Immunosorbent Assay; ELISpot, Enzyme-Linked Immunosorbent Spot; EMA, European Medicine Agency; FDA, US Food and Drugs Administration; G-SCF, Granulocyte Colony Stimulating Factor; GM-CSF, Granulocyte Monocyte Colony-Stimulating Factor; HER2, Human Epidermal Growth Factor Receptor 2; HLA-C, Human Leukocyte Antigen C; ICANS, Immune Effector Cell-Associated Neurotoxicity Syndrome; IDO, Indoleamine 2,3-dioxygenase; IFN, Interferon; Ig, Immunoglobulin; IL, Interleukin; iNOS, induced-Nitric Oxide Synthase; iPC-CM, induced Pluripotent Stem Cell-Derived Cardiomyocyte; iPSC, induced-Pluripotent Stem Cell; irAE, immune-related Adverse Events; KIR, Killer Immunoglobulin-like Receptors; LPS, lipopolysaccharides; LNA, Locked Nucleic Acid; mAb, monoclonal Antibodies; MC, Mast Cell; MDA5, Melanoma Differentiation-Associated Protein 5; mDC, myeloid Dendritic Cell; MHC, Major Histocompatibility Complex; MIP, Macrophage Inflammatory Proteins; miRNA, microRNA; MoA, Mode of Action; moDC, monocyte derived Dendritic Cells; mRNA, messenger RNA; NA, Nucleic Acids; NAM, New Approach Methodology; NF-κB, Nuclear Factor-kappa B; NK, Natural Killer; NLR, Nucleotide Oligomerization Domain Like Receptors; NOD, Nucleotide Oligomerization Domain; PAMP, Pathogen Associated Molecular Patterns; PBMC, Peripheral Blood Mononuclear Cells; PD-1, Programmed Death Protein-1; PD-L1, Programmed Death Ligand 1; PD-L2, Programmed Death Ligand 2; pDC, plasmacytoid Dendritic Cell; PKR, Protein Kinase; PPR, Pathogen Recognition Receptor; PRP, Polyribosylribitol Phosphate; Pt, Platinum; RIG-1, Retinoic Acid-Inducible Gene-1; RISC, RNA-Induced Silencing Complex; RLR, Retinoic Acid-Inducible Gene-1 Like Receptor; siRNA, small interfering RNA; SRID, Single Radial Immunodiffusion Assay; TAA, Tumour-Associated Antigen; TCR, T Cell Receptor; T_{eff}, T effector; T_h, T helper; TLR, Toll-Like Receptor; TME, Tumour Microenvironment; TNF, Tumour Necrosis Factor; T_{reg}, T regulatory; TSA, Tumour-Specific Antigen; WBA, Whole Blood Assay.

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pathologies, but it is not free of adverse effects. Therefore, the understanding of the Mode of Action (MoA) of the pharmaceuticals is of crucial importance to allow a proper use of these drugs avoiding as much as possible adverse effects. Therefore, a proper assessment of their safety must be performed with the use of specific assays. In the field of immunotoxicology, alternative methodologies or New Approach Methodologies (NAMs) are rapidly emerging, and their application to study drug-induced immunostimulation are warranted (Maddalon et al., 2023).

This review aimed to collect and analyse the MoA of different immunostimulatory drugs to define a strategy to study inappropriate immunostimulation induced by drugs using NAMs.

2. Immunostimulants

Immunostimulants are substances (drugs and/or nutrients) that stimulate the immune system by inducing activation or increasing activity of any of its components as a therapeutic effect. They can be classified as specific and non-specific. The **specific immunostimulants** stimulate an immune response to specific antigenic types, as vaccines (Spencer et al., 2017). The **non-specific immunostimulants** act irrespective of antigenic specificity to augment immune response of other antigens or stimulate components of the immune system without antigenic specificity, such as adjuvants and some plant extracts (Shahbazi and Bolhassani, 2017).

2.1. Specific immunostimulants: Vaccines

Immunisation is a process by which immunity to a disease is conferred. The protection from an agent may be passive, if granted by providing antibodies through injection or by passage from mother to child through the placenta; or active, if induced as a result of exposure to the pathogen (natural) or to parts of it capable of stimulating the immune system (Baxter, 2007, and references therein).

Innate immunity has an important role in protecting the body: natural barriers such as the skin and mucous membranes block pathogen entry, but if they are damaged, macrophages and enzymes act immediately, trying to kill the intruder. The presence of adaptive immunity is crucial, as the response of innate immunity sometimes does not allow the host to respond adequately to eliminate the pathogen (Iwasaki and Medzhitov, 2010; Kaur and Secord, 2021; Li et al., 2020; Zhang et al., 2022). **Vaccines** were introduced into medicine in the 18th century as biological tools, used to stimulate the immune response against diseases and prevent their onset by inducing the development of immune memory. The latter allows the body to respond massively and more rapidly to the next exposure, reducing the likelihood of infection. Vaccines have provided important innovation in the field of medicine since they are capable of reducing the second leading cause of death worldwide: infections from pathogens such as malaria, tuberculosis and respiratory infections from pneumonia (Jain et al., 2021).

There are different types of vaccines, and they are divided into different groups, according to their method of production: live attenuated, killed whole organisms, toxoids, subunits, nucleic acid-base vaccines and virus-like particles (Pollard and Bijker, 2021). All the types of vaccines, with the exception of mRNA vaccines that code for a specific antigen, allow great coverage against the pathogen because they let the immune system recognize more antigens and produce more different types of specific antibodies against a specific pathogen (Jain et al., 2021).

Following intravenous or intramuscular administration, the live-attenuated or inactivated pathogen is phagocytosed by the dendritic cells (DCs) and digested through the phagolysosome: several protein fragments (antigens) are obtained, which will be presented on the cell membrane of DCs, via major histocompatibility complex (MHC). The DCs migrate to the lymph nodes, where they will present the antigen to T helper (T_h) lymphocytes. The protein sequence will be recognized through the T cell receptor and stimulate their proliferation. At the same time, the free antigens are recognized by the B cell receptor and internalised, to form the MHC II-antigen complex, which in turn can be recognized and bound by the T_h lymphocyte receptors: this step is very important because it allows a robust immune response. The production by T_h2 of interleukin (IL)-2, IL-4, IL-5, and IL-6 results in the activation, differentiation, and maturation of B lymphocytes into plasma cells, which will first produce immunoglobulin (Ig) type M and then IgG. Memory B lymphocytes will be able to recognize the antigen more quickly at the next infection and to mature and produce antibodies faster than an initial likely infection. Long-lived plasma cells, which produce antibodies for decades, migrate to niches in the bone marrow. When the antigen is presented through MHC I activation of cytotoxic T lymphocytes will produce cytokines and induce apoptosis. Also, some of the cytotoxic T lymphocytes are capable of becoming memory cells. MoA of subunit vaccine is the same, unless the subunit vaccine is polysaccharide-based. The latter can induce B lymphocyte activation, differentiation, and maturation without the help of T_h lymphocytes, therefore, giving a less intense immune response (Abbas et al., 2000; Baxter, 2007; Mak and Saunders, 2006; Pollard and Bijker, 2021).

Vaccines based on nucleic acid inoculation differ from other vaccines as the antigen of interest is produced *in vivo*, directly in the cells of our body. DNA vaccines involve the injection of plasmids, coding for the antigen of interest, directly into the host. After entering the host cells, the plasmids are transcribed in the nucleus into messenger mRNA, which is then translated into the antigen of interest. The type of expressed antigens and the use of signal sequences influence the fate of the antigen, which can be excreted from the cell or presented through the major histocompatibility complex (Pagliari et al., 2023; Tregoning and Kinnear, 2014). mRNA vaccines are a technology consisting of a messenger RNA molecule embedded in a lipid envelope. Together they form a lipid nanoparticle, which can cross the cell membrane and release mRNA, which will be translated into the protein of interest, the antigen (Jain et al., 2021). This class of drugs will be discussed further in the review.

A new therapeutic approach to cancer is ***cancer vaccination***, a tool to activate the immune system to recognize and eliminate cancer cells. The targets of cancer vaccines are tumour-associated antigens (TAAs), expressed by cancer cells. Normally, healthy cells express TAAs but to a lesser extent than tumour cells. However, using them as targets can lead to severe adverse effects. To minimise that, target tumour-specific antigens (TSAs), normally absent in healthy cells, are used. The tumour microenvironment (TME) has a complex composition which can influence the efficacy of the cancer vaccines. Knowing the intricacy of this environment is the best way to develop new more efficient vaccines. Also in cancer vaccines, the use of adjuvants is quite important: Toll-like receptors (TLR) agonists, cytokines, and immune checkpoint inhibitors are used to improve the activity (Kaczmarek et al., 2023).

The MoA depends on the kind of vaccine. The use of DCs loaded with TSAs derived from tumour cells or genetic material is one of the most common types. DCs are administered and migrate to lymphoid organs where they present the tumour antigens to T cells, both cytotoxic and helper, which are activated to identify and suppress tumour cells. Cancer vaccines can be based also on whole-cell preparations, where cancer cells are obtained from the tumour's patient and then modified to make them weaker in growth and strength. As they present TSAs, they trigger immune cells and mediate an immediate nonspecific inflammatory response. When T_h and T_c cells recognize the antigens presented by DCs, or other antigen-presenting cells (APCs) like macrophages and Natural Killer (NK) cells, the adaptive immune response is activated. Induced-pluripotent stem cells (iPSC), virus- or bacteria-based technologies, DNA, RNA, peptides, and exosomes can also be used to generate new cancer vaccines. Their MoA are similar to the ones described before except for the iPSC-based vaccines. iPSC can be differentiated into ME-specific cells: on the surface they can express antigens normally found on TME cells, as endothelial or immune cells. Antigens presented by iPSC

are then processed by APCs and presented to T cells, to activate the adaptive immune response and generate memory. The efficacy of cancer vaccines depends also on the delivery methods: nowadays there are several administration procedures such as intramuscular, subcutaneous, intratumoral injections, and intravenous infusion. A non-invasive method is oral administration, observed for oral polio vaccine, used in the treatment of glioblastoma. Other expedients to deliver vaccines are gene- and nanoparticle-based methods and topical application (as with the T-VEC vaccine used against melanoma lesions) (Kaczmarek et al., 2023).

2.1.1. Safety and side effects of vaccines

Vaccines, as pharmaceutical preparations, need to be tested to assess their safety and efficacy. Unlike drugs, they are administered to healthy people and therefore the risk–benefit assessment is done very strictly.

Because the safety and efficacy of vaccines depend on the immune system's ability to mount an appropriate response, the risk–benefit evaluation for immunosuppressed patients must be approached differently than for healthy individuals but not all initial vaccine trials include testing in immunosuppressed populations. For instance, many studies assessing new vaccines, monoclonal antibodies (mAbs), and antiviral drugs for SARS-CoV-2 have excluded these patients. This is because immunocompromised individuals often exhibit lower vaccine response rates, are at a higher risk of severe disease complications, and tend to have a greater baseline rate of adverse events. Although guidelines have been established for the use of the new mRNA SARS-CoV-2 vaccines, these measures are still limited and could be further enhanced by NAMs. Clinical trials for therapeutic cancer vaccines are conducted on patients who already have cancer, whereas preventive trials involve individuals who do not currently have cancer but are at high risk of developing it, such as those with a previous history of cancer. NAMs can be valuable in evaluating products such as cancer vaccines that rely on human-specific antigens, particularly when predictive models are not available: *in vitro* experiments utilizing human cells may effectively demonstrate initial validation of a concept. Additionally, NAMs can be used to justify the planned starting dose and schedule in phase I studies (Boeckh et al., 2024; Eibl and Wolf, 2015; EMA guidelineEMA/CHMP205/95Rev.6, 2020; Galmiche et al., 2022; Martire et al., 2018; NIH, 2024; Picard et al., 2015; See, 2022).

Their MoA is complex and establishing a predictive assessment of their safety is challenging. Vaccines are generally considered as safe but can induce several adverse effects (Descotes et al., 2002). The most common adverse effects are systemic reactions such as fever, drowsiness, nausea, and irritability, and local reactions, which include pain, redness, and swelling, at the injection site (Spencer et al., 2017). Non-immediate skin rashes are also among the most reported adverse effects, the symptoms of which include itching, redness, swelling (Caubet and Ponvert, 2014). These are common reactions, lasting about 72 h, and develop 24–48 h following injection of an inactivated vaccine, or after 14–21 days following injection of a live-attenuated vaccine. Age, sex, genetics, past infections, and administration of previous doses of the vaccine may influence the development of inflammatory reactions. Inactivated vaccines having adjuvants in their composition often induce local reactions: if painful, they may be Arthus reactions or reactions involving antigen recognition by TLR expressed by macrophages or DCs. In particular, the Arthus reaction develops during the second sensitization for the same antigen, where antibodies already present form complexes with it, at the site of injection (Siegrist, 2007).

Among the most serious adverse effects, there is allergy development. The cause of allergic reactions is rarely the vaccine antigen itself; instead, additional components of the preparation, such as gelatine, are more likely to trigger allergic reactions (Caubet and Ponvert, 2014; Siegrist, 2007). Fortunately, the majority of vaccine-induced allergic reactions are not severe. Serious skin reactions and anaphylactic shock can also occur, although much more rarely. Type I hypersensitivity reactions arise abruptly and are characterised by the rapid and elevated development of IgE, against the component of interest. Symptoms of acute onset of IgE-mediated hypersensitivity may be mild such as itching, or more severe such as angioedema and anaphylaxis. The latter is very dangerous: it involves several organs and is characterised by the development of symptoms such as urticaria, wheezing, swelling of the mouth, tongue, and throat, difficulty breathing, vomiting, diarrhoea, hypotension, decreased level of consciousness, and shock. Allergic reactions that develop later are usually less severe and their MoA consists of complement activation, formation of immune complexes (type 3 hypersensitivity or an Arthus reaction), T cell-mediated processes, or late activation of the IgE system. They arise within hours to days after exposure and the most common symptom is a rash (McNeil and DeStefano, 2018).

Autoimmune side effects can also occur after vaccination and in the worst cases they can induce the generation of autoimmune disease. Genetic characteristics and environmental aspects that interact with each other influence autoimmunity. One important factor that must be considered is infections (Bach, 2002; McDougal, 2006; Wraith et al., 2003). There are two mechanisms by which an infection can trigger an autoimmune disease: antigen-specific or non-specific. Only predisposed individuals are susceptible to the development of these diseases. Molecular mimicry is the most tempting assumption about the mechanism by which autoimmunity can manifest (Wucherpfennig, 2001). Since vaccinations involve exposure to an antigen, similar reasoning can be applied to them (Tishler and Shoenfeld, 2004). Autoimmune disorders that might be stimulated by some vaccines include the development of DNA antibodies, local disorders such as reactive arthritis, and systemic diseases which include transient or life-long disease (Cohen and Shoenfeld, 1996). As with other vaccines, the ones against cancer could have side effects. These types of preparations also have the potential to induce tumour pseudo-progression (Platten et al., 2021). Therefore, it is of crucial importance to monitor immune-related reactions, to have more information about the safety and efficacy of cancer vaccines.

2.1.2. From animals to in vitro models

A vaccine can be considered effective when it induces a consistent immune stimulation, which allows protection against the disease. Before the release of any new vaccine batch, tests that can confirm their immunogenic potential must be performed. Currently, the assays handled to evaluate the efficacy of vaccines include the use of animals: even though they are specific and sensitive, they have disadvantages, such as low precision, low reproducibility, and high variability. Although these kinds of models are representative of the human immune response, they involve a large number of animals together with important animal distress. Therefore, the development of new approaches that can reduce, refine, and replace (3R) the use of animals is also promoted by various regulatory agencies (Hoefnagel et al., 2011; McFarland et al., 2011).

The Food and Drug Administration (FDA) regulates human vaccines in the United States (U.S.), with oversight requiring that biologics meet standards of purity, safety, and potency as outlined in Section 351 of the Public Health Service Act. These requirements are typically met through testing of the final bulk sample using *in vivo*, *in vitro*, or both types of tests. However, with the exception of the general safety test, the regulations do not mandate specific tests for potency and safety. This flexibility allows manufacturers to propose alternative tests that may reduce, refine, or replace animal testing. When manufacturers propose using an alternative test, they must demonstrate its relevance, provide supporting data, and ensure it is adequately validated for its intended purpose. The goal is to ensure the approval and continued availability of vaccines that are safe, pure, and effective. If a manufacturer wishes to use an alternative safety or potency test not specified in the original license, they must submit a supplement to the license, along with data showing that the modification does not negatively impact the product's safety or efficacy. Requirements for reporting these changes to the FDA are detailed in § 601.12, which covers licensed biological products, including

vaccines (FDA, 2017; Finn, 2011; McFarland et al., 2011).

Regarding NAMs, they consist of the study of vaccine activity through *in vitro* studies and new technologies that do not involve the use of animals. *In vitro* tests become necessary to get a better resolution in assessing the biological activity of the vaccine. Certain vaccines are high-priority candidates for the development of *in vitro* models for potency studies due to their reliance on large numbers of animals or the significant pain and distress involved (Hoefnagel et al., 2011). However, several other criteria must also be considered to identify these vaccines. Examples include diphtheria and tetanus toxoid, whole-cell and acellular pertussis, rabies, anthrax, complex combination vaccines, and inactivated polio vaccines (McFarland et al., 2011). Development of an *in vitro* assay should require the use of primary human cells or immortalised cell lines, but these cannot provide a complete evaluation of what occurs *in vivo*, after vaccine inoculation (Hoefnagel et al., 2011).

Some of the most used *in vitro* assays to test vaccine safety and potency are reported in Table 1**.**

Safety, immunogenicity, and protective efficacy must be demonstrated in subjects before obtaining regulatory licensure for new vaccines. New vaccines go through two main trials: immunogenicity and vaccine field trials, which also include safety assessment (M. Liu et al., 2021). To test the immunogenicity of a new vaccine, alternative *in vitro* methods suggest the use of DC models to evaluate cell maturation after exposure to the novel vaccine (Vandebriel and Hoefnagel, 2012). Next, other key immune functions should be evaluated as the ability to stimulate T cells, the induction of cytotoxic T cell responses, and the measurement of cytokine release. If a vaccine was already tested for immunogenicity, understanding its efficacy towards the target *in vitro* can be done with new methods that include the use of culture-derived human B cells, obtained from Peripheral Blood Mononuclear Cells (PBMCs) isolation from blood samples collected after vaccination, as described by Su et al. (2016).The researchers successfully activated and expanded antigen-specific memory B cells. Additionally, the T cell receptor (TCR) repertoire of rare antigen-specific $CD4^+$ T cells, which proliferated in response to tetanus toxoid presented by autologous CD B cells, was also characterized (Su et al., 2016).

To eliminate the need for preclinical safety testing in animals and rely solely on non-animal methods, *in vitro* safety testing can be conducted using PBMCs from human blood donors treated directly with the vaccine under investigation. In a recent study, PBMCs were used to initiate the evaluation of specific RNA drugs, including an RNA vaccine, by analyzing the toxicity pathways activated by various chemicals. After identifying these pathways with appropriate positive controls, the RNA vaccine can then be assessed for safety (Bettinsoli et al., 2024). Some of the *in vitro* studies reported methods using human DCs to test the immunogenicity of vaccines. The activation of naïve T cells by DCs indicates how much the latter are important and why they were used to develop some *in vitro* assays to study the efficacy of vaccines (Vandebriel and Hoefnagel, 2012). DCs derive from their myeloid precursors, which move from the blood to the peripheral tissues. DCs mature when they encounter a danger signal, and after exposure to an antigen, maturation includes the upregulation of MHC and costimulatory molecules. After that, DCs migrate to the lymph node and interact with T and B cells (van Helden et al., 2008). There are different types of DCs: myeloid and plasmacytoid DCs. The first, when they are immature, have a high phagocytic activity; on the other hand, when they are mature, they tend to have an elevated cytokine-producing capacity. Plasmacytoid ones produce type I interferons after exposure to viruses. Both types have an antigen-presenting activity.

Myeloid DCs can be obtained from human peripheral blood, but there are problems in terms of isolation efficacy and life span. *In vitro* differentiation of bone marrow stem cells or PBMCs or monocytes can be another way to obtain larger number of cells. Other way to replicate DCs biological aspects is using different human cell lines such as MUTZ-3, HL60, and THP-1. As mouse models murine cell lines such as Raw264.7, J774 and D1, can be used (Bagwe et al., 2022; van Helden et al., 2008; Vandebriel and Hoefnagel, 2012). Both cellular models must undergo differentiation processes to more accurately resemble dendritic cells (DCs) and mimic their biological characteristics.

Murine macrophage cell lines, such as Raw264.7 and J774, share notable similarities with DCs in their responses to pathogen exposure. These cell models can therefore be used to simulate DC responses when stimulated with lipopolysaccharides (LPS), IL-4, or granulocyte–macrophage colony-stimulating factor (GM-CSF). The D1 cell

Table 1
Alternative assays used to test vaccines potency.

Type of vaccine	Methods	Experimental model	Efficacy of the method	Aim of the test	Reference
Recombinant *Hepatitis B* vaccines	An improved Hepatitis B virus surface antigen assay on the fully automated Abbott ARCHITECT® platform was developed to improve sensitivity, mutant and genotype detection. Enzyme-linked immunosorbent assay (ELISA) to quantify the content of recombinant hepatitis B surface antigen which comprises the vaccine and induces the immune response.	Human serum and plasma	*In vitro* assay is superior to *in vivo* assay in sensitively detecting changes in sample potency.	Quantify the content of recombinant Hepatitis B antigen (after testing in animals)	Lou et al., 2018
Inactivated *Hepatitis A* vaccine	Quantification of the Hepatitis A antigen content carried out with an indirect ELISA immunocapture method developed and validated.	Hepatitis A references or vaccines	*In vitro* assay could detect changes in vaccine potency that the *in vivo* assay failed to detect.	Immunogenicity testing (after testing in animals)	Poirier et al., 2010; Asgary et al., 2017
Inactivated *Rabies* vaccine	Immunochemical methods that can quantitatively determine the antigen content in vaccines, including antibody binding assay, single radial immunodiffusion (SRID) assay, and ELISA, have been established.	Serum	SRID assay is inferior to ELISA in terms of sensitivity.	Immunogenicity testing (after testing in animals)	Ferguson and Schild, 1982; Jallet and Tordo, 2020
Inactivated *N. gonorrhoeae* vaccine	Murine macrophages and DCs were used for an *in vitro* assay for evaluation of autophagy, nitric oxide release, cytotoxicity, uptake of the microparticles of the vaccine, and expression of MHCI, MHCII, CD40, and CD86 on the surface of these cells.	Murine macrophages and DCs	Every test performed succeeded in the evaluation of immunostimulatory responses.	Evaluation of different formulations of the same vaccine after already tested vaccines in animal (in place to animal test)	Bagwe et al., 2022
Recombinant HIV vaccine (Ad5-HIV gag)	Use of ELISPOT assay to quantify the IFN-γ secreted by lymphocytes in HIV-infected donors.	Lymphocytes	Compared to *in vivo* methods, it's safer, more efficient, and intuitive.	To confirm efficacy before clinical trial (after testing in animals, in pace to animal test)	He et al., 2017

line is also capable of mimicking immature DCs (iDCs) and, like Raw264.7, expresses the DC-specific marker CD11c following stimulation. Compared to J774, Raw264.7, and D1 are superior models, with D1 cells most closely resembling primary DCs. Additionally, both Raw264.7 and D1 cells can process and present antigens to T cells, with D1 showing greater efficacy. When choosing the ideal DC model, adhesion and migration properties are crucial considerations. Immature DCs are highly adherent due to podosome formation, and both Raw264.7 and J774 cells exhibit strong adhesion, which increases upon exposure to IL-4/GM-CSF and LPS. In contrast, D1 cells are more akin to primary DCs as they acquire a highly migratory phenotype in response to stimulation (van Helden et al., 2008, and references therein).

Human cell lines that mimic DC characteristics include THP-1, MUTZ-3, and HL-60. These lines exhibit a DC-like phenotype when stimulated with cytokines, LPS, and/or GM-CSF. The receptor expression profile (e.g., MHCI, MHCII, CD80, CD86, CD40) of stimulated THP-1 cells more closely aligns with that of DCs compared to HL-60 cells. However, MUTZ-3 cells have a receptor profile that most closely matches primary DCs and better emulates normal DC differentiation and maturation, despite being more challenging to culture. While THP-1 and HL-60 cells can uptake and present antigens and secrete IL-6 and IL-8 for T cell recruitment, their ability is less pronounced compared to immature and mature MUTZ-DCs, which produce IL-12 and effectively stimulate T cells. Additionally, MUTZ-DCs can induce strong cytotoxic T lymphocyte (CTL) responses. Adhesion properties are better studied using THP-1 and HL-60 cells due to the poor adhesion of MUTZ-DCs, while MUTZ-3 cells are preferable for studying migration, as they more accurately reflect high-speed migratory behavior. Ultimately, MUTZ-3 cells require more intensive culturing efforts and costlier cytokines, whereas THP-1 and HL-60 cells are easier to culture and less resource-intensive. The choice of cell line should be guided by the specific endpoint being evaluated (van Helden et al., 2008, and references therein).

MUTZ-3 cell line was used to evaluate the differences between the *Hemophilus influenzae* type b antigen polyribosylribitol phosphate (PRP), the outer membrane protein of *Neisseria meningitides* (OMP) and a vaccine composed by a conjugate between PRP and OMP (Vandebriel and Hoefnagel, 2012).

Also, some studies demonstrated the efficacy of YF-17D, a yellow fever vaccine, by the evaluation of CD80 and CD86 expression and the secretion of different cytokines, such as IL-6, TNF-α, MCP-1 (CCL2), IP-10 (CXCL10) and IL-12p40, on moDCs (Querec et al., 2006; Vandebriel and Hoefnagel, 2012).

A comparative efficacy study was conducted to evaluate the immunostimulatory potential of different vaccine formulations against *Neisseria gonorrhoeae*. The research team decided to assess the immunogenic and immunostimulatory activity of these formulations through *in vitro* tests. These vaccines utilized microparticle technology to deliver the inactivated whole cell of *N. gonorrhoeae* and differed in the adjuvants used to enhance their activity. The efficacy of the vaccine without adjuvants had already been demonstrated through *in vivo* testing, where it was shown to induce the generation of antigen-specific CD4 and CD8 T lymphocytes. The study has been conducted using murine cell lines such as Raw264.7 (macrophages) and DC2.4 (DCs). The evaluation of autophagy induction and nitric oxide release was conducted on Raw264.7 murine macrophages cell line: although the cells used derived from animals, no live animal testing is involved, making this another step toward NAMs. This is true also for the use of murine DCs (DC 2.4 murine cell line), required in the evaluation of cytotoxicity, uptake of the microparticles of the vaccine, and expression of MHCI, MHCII, CD40, and CD86 on the surface of these cells. Compared to untreated cells and cells treated only with Gonococcal vaccine microparticles (Gc-MP, without adjuvants), adjuvated, inactivated gonococcal microparticle vaccine promotes more autophagy, and increased immunostimulation in macrophages, and higher expression of MHCI, MHCII and co-stimulatory molecules in DCs. Autophagy induction in APCs was evaluated since it is important for enhancing antigen presentation. The maximization of efficiency and specificity in the adaptive immune response can be ensured by the process of antigen presentation by APCs, which leads to the stimulation of helper T lymphocytes, thereby triggering the activation of the immune response. Current experiments are being conducted *in vivo* with a mouse model to assess the immunogenicity of this gonococcal vaccine formulation containing an adjuvant (Bagwe et al., 2022). This suggest that the above mentioned *in vitro* model can be used to predict vaccine immunostimulatory potential without the use of animals.

The evaluation of the efficacy of Ad5-HIV gag (a vaccine against HIV) was done before a clinical trial, using an *in vitro* method based on the use of PBMCs, derived from HIV-infected individuals and a healthy donor. The evaluation of the immunogenicity of the vaccine was conducted through an enzyme-linked immunosorbent spot (ELISpot) assay measuring the level of activated Interferon (IFN)-γ production lymphocytes in PBMCs of HIV-infected donors (He et al., 2017). This method allows the measuring of antigen-specific T cells and it is commonly used in human clinical trials of vaccines (Slota et al., 2011).

What was observed is that Ad5-HIVgag vaccine can induce a greater response of the immune system, measured by the level of IFN-γ-secreting lymphocytes, than the Ad5-null control. This method, compared with the ones that use animals, is safer, more efficient, and intuitive, and could be used to support the existing evaluation methods (He et al., 2017).

2.2. Non-specific immunostimulants: Therapy and adverse effects

Non-specific immunostimulants do not have antigenic specificity and represent a wide class of drugs with promising use in the treatment of chronic infections, immunodeficiency, autoimmunity, and neoplastic diseases (Shahbazi and Bolhassani, 2017).

As reported in the first chapter of this review, the main objectives of vaccine-induced immunostimulation are to provide a immunity against the target antigen and lasting memory for protection against pathogens. To increase antigen visibility and antibody production, adjuvants are often added in the vaccine formulation (Young, 2019). Adjuvants enhance the immunogenicity of the vaccine, allowing for a reduction in the amount of antigen required in the vaccine dose. The different adjuvants induce a characteristic immunological differentiation program that may rely on T_h type 1, T_h2, or T_h17 cells, independently of the antigen (Caucheteux and Piguet, 2016). The molecular mechanisms underlying the immune boosting are not fully understood and adjuvants like alum, MF59, and AS04 (adjuvant combination of monophosphoryl lipid A and alum) can boost the immune response through more than one mechanism.

One MoA of adjuvants is the formation of a depot at the injection site, ensuring a slow release of the trapped and sustained immune system stimulation for antibody production (Awate et al., 2013; Young, 2019). Various adjuvants, such as water-in-oil emulsions and biodegradable micro- and nanoparticles, have been demonstrated to act with this mechanism to generate prolonged and sustained high antibody titres (Awate et al., 2013). Another mechanism by which adjuvants can elicit their function is the generation of a local proinflammatory environment to recruit immune cells at the site of injection, where genes encoding cytokines, chemokines, innate immune receptors, interferon-induced genes, and gene encoding adhesion molecules defined as "adjuvant core response genes" were found upregulated. A similar pattern of gene regulation was also found in the lymph nodes (Awate et al., 2013). The recruitment of immune cells at the site of injection generates an environment rich in neutrophils, monocytes, eosinophils, macrophages, DCs, and NK cells. The generation of the inflammatory state at the site of injection is the trigger signal for the non-specific activation of the immune system, and the following activation of the adaptive immunity. Neutrophils are the first type of cells arriving, and they attract the other immune cells by secreting chemokines. Different types of Pathogen

Recognition Receptors (PRRs) expressed in APCs, as TLRs, regulate the uptake of the antigens. Once matured, APCs as DCs, up-regulate the expression of MHC of class II, the activation marker CD86, and the maturation marker CD83 and migrate to the lymph nodes to interact with B and T cells (Awate et al., 2013).

Molecular adjuvants, such as the CD40L molecule, have been studied in recent years and included in some vaccine formulations under study (as a DNA based Vaccine Expressing SARS-CoV-2 Spike-CD40L Fusion; Tamming et al., 2022). CD40L is a key molecule that is expressed on activated $CD4^+$ T cell membranes, and it helps dendritic cells to activate $CD8^+$ T cells. Moreover, CD40/CD40L interaction on B cells is responsible for promoting B cell activation and immunoglobulin class switching (Young, 2019).

Despite the large use and advantages of adjuvants, adverse reactions may still occur after vaccine administration, as many adjuvants induce skin inflammation that significantly impacts epithelial integrity and patient tolerance (Caucheteux and Piguet, 2016). Some adjuvants can cause local tissue damage, release of pro-inflammatory mediators, and cytotoxicity at the site of injection (Calabro et al., 2011; Didierlaurent et al., 2009). This is the case of alum hydroxide that could induce necrosis of muscle fibres and generate sterile inflammation. Also, MF59 increases inflammatory mediators' productions and recruitments of white cells as monocytes and neutrophils to the site of injection (Caucheteux and Piguet, 2016).

Another class of non-specific immunostimulants are the **recombinant cytokines**. Cytokines are a wide group of glycoproteins able to regulate biological functions playing a crucial role in autocrine, paracrine, and endocrine signalling. They regulate the function of immune cells acting on intracellular messengers following their binding with membrane receptors. Cytokines administration is also one of the oldest immuno-oncology therapies and is based on the peculiarity of these small proteins to activate the patient's immune system (Jain, 2021). Recombinant cytokines have been studied both alone and in combination with other immunotherapeutic agents to trigger/boost the immune response, not only for anti-cancer treatment (García-Martínez et al., 2018). The cytokines used in immune-oncology therapy are summarized in Table 2.

IFNs are a group of cytokines classified into type I, type II, and type III based on their cell membrane receptors. Type I IFNs encompasses IFN-α and −β, two types of interferons with a well understood role in anti-tumour activity (Blaauboer et al., 2021). The antitumoral activity consists of direct and indirect mechanisms. The indirect mechanism occurs through the modulation of immune cells, while the direct mechanism involves apoptosis and inhibition of cancer cell growth (Chawla-Sarkar et al., 2003). The immune system cells are reduced and functionally impaired in cancer, while type I IFNs enhance their function and stimulate their proliferation. IFN-α/β induces the activation of $CD4^+$ T_h cells and the direct stimulation of DCs by the cross-priming mechanism; thus, the expansion phase of $CD8^+$ T-cells and the proliferation is prolonged. These interferons act on DCs, providing a signal for maturation and activation; regarding NK cells, IFN-α/β stimulate their maturation and the ability to bind to tumour cells (Jain, 2021), and the release of NK cytotoxic factors (Blaauboer et al., 2021).

The main issue which comes from the use of recombinant cytokines for therapy, not only in cancer, is their pleiotropic effect. A single cytokine can bind multiple receptors and induce the secretion of other inflammatory mediators, and the actual response is profoundly influenced by contextual variables, such as the concentration of the mediator, receptor type or isoform, target cell and differentiation of it, and presence of additional mediators (Deckers et al., 2023; Vacchelli et al., 2016). Therapies that use cytokines could be promising if applied for modulating dysregulated immune responses in various disorders (i.e., cancer or autoimmune diseases) (Deckers et al., 2023). Therapies that use cytokines can have adverse effects, including generation of chronic inflammation that may evolve into oncogenesis and tumour progression, or stimulate the proliferation of non-dividing cells, potentially fixing oncogenic genetic or epigenetic defects.

An innovative approach used to bypass the adverse effects due to pleiotropism of the cytokines is to genetically fuse the molecule with other proteins, to better define its distribution profile (Deckers et al., 2023). Cytokines can be linked to other proteins, such as antibodies, to specifically target tissue or organ and reduce off-target adverse effects. Immunocytokines have been engineered and studied for several types of cancer. For example, immunocytokine generated with IL-12 fused to L19 antibody are currently under clinical trial as therapy against tumour (i.e., epithelial ovarian cancer). This approach has enhanced therapeutic effects and reduced toxicity, as the L19 antibody targets fibronectin's extra-domain B, which is overexpressed on angiogenic blood vessels. Some side effects are still registered in some cases due to off-target effects (Halin et al., 2002; Orecchia et al., 2019).

2.2.1. In silico tools to design and validate non-specific immunostimulants

The knowledge (Fig. 1) acquired on the adjuvant MoA are useful to develop new *in silico* tools and approaches to design and validate new small molecule adjuvants. An example of *in silico* methods is the identification and validation of small molecule antagonists for C-C chemokine receptor type 4 (CCR4) (Davies et al., 2017). To target regulatory T cells (T_{regs}) is a helpful method to boost the immunogenicity of vaccines since an enhancement of the primary and secondary response to vaccines has been shown following their depletion, even though it is not free of adverse effects (Pere et al., 2012). CCR4 is a chemokine receptor for CCL22 and CCL17 produced mainly by DCs, and it is expressed on T_{regs} and not on naïve and T_h 1 cells (Iellem et al., 2001). Davies and colleagues (2017) have developed an *in silico* method to target T_{regs}, without depleting them, to boost immune response to vaccination, identifying a small molecule antagonist to CCR4 (Fig. 2). They generated a 3D structure of the CCR4 using an existing crystal structure of a G protein coupled receptor model with WHATIF and AMBER programs (Case et al., 2005; Vriend, 1990). A first virtual screening is performed using different tolls (e.g., UNITY, CORINA, and GOLD docking program) (Davies et al., 2017). They proceed with human PBMC from where the T_{regs} and T_h2 cells are isolated separately. At this point, T_{regs}, T_h2, CCRF-CEM cell line or B9.1 cell line are seeded into the upper layer of a transwell, and in the basolateral layer the optimal dose of CCL22 and CCL17 are added. Then an *in vitro* chemotaxis assay is performed to measure CCR4 antagonism, and the ideal concentration of chemokines is determined following the addition of doses of the small molecule antagonists to CCR4. To further validation, they proceed with *in vivo*

Table 2
Summary of the cytokines used in therapy and their properties.

Cytokine	Properties and use	Reference
Interferons (IFNs)	Direct and indirect anti-tumour activity of IFN-α and −β: Direct – apoptosis and inhibition of cancer cell growth. Indirect – modulation of immune cells enhancing their functions and stimulating their proliferation.	Blaauboer et al., 2021; Chawla-Sarkar et al., 2003; Jain, 2021
Interleukin-2 (IL-2)	Induction of $CD4^+$ T cells proliferation and differentiation into T_h1 and T_h2 cells. Increase in number and activity of $CD8^+$ effector T cells and NK cells. Enhancement of the differentiation of immunosuppressive regulatory T cells.	Herzog et al., 2023; Overwijk et al., 2021
Granulocyte Monocyte Colony Stimulating Factor (GM-CSF)	Trigger of cellular and humoral immune responses by activating neutrophils, macrophages, and DCs. Activation of T cells, influencing the balance of T_h1/T_h2 cytokines.	(Tarhini et al., 2021)

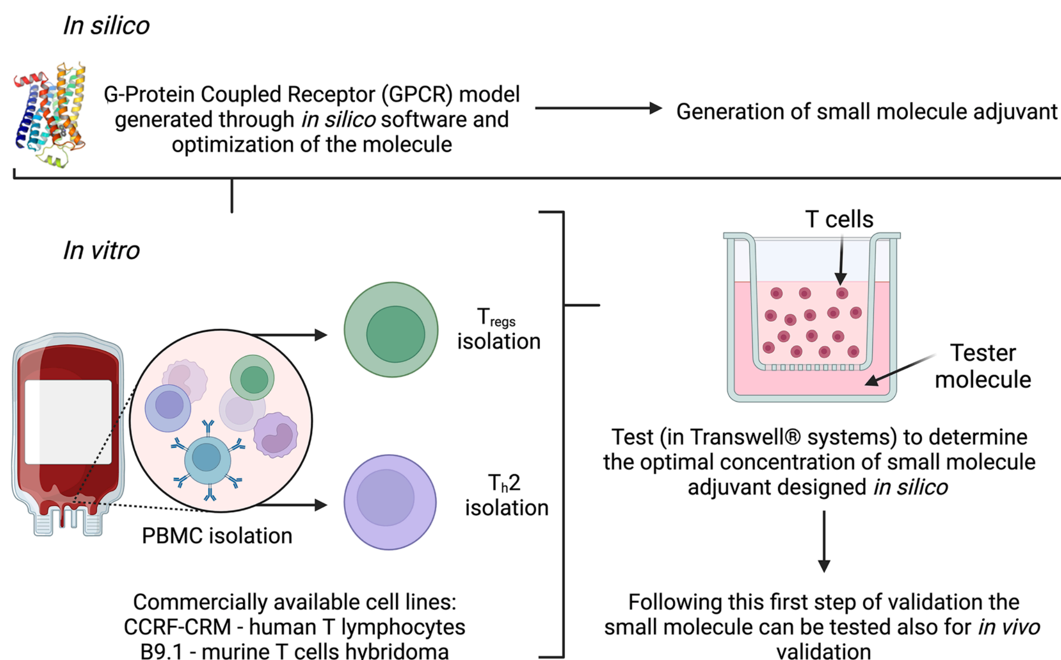

Fig. 1. Protocol developed by Davies and colleagues to identify a new small molecule antagonist to CCR4. The combination of *in silico* and *in vitro* method allows the targeting of T_{regs} without depleting them, enhancing the immune response to vaccination (Davies et al., 2017). Created in BioRender. https://BioRender.com/d35t486.

testing (Davies et al., 2017).

Not only *in silico* approaches are useful to study the effects of these drugs.

2.2.2. NAM for non-specific immunostimulants

Understanding the MoA of different drugs that induce immunostimulation is crucial for determining the most appropriate assay to evaluate their potential hazards. New assays and technologies continue to be developed alongside traditional ones. Cytokine measurement by ELISA is commonly used in several assays and is typically employed as a screening tool before conducting more in-depth tests. ELISAs are widely used due to their specificity and ease of performance. This test can detect a broad range of cytokines and other factors. It is versatile and applied in various settings with a wide array of applications. The standard assay can be performed on PBMCs, whole blood, as well as on different cell types and lines, both primary and immortalized. It serves as a standard screening method for multiple purposes, such as detecting infectious diseases and monitoring drug use

Specific chemotherapeutic drugs

Cancer treatment often involves chemotherapeutic drugs that inhibit the growth of excessively proliferating cells.

2.3. Chemical compounds: MoA

Chemical compounds such as Platinum compounds (Cisplatin, Carboplatin and Oxaliplatin) are conventional anticancer drugs that can interfere with normal DNA functions and generate cross links between DNA and proteins. In recent years, the immunomodulating properties of Pt compounds have emerged. Studies have shown an increased expression of MHC call I via IFN-β signalling following the administration of platinum (Pt) compounds and a decreased expression of the immunosuppressive molecule programmed death receptor 1 (PD-L1) both in DCs and in tumour cells. A detailed study of how Pt drugs interact with the immune system could be to improve the efficacy of Pt-based chemotherapeutic treatments (Corno and Perego, 2018).

Imiquimod is a chemical compound used for the treatment of basal cell carcinoma that acts through three different mechanisms: augmentation of adenosine receptor-associated inflammation, direct pro-apoptotic activity, and induction of proinflammatory cytokines. Imiquimod induces the activation of TLR 7 and 8 which leads to the activation of nuclear factor-kappa B (NF-κB). This NF-κB stimulates the production of IL-1, TNF-α, IFN-α and IFN-β (Singal et al., 2016).

2.4. Immune checkpoint inhibitors (ICIs): IDO and mAb

ICIs are cancer immunotherapies that target receptors located on the surface of T-lymphocytes (Shiravand et al., 2022). One of the ICIs attracting researchers' attention is Indoleamine 2,3-dioxygenasi (IDO), categorized into IDO1 and IDO2. These are tryptophan catabolic enzymes which degrade tryptophan to its metabolites causing an elevated level of immunosuppressive cells. For this reason, the role of IDO1 has been investigated in anticancer therapy (Fujiwara et al., 2022). The catabolism of tryptophan in tumour cells implies the generation of kynurenine enabling the suppression of T_{eff} cells, the recruitment of myeloid-derived suppressor cells and the vascularisation of the tumour cells. Studies show that Imoximod attenuates the suppression of T_{effs} in tumours, decreases T_{regs} and increases T_h17 in draining lymph nodes (Fujiwara et al., 2022). Epacadostat is a selective inhibitor of IDO1. It promotes the growth of T_{effs} and NKs reduces the transformation of naïve T cells into T_{regs}, and increases the number of CD86 DC. This promising anticancer drug is in first-in-human Phase I study to evaluate its antitumor activity and its safety (Prendergast et al., 2018).

In recent years, substantial improvements have been achieved in the field of immunotherapy. The use of mAb to induce immunostimulation against tumour cells can be particularly effective, as it leverages the specificity of antibodies to selectively recognize cancer cells. This targeted approach minimises damage to surrounding normal cells and can enhance treatment efficacy by providing a specific immune response against the tumour (Menon et al., 2016). ICIs are able to increase antitumor activity by blocking intrinsic downregulators of the immune system, including T cell lymphocyte costimulation inhibitor (CTLA-4), programmed death protein-1 (PD-1), and PD-L1 (Topalian et al., 2015). Normally, these regulatory receptors, or checkpoints, maintain the

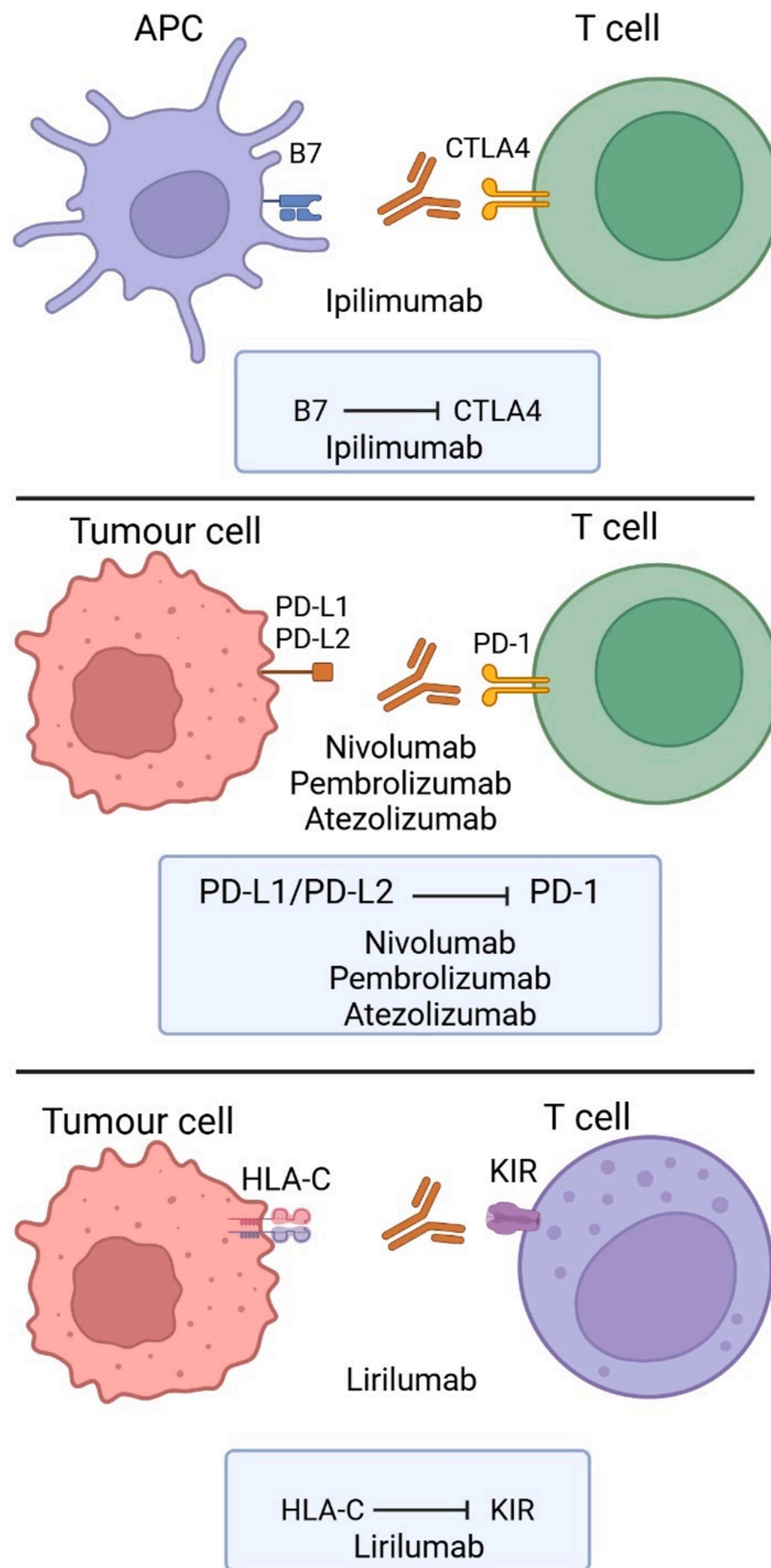

Fig. 2. MoA of Mab in tumour therapy. The focus is on the modulation of the immune checkpoint pathways. Ipilimumab (**A**) blocks the inhibitory signal of T cells induced by the CTLA-4 pathway. Nivolumab, Pembrolizumab and Atezolizumab (**B**) inhibit the interactions between PD-1 receptor and the ligands PD-L1 and PD-L2. Lirilumab (**C**) blocks the interaction between the ligand HLA-C from associating with KIR2DL-1, L-2, L-3 receptors. Created in BioRender. https://BioRender.com/d35t486.

balance between T cell activation and inhibition. The primary aim of ICI is to attenuate a variety of activated T cell functions, including cellular proliferation, cytokine secretion, and cytolysis reducing the suppression of effector T cells, particularly $CD8^+$ T cells, improving their ability to mount tumour-specific immune responses (Chen and Flies, 2013). The two most common checkpoint inhibition strategies are CTLA-4 inhibition and PD-1/PD-L1 blocking.

CTLA-4 is upregulated early after T cell activation in central tissues including lymphoid and thymic tissues (Jamal et al., 2020). It transmits an inhibitory signal to activated T cells at a proximal step in the immune response, by preferentially binding to CD80/86 expressed by APC and blocking the second signal required for T cell activation. Antibodies targeting CTLA-4 neutralise this central checkpoint, allowing ongoing T cell activation and thereby enhancing their anti-tumour activity (Jamal et al., 2020).

The use of mAb as a tumour therapy, with a specific focus on their action in checkpoint pathways, represents an innovative strategy in the fight against cancer. Immune checkpoints serve as key regulators of the immune system, and their modulation through mAb provides a targeted approach to enhance the immune response against tumour cells. This treatment modality relies on the capability of mAb drugs to block signalling pathways that inhibit the activation of immune cells, enabling them to recognize and attack cancerous cells more effectively. In the context of anti-tumour therapy, this approach aims to improve the specificity and efficacy of the immune system while concurrently reducing the risk of collateral damage to normal cells (Milling et al., 2017). Many treatments available are mAb, while there is also a small amount of chemical compounds able to act on checkpoints. Chemotherapy drugs are used for the treatment of cancer disease and are routinely prescribed.

Despite their efficacy, they can induce ~~an~~ excessive immunostimulation and hypersensitivity reactions (HSRs). Analysing the family of taxanes, Paclitaxel, Decetaxel and Cabazitaxel are known to be responsible for HRSs during the first or the second infusion. Probably the majority of HSRs is caused by the direct complement activation by cremophor or polysorbate, emulsifying agents used in the formulation of this drug. However, it has been proposed as an IgE-mediated mechanism due to the positivity of skin tests performed on patients with a reaction to taxanes. To demonstrate this hypersensitivity reaction, an *in vitro* study conducted by Prieto García and Pineda de la Losa (2010) shows the development of an IgE-mediated mechanism through an IgE dot-blot assay (Prieto García and Pineda de la Losa, 2010). HSRs can occur also after multiple administration of Pt compounds (Cisplatin, Carboplatin, Oxaliplatin). Although the underlying mechanism of the hypersensitivity reaction is not fully understood, it appears to be related to type I IgE-mediated hypersensitivity. The symptoms are consistent with those of type I reactions, and prior exposure is essential, as a second exposure typically triggers the reaction. Additionally, skin tests are often positive in such cases. (Pagani et al., 2022).

CTLA-4 is a key regulator of T cell activity. Ipilimumab (IgG1) is an ICI targeting CTLA-4 that blocks the inhibitory signal of T cells induced by the CTLA-4 pathway, thereby increasing the number of reactive effector T cells mobilising for a direct immune attack on tumour cells. CTLA-4 blockade can also diminish the function of regulatory T cells, contributing to an anti-tumour immune response. This mAb can selectively eliminate regulatory T cells at the tumour site, leading to an increased intratumoral ratio of effector T cells to regulatory T cells, ultimately resulting in tumour cell death. It is indicated for the treatment of melanoma and, in combination with Nivolumab, for renal cell carcinoma (Hodi et al., 2010).

The second immune checkpoint receptor to be targeted for immune checkpoint blockade therapy was PD-1. Upon activation, such as in response to inflammation, T cells express PD-1, allowing them to identify abnormal and cancerous cells (Munn and Bronte, 2016). PD-1 is believed to play a role in T cell inhibition in the peripheral tissues during the later stage of the immune response. PD-1 provides a potent inhibitory signal to T cells (Alsaab et al., 2017; Jamal et al., 2020).

PD-L1 is expressed by many tumours and can be expressed by APCs. PD-1 is more broadly expressed than CTLA-4. PD-L1 is expressed not only on T cells but also on B cells and NK cells. Therefore, although PD-1 blockade is typically viewed as enhancing the activity of effector T cells in tissues and in the TME, it also probably enhances NK cell activity in tumours and tissues and may also enhance antibody production either indirectly or through direct effects on $PD1^+$ B cells (Alsaab et al., 2017).

PD-1 ligands are PD-L1 (B7-H1) and PD-L2 (B7-DC). They are members of the B7 family of costimulatory molecules. Further, when bound to PD-L1 or PD-L2, PD-1 causes the inhibition of phosphatases involved in effector T cell activation (Keir et al., 2008).

Also, in light of clinical observations linking elevated serum levels of IL-18 with impaired functions of NK cells and the potential involvement of tumour-derived IL-18 in cell-autonomous tumour progression, an investigation was conducted on the potential immunosuppressive effect of IL-18 in tumours controlled by NK cells. It has been reported that IL-18 could induce the upregulation of PD-1 expression on NK cells, facilitating the metastatic dissemination of NK cell-dependent tumours in a PD-1–dependent manner. The depletion or neutralisation of IL-18 produced by tumour cells significantly enhanced NK cell-mediated immune surveillance against cancer, as documented in the referenced study (Terme et al., 2011).

When T_{effs} cells encounter antigen chronically (as in chronic infection or cancer), they lose the ability to respond to the antigen and this condition is known as T cell exhaustion. This is partly mediated by the enhanced expression of immune checkpoints such as PD-1. The binding of PD-1 to its ligands, PD-L1 and PD-L2 (which are expressed by many tumour cells), interferes with downstream signalling and can lead to T cell exhaustion. Antibodies that block the interaction of PD-1 with PD-L1/PD-L2 serve to neutralise this checkpoint, restoring T cell effector function (Buder-Bakhaya et al., 2018; Dyck and Mills, 2017).

Nivolumab is a fully human IgG4 antibody targeting the PD-1 immune checkpoint. It selectively inhibits the interaction between the PD-1 receptor and its two known ligands, PD-L1 and PD-L2, thereby disrupting the negative signal that regulates T cell activation and proliferation. The interaction with PD-L1 and PD-L2, expressed by APCs and potentially by tumour cells or other cells in the TME, results in the inhibition of T cell proliferation and cytokines secretion (Wang et al., 2014). In a 2015 phase 1 study~~,~~ Nivolumab demonstrated promising anti-tumour activity and a favourable safety profile in patients with solid tumours, including advanced melanoma (Robert et al., 2015), this antibody is currently in clinical use.

Experiments conducted *in vitro* using a combination of Ipilimumab and Nivolumab demonstrated increased secretion of cytokines (IFN-γ, IL-2) in the stimulation of superantigens in human peripheral blood lymphocytes and in assays measuring mixed lymphocyte responses compared to those observed with nivolumab alone, as assessed by ELISA for IL-2 and IFN-γ (Pelster et al., 2021; Selby et al., 2016).

Pembrolizumab is an IgG4 antibody that prevents the interaction between PD-1 and its ligands. It is indicated for melanoma and colorectal carcinoma (Winer et al., 2021). Atezolizumab targets PD-L1, which can reactivate suppressed immune cells while preserving PD-L1/PD-1 interactions that may maintain immune homeostasis. It is indicated for urothelial carcinoma, triple-negative breast cancer, and melanoma (Herbst et al., 2014). The MoA of these mAbs is detailed in Fig. 2.

NK cells are integral components of the host's defence mechanism against infections and tumours. They contribute to this defence by releasing immunoregulatory cytokines and carrying out the elimination of infected or transformed cells. The activation of NK cell effector function is intricately governed by a variety of activating and inhibitory receptors, such as Killer Immunoglobulin-like Receptors (KIR). These receptors specifically recognize ligands expressed on potential target cells. The intricate interplay between positive and negative signals transmitted through these NK receptors determinates the fate of the

target cell, deciding whether it succumbs to the cytotoxic activity of NK cells (Thielens et al., 2012).

NK cells, when activated by surface receptor signals, play a key role against malignant tumours by directly killing transformed cells through proteolytic granzymes and secreting immunoregulatory cytokines, such as IFN-γ, macrophage inflammatory proteins (MIP), interleukins (IL-8, IL-10), and TNF-α. The release of these cytokines attracts other immune cells (e.g., T_h1 cells, myeloid cells) to the site and induce their antitumor response (Morvan and Lanier, 2016; Sabry et al., 2019).

KIR and their ligands, human leukocyte antigen C (HLA-C), interact to suppress NK cell functions. Patients with invasive cervical cancer positive for HPV show upregulation in the HLA-C/KIR2DL2 and HLA-C/KIR2DL3 antigen pairs, enhancing the HLA-C/KIR interaction may suppress the normal functions of NK cells in patients with HPV-positive cervical cancer (Rizzo et al., 2014).

The human anti-KIR (IgG4) mAb Lìirilumab has been demonstrated to block HLA-C from associating with KIR2DL1, −L2, and −L3 receptors, thereby compromising the inhibitory signal on NK cells (Kohrt et al., 2014). Immunotherapy with the anti-KIR antibody leads to the upregulation of various key cytokines and has shown positive benefits in several types of tumours (Benson et al., 2015; Sola et al., 2014; Vey et al., 2012).

ICIs have proven effective in treating diverse cancers; however, they may trigger irAEs as a potential side effect. Myocarditis, though rare, constitutes a serious irAE that can occur following ICI treatments with a reported mortality rate of 40 %, highlighting its severity (Won et al., 2022).

An example of this loss of self-tolerance is Trastuzumab. Trastuzumab is a mAb that targets the human epidermal growth factor receptor 2 (HER2), which is overexpressed in certain tumour types, particularly in breast cancer. The primary MoA of Trastuzumab is to inhibit signalling through the HER2 receptor, thereby impeding the growth and proliferation of tumour cells dependent on this signalling pathway (Swain et al., 2023).

However, a known side effect of Trastuzumab is its association with the risk of myocarditis. This side effect has been observed in a relatively low percentage of patients treated with Trastuzumab, but it is an important consideration during clinical use (Sunny et al., 2022).

The exact mechanism through which Trastuzumab induced myocarditis is not fully understood, but it is hypothesised to involve an aberrant immune response against cardiac cells.

An *in vitro* study employed human induced pluripotent stem cell-derived cardiomyocytes (iPS-CMs) to model Trastuzumab-related cardiotoxicity, suggesting their potential utility in exploring additional modes of toxicity associated with Trastuzumab and related compounds (Kurokawa et al., 2018). This study contributes valuable insights into the *in vitro* modelling of Trastuzumab-induced cardiotoxicity, emphasising the broader applicability of such methodologies in pharmaceutical research and development.

2.5. Chimeric antigen Receptor-T (CAR-T)

Among cellular immunotherapies for cancer, **Chimeric antigen receptor T (CAR-T)** therapy is widely utilised, especially for haematological malignancies. This technology is based on the isolation of T lymphocytes from the patient's blood, which are engineered to express chimeric antigen receptors (CARs) to enable modified T lymphocytes to recognise and attack cancer cells. These cells undergo *in vitro* proliferation and are re-infused into the patient to start the immune response. Over the years, three generations of CAR haves been formulated but the second generation, the one that includes a costimulatory molecule to promote CAR-T cell survival and *in vivo* functionality, is the one that has shown to be the most effective and for this reason is the most used (Lin et al., 2021). Kymriah (Tisagenlecleucel) was the first CAR-T CD19 approved by the FDA for the treatment of acute lymphoblastic leukaemia. The immunostimulatory effect and the sustained activation are facilitated by the costimulatory domain 4-1BB, which that interacts with endogenous TCR signalling. This action entails T-cell proliferation, cytokine secretion such as IL-2, IL-4, IL-5 IFN-γ, prevention of clonal deletion and increased cytolytic potential. The effectiveness of this therapy has been demonstrated in some clinical trials; in ELIANA trials (a phase II multicentre trial in paediatric and young adults) event-free survival rate was 50 % and overall survival was 76 % (Vairy et al., 2018). Yescarta (Axicabtagene ciloleucel) was the second CAR-T therapy approved by FDA in 2017. It belongs to the second generation and his target is CD19; it is used against B cells lymphoma. Yescarta binds to CD19 expressing cancer cells and this interaction causes the activation of T-cell proliferation and cytokines release. The combination of these events causes the CD19-expressing cell apoptosis. In the clinical trial ZUMA-1 is reported the efficacy of the treatment: the 49 % of the enrolled patient shows a six-month progression-free survival and relapses seemed to occur rarely after a duration of six months (AlDallal, 2020). A major drawback of this therapy is the potential for serious side effects. For this reason, there is an increasing interest in developing CAR-NK cells for immunotherapy. The feature that makes them safer is the spectrum of cytokines released: CAR-NK cells produce IFN-γ and GM-CSF, while CAR-T cells produce IL1, IL-2, IL-6, IL-8, IL-10, IL-15 and TNF-α. NK cells can be obtained both from the patient PBMC and from the NK92 cell line, as CAR-T cells undergo gene transfer and *in vitro* expansion. The results obtained from clinical trials show safety and clinical activity in patients with B cell lymphoma (Xie et al., 2020); the trial conducted by Liu et al. (2020) show that seven out of eleven patients with non-Hodgkin's lymphoma or chronic lymphocytic leukaemia attain a complete remission and a high expansion of CAR-NK cells.

Many preclinical studies are conducted in mice, but these models scarcely represent the human disease because of the variability in cross-species reactivity (Ramos-Cardona et al., 2022); for this reason, in the latest years, alternative *in vitro* methods are being developed. Using *in vitro* methods, the researchers have access to most of the required information they need: it is possible to harvest the cell supernatants to analyse the cytokines release, the target cells and CAR-T cells can be collected to study respectively the killing potency and the cell proliferation through flow cytometric analysis (Si et al., 2022). Examples include a study conducted by Walker et al. (2017), in which Protein L was used to monitor the internalization of CAR molecules. An alternative is the use of a culture plate or nanobead coated with recombinant TAAs that are recognized by CAR-T cells (Walker et al., 2017).

2.6. Chemotherapeutics drugs and adverse effects

Cytokine release syndrome (CRS) is a systemic inflammatory response that frequently occurs after the infusion of monoclonal antibodies. With the growing success of T cell-engaging immunotherapies, such as CAR-T, CRS is increasingly studied because it is one of the most critical adverse effects (Shimabukuro-Vornhagen et al., 2018). The symptoms of CRS can occur immediately following CAR-T therapy or may appear days or weeks after treatment. The clinical manifestations are varied and include fever, myalgia, nausea, vomiting, hypotension, tachycardia, respiratory distress syndrome, renal and hepatic failure, cardiac dysfunction, neurologic toxicity, hallucinations and intravascular coagulation. The onset of this side effect is related to the MoA of CAR-T. CAR-T cells target tumour cells, and upon binding, the tumour cells release cytokines such as IFN-γ and TNF-α. These cytokines activate immune system cells, including T cells, DCs, macrophages, monocytes, and NK cells, which in turn release additional cytokines (IL-1, IL-5, IL-6, IL-10, IFN-γ, TNF-α, and TGF-β), leading to a cytokine storm. IL-6 appears to play a central role in the pathophysiology of CRS, given the elevated levels of this cytokine in patients. IL-6 is secreted by T lymphocytes, macrophages, and DCs, and it has multiple biological functions, including the production of proinflammatory cytokines, the proliferation of antibody-producing B cells, and the synthesis of acute-phase reactive proteins. In addition to CRS, another side effect of

excessive immunostimulation induced by CAR-T cells is immune effector cell-associated neurotoxicity syndrome (ICANS) (Gu et al., 2022; Sterner and Sterner, 2022). It usually occurs 4–6 days after the infusion; in a few cases, patients develop a delayed neurotoxicity 3–4 weeks after the administration of the therapy. In mild cases, symptoms include aphasia, dysregulation of fine motor function, and reduced attention levels. In more severe cases, elevated intracranial pressure, seizures, and cerebral oedema may occur. The mechanism of syndrome onset is explained in Fig. 3. After the infusion of CAR-T into the patient, the interaction with the tumour cells induces activation of host myeloid cells. This interaction leads to the production of an excessive amount of cytokines (IL-1, IL-6, IFN-γ, TNF-α, and GM-CSF) that participate in the endothelial activation-induced blood–brain barrier (BBB) dysfunction. The increased permeability of BBB allows cytokines to infiltrate the central nervous system causing inflammation and abnormal neural function, which results in ICANS. The management of ICANS involves corticosteroids that exert anti-inflammatory effects on immune cells, and on biological agents such as the monoclonal antibody Siltuximab that targets IL-6. Other biological agents could be used to target other cytokines (e.g., Lenzilumab for GM-CSF or Anakinra for IL-1) or adhesion molecules (Gu et al., 2022).

3. Monoclonal antibodies

Monoclonal antibodies represent an innovative class of biotechnological drugs with a wide range of applications in the field of medicine. Their uniqueness lies in the ability to specifically target molecular entities, serving as precision tools in the treatment of a diverse array of pathological conditions. mAbs, owing to their capacity to target specific cells or molecules precisely and selectively, have the potential to substantially enhance immune responses. This phenomenon manifests through the activation of immune system cells, such as T cells and NK cells, alongside an escalation in the production of pro-inflammatory cytokines.

The use of mAb is recently developing for the treatment of tumours, as previously described in this review.

The ongoing exploration of the molecular mechanisms underlying immunostimulation will provide a fundamental scientific foundation for the evolution and optimization of mAbs as innovative therapeutic agents.

These biotherapeutics have demonstrated effectiveness in multiple therapeutic contexts, from cancer treatment to autoimmune diseases, providing new perspectives in the control and management of complex disorders. Their relevance in modern medical practice extends beyond mere therapy, as they interact crucially with the human immune system (Riley et al., 2019).

mAb acts through various mechanisms, interacting with cellular receptors, signalling molecules, and other components of the immune system, with implications that extend beyond merely targeting of specific target molecules. In this perspective, attention is focused on a crucial and complex aspect related to the use of these drugs: their ability to modulate immunostimulation. On one hand, they play a key role in

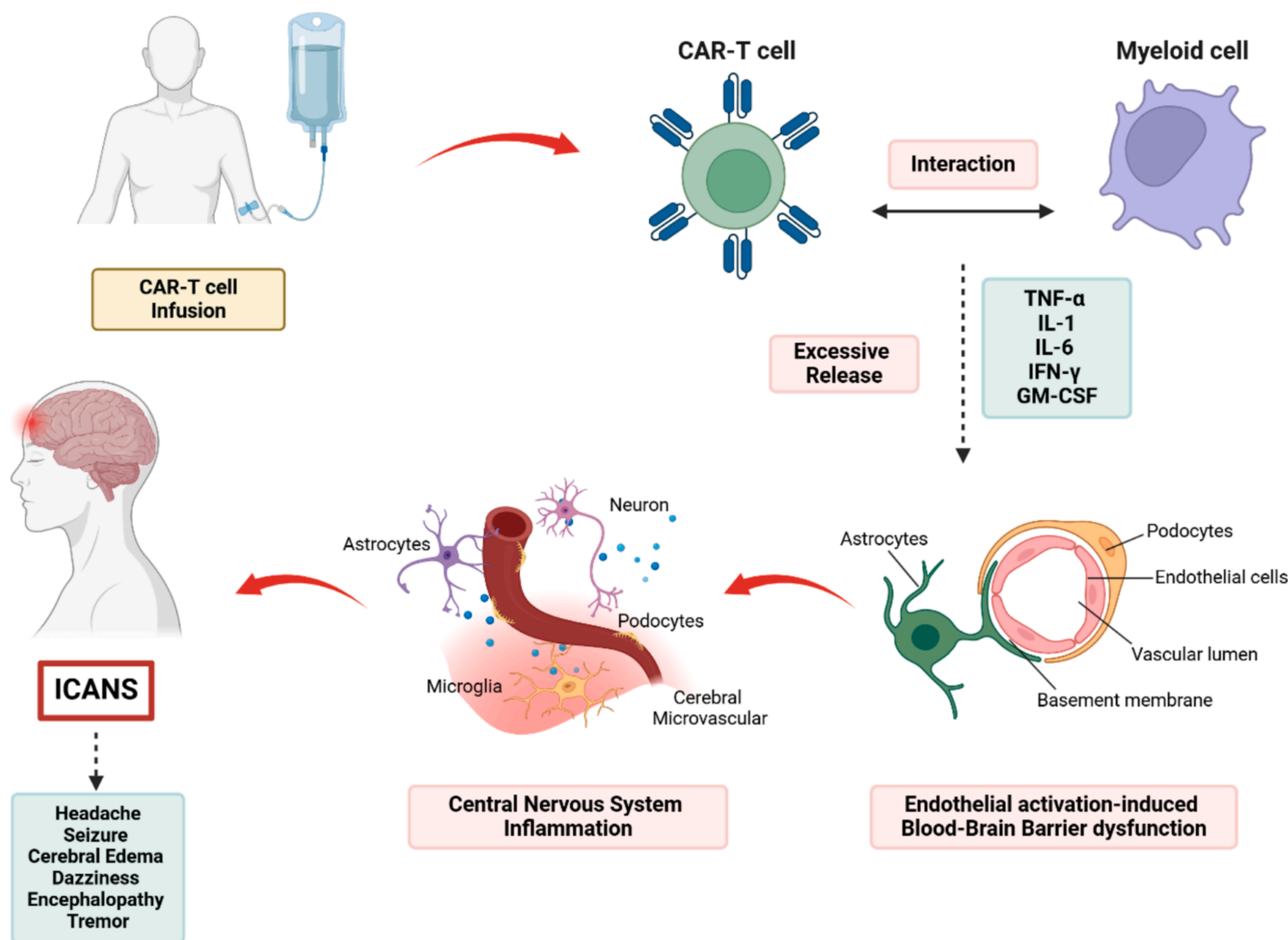

Fig. 3. Illustration of immune effector cell-associated neurotoxicity syndrome (ICANS) pathogenesis after treatment with CAR-T cells. After the infusion into the patient, CAR-T cells is activated by tumour cells (not shown) and induce myeloid cells activation. This interaction causes excessive circulating cytokines that imply the endothelial activation-induced blood brain barrier (BBB) dysfunction. The increased permeability of BBB allows the penetration of cytokines into the central nervous system (CNS) that results in the typical symptoms of ICANS. Modified from (Gu et al., 2022). Created in BioRender. https://BioRender.com/d35t486.

regulating the immune response by providing targeted therapies against specific pathologies. On the other hand, interaction with the immune system can generate adverse effects, triggering unwanted reactions or compromising the natural immune response.

The increasing use of mAb as therapeutic agents has revolutionised the clinical landscape, leading to significant advancements in the management of various pathologies. However, with this therapeutic evolution, challenges related to immune-related adverse effects associated with mAb use also emerge.

One critical challenge associated with mAb therapy is patient immunization, resulting from the development of anti-drug antibodies (ADAs). These ADAs are generated by the immune system in response to therapeutic mAbs, potentially leading to significant clinical consequences. Depending on their characteristics, particularly their neutralizing capacity, ADAs can form drug-ADA complexes that interfere with the therapeutic efficacy of mAbs or trigger adverse effects such as hypersensitivity reactions. This immunogenicity not only compromises the intended clinical benefits of the therapy but also poses safety concerns, highlighting the need for strategies to minimize ADA formation and manage its effects in clinical practice. To address issues related to ADA formation, NAMs could include neutralization assays to evaluate the neutralizing activity of ADAs, high-throughput screening techniques such as ELISA or Luminex for detection and quantification, mass spectrometry for detailed analysis of ADA variants, and predictive computational models based on machine learning to identify potential immunogenicity risk factors.

Immune hyperstimulation can lead to cytotoxic phenomena and excessive inflammatory reactions, which may, in turn, contribute to the development of autoimmune pathologies or inflict damage upon healthy tissues. Accurate assessment of the risks and benefits associated with the use of mAbs is thus crucial to ensure their appropriate therapeutic application. Technologies such as multiplex assays for cytokine quantification provide an innovative tool for monitoring inflammation levels, particularly in response to mAb treatments. These assays allow for the simultaneous measurement of multiple cytokines and other immune mediators in a single sample, thereby enabling a comprehensive profile of the inflammatory response *in vitro*. This methodology is particularly valuable for observing potential increases in pro-inflammatory cytokines, such as IL-6, TNF-α, and other key molecules that may signal an excessive or undesirable immune reaction. Multiplex assays offer a rapid and efficient solution to quantify these responses, providing essential support in studying interactions between mAbs and the human immune system, with applications ranging from preclinical research to clinical monitoring (Leng et al., 2008).

Activating the immune system to fight cancer can also lead to serious, undesirable off-target immune and inflammatory events known as immune-related adverse events (irAE). The most common irAE include rash, colitis, thyroiditis, hypophysitis, hepatitis, pneumonitis, and arthritis, with clinical severity ranging from mild to severe and occasionally fatal. More than 70 % of cancer patients undergoing treatment with ICIs encountered irAEs affecting the heart, lungs, liver, intestines, and skin, with severity ranging from mild to severe grades (Esfahani et al., 2020; Martins et al., 2019; Song et al., 2020).

Most events occur within the first 3–4 months of therapy, but can occur anytime during treatment, and even years after cessation of immunotherapy. It remains unclear whether the effects result from T cells specifically acting against antigens shared by tumour and normal cells, or the concomitant activation of multiple T cell populations with separate host-adverse and anti-tumour activity. Systemic therapy with high doses of corticosteroids, with or without additional immunosuppressive therapy, may be necessary to manage severe immune-related adverse reactions (Horvat et al., 2015).

Hypersensitivity reactions, occurring in response to the administration of mAb, are particularly relevant in the context of murine and chimeric formulations (June et al., 2017). The use of such antibodies, characterised by their biological origin and significant interspecificity, introduces greater complexity in their interaction with the human immune system. The heterogeneity of protein sequences and three-dimensional structures inherent in mAb of this nature can trigger adverse hypersensitive immunological responses (Milling et al., 2017). A comprehensive understanding of these phenomena necessitates careful analysis of the molecular interactions involved, coupled with the identification of specific subtypes of immunological reactions induced by these biotherapeutic entities.

In this context, an illustrative example is the chimeric mAb, Infliximab. This IgG1-type mAb is designed to intercept and neutralise TNF-α and is used for the treatment of autoimmune diseases such as rheumatoid arthritis, psoriasis, Crohn's disease, and ulcerative colitis. However, its administration is associated with a well-recognized risk of infusion-related adverse events (Lichtenstein et al., 2015).

Possible etiological mechanisms of Infliximab infusion-related reactions include:

- CRS: is a systemic inflammatory syndrome whose symptoms and characteristics are described in paragraph 3.4.

  CRS is also associated with other mAb such as Rituximab. Rituximab, primarily used in patients with non-Hodgkin lymphoma, acts through selective binding to the CD20 antigen on the surface of B lymphocytes, leading to their destruction (Weiner, 2010).

- IgE-Mediated Anaphylactic Reactions: infusion hypersensitivity reactions are often associated with mAb produced from the SP2/0 cell line, characterised by the addition of an α-1,3-galactose moiety not present in human proteins (Matucci et al., 2013). Consequently, upon administration, these mAbs can trigger an immune response through the release of IgE (Chung et al., 2008). IgE-mediated reactions require pre-sensitisation and should not occur during the first infusion. This reaction is also associated with other mAbs, such as Adalimumab. Adalimumab, another anti-TNF-α mAb widely used in autoimmune conditions such as rheumatoid arthritis, Crohn's disease, and ulcerative colitis, can trigger adverse reactions such as itching, swelling, and occasionally anaphylaxis. These responses can disrupt the immunological balance, questioning the efficacy of the therapy and the patient's tolerability. Other examples of such etiological mechanisms include the mAb Ustekinumab, used for the treatment of psoriasis and psoriatic arthritis as it prevents the binding of IL-12 to its receptor, disrupting the cascade of inflammation mediator production (Sunny et al., 2022).
- Complement system activation: in patients with pre-existing anti-Infliximab antibodies, the administration of Infliximab may result in the formation of circulating complexes. These complexes have the potential to activate the complement system, triggering an immediate infusion reaction. Additionally, complement activation may also occur through the aggregation of Infliximab molecules (van der Laken et al., 2006).

To better understand the etiological mechanisms underlying infusion-related reactions with mAbs like Infliximab, *in vitro* NAMs focused on drug-complement interactions can be instrumental. Complement activation is a key component in immune-related adverse events, often triggered by immune complex formation or direct complement binding by therapeutic antibodies. Developing NAMs that simulate these interactions *in vitro* could provide valuable insights into the specific pathways and cellular responses involved in such reactions. For example, using complement-targeted *in vitro* models could aid in identifying the role of various complement proteins in adverse immune reactions and guide the design of mAbs with minimized complement activation risks (Ricklin and Lambris, 2007).

In the future, iPSCs could offer a promising method for modelling hypersensitivity reactions, enabling patient-specific studies of immune

responses, especially in cases of allergies or immunodeficiencies. By generating immune cells from patient-derived iPSCs, researchers may be able to create personalized models to explore unique cellular triggers and safely test therapeutic interventions, potentially transforming approaches to immunological disorders with tailored, low-risk treatments. The conclusive chapter of this review explain the *in vitro* methods useful to study the wanted or unwanted immunostimulation effects triggered by mAbs.

3.1. mAb and NAMs

An important aspect to consider is the evaluation of cytolytic processes using assays such as complement-dependent cytotoxicity (CDC) or antibody-dependent cell-mediated cytotoxicity (ADCC) assays. These assays are primarily conducted to assess the safety of mAbs. They help detect potential immune responses in which target cells may be lysed through the complement cascade. ADCC, an adaptive immune response primarily mediated by NK cells, binds to the constant region of IgG antibodies, triggering the lysis of targeted cells. Additionally, complement activation assays, typically performed in serum, provide a comprehensive understanding of the effects of different drugs.

Several assays are designed to assess cell proliferation, such as the carboxyfluorescein succinimidyl ester (CFSE) assay and the 5-bromo-2′-deoxyuridine (BrdU) incorporation assay. These assays are commonly applied to T-cell populations.

These assays are usually performed in a battery of tests. If a drug is found to be positive in the cytokine release assay and another of these assays, further testing may not be necessary, as the hazard has already been identified. Subsequent studies and drug development can proceed after a positive result in cytokine release assays, as it is crucial to analyse the types of cytokines released, their effects, and their duration. Furthermore, selecting an appropriate dose and monitoring its effects are essential components of the safety assessment process (U.S. Department of Health and Human Services Food and Drug Administration, 2023).

4. Nucleic acid-based drugs

In the last few decades, the treatment~~s~~ of human disorders have been undergoing modifications, focusing more and more on personalising the treatments. This evolution is happening thanks to the identification of molecular targets involved in the development of diseases, with the consequent development of highly specificity drugs as a treatment for these disorders. The molecular diversity involved in the development of diseases is not a peculiarity of the rare diseases but also common human illnesses. It is important to focus on new therapeutic approaches that do not concern only the study of small molecules interacting with specific domains of transport channels or enzymes but involve the development of classes of drugs that can act on different classes of protein with high specificity.

The great power and flexibility of **nucleic acids (NA)** make these molecules ideal candidates in this field with a wide range of possible applications. In fact, NAs are a versatile class of sequence-programmable drugs that can be used for therapy. The potentials of using these molecules in therapy is that they can modulate the expression or correct the genetic defects that cause diseases (Sarli and Watts, 2022); they can also targets molecules that are undruggable using other type of drugs such as small molecule or protein/antibody- based biologics (Yamada, 2021). NA drugs can be used to treat various types of human diseases such as, viral infections, genetic disorders, and cancer. The biological functions of cells are controlled by nucleic acid drugs, based on the information of the nucleotide sequence. These drugs can function through their expression in cells or by modifying genes that have complementary sequences. These molecules provide a major advantage because they can be designed independently of the localization or the structure of the molecular target, allowing approaches that were not possible with antibodies or small molecules (Yamada, 2021). Another peculiarity of these drugs is that once the main structure has been defined, it is possible to change the drug modifying the nucleotide sequence of the target gene (Yamada, 2021).

4.1. RNA-based drugs

Focusing on RNA-based drugs, the most common classes are messenger RNA, microRNA, antisense oligonucleotides, small interfering RNA, aptamers, and ribozymes.

Messenger RNA (mRNA) was discovered in 1961 and is a single-stranded molecule composed of 2000–2500 bases (Ota et al., 2004). Therapeutic mRNA is composed of over 1000 bases, it can be used in the replacement therapy when there is a defect~~ion~~ or a reduction in the protein transcription or it can be used to induce production of a new protein (e.g., Tozinameran and Elasomeran have been used for Covid-19 disease). These types of drugs act without entering the nucleus and changing the DNA of the cell because their translation occur in the cytosol (Sahin et al., 2014). Once translated into protein, the encoded protein works as an antigen in case of an mRNA vaccine and can be recognized by immune system cells, generating the immune response described in the first chapter of this review; otherwise, it performs its action in the cytoplasm or in the extracellular compartment.

MicroRNA (miRNAs) are single-stranded noncoding RNAs composed of 15–22 nucleotides (L. Liu et al., 2021); they can be involved in different functions of the cells through the regulation of gene expression involved in apoptosis, differentiation, proliferation, and metabolism (Hausser and Zavolan, 2014; Jonas and Izaurralde, 2015; Makarova et al., 2014; Meng and Lu, 2017; Tonevitsky et al., 2013). The field of miRNA drugs is divided into two categories: miRNA mimics and miRNA antagonists. Anti-miRNA binds to the active miRNA and inhibits its overexpression (Krützfeldt et al., 2005). On the other hand, miRNA mimics have the same sequences as the endogenous miRNA and their function is to fix in the diseased tissues the function of miRNA (Wiggins et al., 2010).

Small interfering RNAs (siRNAs) are double-stranded RNAs, with each strand consisting of 20–30 nucleotides (Yamada, 2021). Once siRNA is taken up by cells, its activity is triggered through the formation of a complex known as the RNA-induced silencing complex (RISC) in the cytoplasm (Rana, 2007). The primary function of siRNAs is gene silencing; they exert their effect by binding to the complementary target mRNA region (Claycomb, 2014). In recent decades, siRNA-based drugs have demonstrated potential applications in treating various genetic diseases, cancer, and viral infections, due to their ability to silence target genes by binding to complementary mRNA sequences (de Fougerolles et al., 2007).

Another class of NA drugs is antisense oligonucleotide (ASO). These molecules are short single-stranded RNA or DNA, composed of 3–30 nucleotides. The oligonucleotide interacts with the complementary sequence of the target gene, forming a DNA/RNA double-stranded structure at the target site, leading to the inhibition of gene function (Krützfeldt et al., 2005). ASOs can act through three different mechanisms: RNase H-mediated degradation, miRNA inhibition, and mRNA modification (Garber, 2018). ASO can interact with target mRNA mimicking the DNA-RNA binding and induces the recruitment of RNase H enzymes (Wu et al., 2004). These enzymes stimulate the cleavage and degradation of the mRNA target without interacting with the non-target RNA. In addition, RNase H keeps working on the degradation of other mRNA target to reduce the production of the target protein promoting the knockdown of the target gene (Cerritelli and Crouch, 2009). The second MoA of ASOs is the inhibition of miRNA activity through complementation with them. The MoA is similar to the one of anti-miRNA oligonucleotides (AMOs). AMO interacts with target miRNA, forming the complex AMO-miRNA that induces RNase H-mediate gene-targeting cleavage (Nagahama et al., 2017). The last MoA is mRNA modification. This mechanism occurs when ASOs bind to and modify the

5′ cap or the polyadenylated tail of mRNAs. This interaction can alter the stability of the mRNA or inhibit the translation leading to a reduction in the expression of the target mRNA and protein (Bennett and Swayze, 2010). The splicing mode of mRNAs can also be altered by ASOs. These drugs, forming a complex with the pre-mRNA intron/exon junctions, can make the splice site unstable or influence the recruitment and the binding of the splicing factors causing an alteration on the attachment of the spliceosome to the splice site altering the normal splicing pattern of the target mRNA. This strategy could be used to correct splicing defects and restore the normal function of proteins (Havens and Hastings, 2016).

Aptamers are another class of NA drugs. They are RNA or DNA (ssDNA or ssRNA) single stranded with tertiary structures that allow them to interact and bind with the molecular targets (Ellington and Szostak, 1990). Aptamers have similar properties to the antibodies in molecular recognition. Aptamers showed greater advantages over antibodies because they can be synthesised through economical chemical synthesis methods, they also exhibit higher stability and lower immunogenicity (L. Liu et al., 2021). These drugs have different types of targets such as ions, peptides, proteins, viruses, small molecules, bacteria, parasites, and living cells (L. Liu et al., 2021). Aptamers are synthetically screened *in vitro* by a method called systematic evolution of ligands by exponential enrichment (SELEX) (Nimjee et al., 2017).

The last class of NA (Table 3) drugs is the **ribozymes**, that was discovered as natural components; they are a portion of RNAs that can act as enzymes in absence of protein. They can be used as suppressor of the gene function due to their high specificity, target selection, and action before protein translation. They can be artificially produced and have applications in cancer and virology (Abera et al., 2021).

4.2. Adverse effects

It is known that most of the RNA drugs, once administered in the human body can lead to an unwanted activation of the immune system causing undesired effects. RNA molecules can be recognized by different pathways that can induce the production of pro-inflammatory cytokines and type I of IFN. This innate immune response can be mediated by TLR or non-TLR. In humans, there are 10 different types of TLRs; all the types of these receptors recognize specific patterns of pathogen that are called PAMPs; the interaction between PAMPs and TLR induce the activation of the innate immune system and the consequent activation of antigen-specific adaptive immunity. The receptor involved in the recognition of the RNA based drugs include TLR3, TLR7 and TLR8 which are located inside the cells (Meng and Lu, 2017). Regarding the innate response non TLR-mediated, it can be triggered by different mechanisms involving different types of receptors, such as retinoic acid-inducible gene-I (RIG-I)/melanoma differentiation-associated protein 5 (MDA5), and dsRNA-dependent protein kinase (PKR) (Meng and Lu, 2017). TLR3 is expressed in immune cells like myeloid DCs, macrophages, mast cells (MCs), as well as in fibroblasts, and various epithelial cells. Following TLR3 activation, the production of pro-inflammatory cytokines, type I of IFNs, and chemokines is enhanced. TLR7 is constitutively expressed in plasmacytoid DCs (pDC); these cells are responsible for a large production IFN 1. TLR8 is expressed by macrophages, monocytes, and mDC in humans (Zarember and Godowski, 2002). The soluble factors released by these cells, including IFN-α, are all responsible for the inflammatory response. In fact, the signal of IFN-α can also induce the production of the enzymes cyclooxygenase-2 (COX2) and nitric oxide synthase (iNOS) that are correlated with the production of eicosanoids and nitric oxide respectively (Sharara et al., 1997; Yeo et al., 2003).

Regarding the ASOs, this class of drugs can be recognized from the immune system causing immunostimulatory effects that depend on the chemistry, the design and on the nucleotide sequence of the drug (Agrawal and Kandimalla, 2004). In some cases, the effects on the immune system are wanted and are used in therapy such as in autoimmune and cancer treatment (Kline and Krieg, 2008; Krieg and Davis, 2001), but when ASOs are not used for the treatment mentioned above, these effects are unwanted. Also in this case, the activation of the immune system is mediated by the binding to the PRRs, particularly the TLR3, TLR7, and TLR8. To avoid the side effects connected with the activation of the immune system, chemical modifications have been introduced over the years. For example, the 5′-methylation of cytosine residues has been introduced to reduce the immune stimulatory effect of CpG DNA sequences. Also, the modification 2′F,2′ methoxyethyl and the locked nucleic acid (LNA), that were introduced to increase the affinity with the target, showed the ability to reduce the stimulation of the immune system induced by ASO drugs (Henry et al., 2000; Sewing et al., 2017; Sioud, 2006).

Despite efforts to introduce chemical changes in structure of ASOs drugs, some of them still interact with the immune system causing overactivation; the result of this activation induces proinflammatory effects such as flu-like symptoms, thrombocytopenia and injection site reactions (Crooke et al., 2017; Rudin et al., 2001; Thomas et al., 2013; Voit et al., 2014).

Aptamers are another class of RNA based drugs. Also in this case, therapies that use aptamer drugs can develop off-target effects that involve the immune system causing an immunostimulation. The study of the adverse effects that occur on the immune system associated with therapeutic aptamers is focused on the interaction with the TLR9 (Keefe et al., 2010). Once the pathway TLR9 interacts with the aptamer drugs, it can induce the production of cytokines in particular IL-6 and IFN-α and also the activation of antigen-specific B cells (Cooper et al., 2008; Krieg, 2006).

It is known that the unmethylated 2′-deoxycytidine-phosphate-2′-guanine (CpG) sequence is responsible for the interaction with TLR (3,7,8,9) and induce the activation of the innate immune system.

mRNA drugs can be used to activate the innate immune system such as the field of the vaccination but in the same case the activation of the immune system is unwanted such as in protein-replacement therapies. The activation of the immune system is mediated by the interaction with the pattern recognition receptors such as the TLR, in particular, TLR3, TLR7, TLR8. The result of the activation of these TLR is the secretion of INF, TNF, IL-6, IL-12 (Sahin et al., 2014). Regarding protein-

Table 3
Examples of NA-based drugs approved in recent years.

Type	Drug	Approval	Reference
mRNA	Comirnaty (BNT162b2) – vaccine against Covid-19	2020	Feng et al., 2021
	Spikevax (mRNA-1273) – vaccine against Covid-19	2020	Feng et al., 2021
siRNA	Patisiran – treatment of polyneuropathy in people with hereditary transthyretin-mediated amyloidosis	2018	Al Shaer et al., 2019
	Givosiran – treatment of adults with acute hepatic porphyria	2019	Scott, 2020
	Lumasiran – treatment of primary hyperoxaluria type 1	2020	Scott and Keam, 2021
ASO	Formivirsen – treatment of cytomegalovirus retinitis in immunocompromised patients	1998	Roehr, 1998
	Mipomersen – treatment of familial hypercholesterolemia	2013	Hair et al., 2013
	Defibrotide – treatment of veno-occlusive disease of the liver of people having had a bone marrow transplant	2016	Kaufman, 2017
	Inotersen – treatment of nerve damage in adults with hereditary transthyretin-mediated amyloidosis	2018	Keam, 2018
	Casimersen – treatment of Duchenne muscular dystrophy	2021	Shirley, 2021
Aptamer	Avacincaptad Pegol – treatment of age-related macular degeneration	2023	Al Shaer et al., 2024
	Pegaptanib – treatment of neovascular age-related macular degeneration	2004	Vinores, 2006

replacement therapies, mRNA drugs can, as a side effect, activate the innate immune system through the receptors mentioned above. Modifying the structure of the mRNA can reduce its recognition and interaction with TLRs. Karikó et al. (2008) studied the replacement of uridine residues with pseudouridine, which induces changes in the secondary structure of the mRNA, leading to alterations that reduce the unwanted activation of the immune system. miRNA drugs can find a large application in the field of cancer immunotherapy, in particular, they can act as miRNA mimics when there are defections of the miRNA or antagonists that are able to inhibit the hyperfunctioning miRNA. The drug MRX34 is a miRNA mimic used for the treatment of lung cancer. During the trial, severe immune-related toxicities were developed by few patients due to the ability of the miR-34a to regulate the immune cell function (Cortez et al., 2016). The activation of the innate immune response is mediated by the interaction with the TLR7.

4.3. Na-drugs and NAMs

It has been demonstrated that siRNA can induce the production of cytokines, in PBMCs; the cytokines involved are IL-6, TNF-α, and IFN-α. The pathways that are involved in these mechanisms are the TLR7 in mice and TLR7/8 in humans (Meng and Lu, 2017). This immunostimulation that develop following exposition to the siRNA drug is dependent on the sequence of nitrogenous bases that compose the drug, in particular, the presence of the nucleoside uridine in the sequence can induce the production of the cytokines through the via TLR7/8. In this way, siRNA can activate an inflammatory cascade that induces the production of cytokines, inflammatory and vasoactive mediators (Robbins et al., 2009).

It has been shown that modifications on the nucleosides can reduce the development of these side effects as previously explained(Sioud, 2006). An *in vitro* test useful to analyse the response of the immune system against the therapeutic oligonucleotides is the use of the whole blood. This test allows identification of immune system activation induced by oligonucleotides, which can lead to severe conditions of excessive cytokine release (i.e., cytokine storm). In particular, this test involves the use of human whole blood assay (WBA) anticoagulated with Hirudin; the cells are incubated for 48 h with the siRNA drugs and the concentration of IFN-α and TNF-α in the supernatant is analysed developing the ELISA test (Coch et al., 2013). The same test can be performed using human PBMC (Fig. 4). Coch et al. (2013) compared the release of cytokine induced by a non-methylated and a methylated siRNA developing the same test using WBA or PBMC. The inhibition of cytokine released obtained with the methylation of the siRNA was observed in the experiments performed using both PBMC and WBA.

Regarding the study of the unwanted effects of ASO drugs, *in vivo* screenings with rodents are difficult to translate to humans because rodents are particularly sensitive to immune stimulation (Monteith et al., 1997; Paz et al., 2017) but on the other side, *in vitro* tests has been performed. The use of isolated PBMC or whole blood assay are useful to study the cytokine release correlated to the interaction with ASO (Burel et al., 2022; Coch et al., 2013; Lankveld et al., 2010; Sewing et al., 2017). The cells are treated with different doses of ASOs, after incubation, the release of cytokines is analysed by performing ELISA on the cell culture supernatants to allow easy, fast, and reliable predictions. The cytokines involved are IL-6, IFN-α, and TNF-α.

Another *in vitro* test suitable for studying the immunotoxicity of this class of RNA-based drugs involves the use of BJAB cells, an Epstein-Barr virus (EBV)-negative Burkitt-like lymphoma cell line. These cells are sensitive to stimulation by CpG and non-CpG oligonucleotides in a sequence and TLR9-dependent manner (Anderson et al., 2021). The release of the cytokine CCL22 from BJAB cells treated with ASOs drugs, measured with qRT-PCR, is a predictive way to investigate the proinflammatory profile of ASOs drugs. The use of the BJAB cells is an emergent tool for inflammation screening because of the high-level expression of TLR9 (Pollak et al., 2022).

To investigate the potential immune activation of aptamers, Avci-Adali et al. (2013) performed a study using fresh human whole blood.

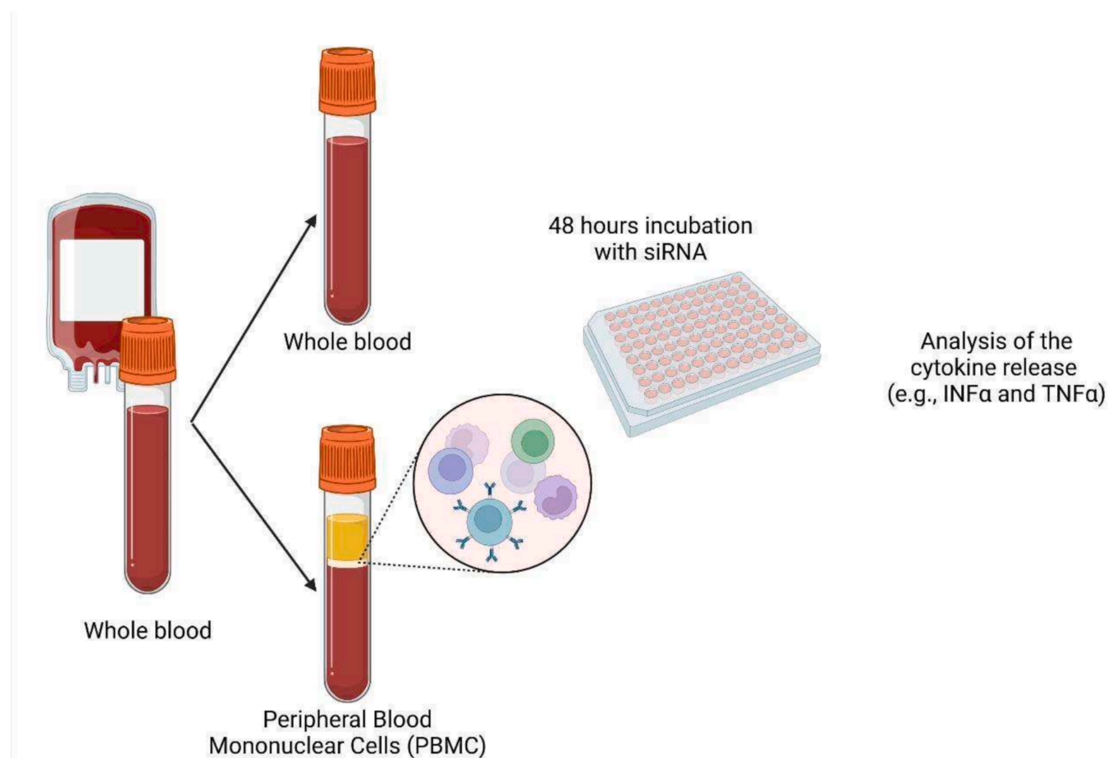

Fig. 4. Protocol of the Whole Blood Assay and the Peripheral Blood Mononuclear Cells (PBMCs) Assay. The cells (PBMCs or Whole blood) are incubated for 48 h with siRNA drugs. After the exposure time, the release of IFN-α and TNF-α in the supernatant is analysed developing the ELISA test. Created in BioRender. https://BioRender.com/d35t486.

The experiments were performed using human peripheral blood cells incubated for 2 and 4 h with the selected ssDNA oligonucleotides in an *in vitro* closed-loop model (modified Chandler-Loop) (Chandler, 1958); this model allows to study of blood cell activation under dynamic flow conditions and mimic the blood movement in a vessel. For this study, two ssODN were used: SB_ODN and CpG ODN (as a positive control), and microarray analyses were performed to analyse the changes in gene expression. This study shows that the treatment with SB_ODN affects the expression of the genes CCL8, CXCL10, CCL7 and CXCL1. The chemokine CCL8 is produced by monocytes and its chemotactic activity regards different cells, in particular NK cells, T cells and monocytes. The chemokine CXCL10 is produced by different types of cells: fibroblast, monocytes, and endothelial cells. Like CCL8, CXCL10 has chemotactic activity to monocytes, macrophages, T cells, NK cells and DCs. It also acts on T cells promoting their adhesion to endothelial cells. The chemokine CCL7 is secreted by various cells, including keratinocytes, stromal cells, airway smooth muscle cells, fibroblasts, parenchymal cells, leukocytes, and tumour cells. The function of this chemokine is to chemo-attract monocytes, eosinophiles, basophiles, monocytes, dendritic cells, NK, neutrophiles and activated T cells. CXCL1 is a chemokine expressed in leukocytes and it is chemotactic for activated T cells. The analyses with Gene ontology terms and KEGG pathways revealed that the genes modulated after incubation with SB_ODN are the ones that are involved in an immune and inflammatory response. Avci-Adali et al. (2013) demonstrated the potential of aptamers to activate the immune system after systemic application to the human blood.

5. Conclusion

The transition toward the reduction in the use of animal models in immunotoxicity testing marks a significant advancement in therapeutic development, reflecting both ethical considerations and scientific progress. This review highlights several promising alternatives to animal testing, including *in silico* and *in vitro* assays, as well as dynamic systems such as PBMC-based cytokine release studies, WBA, and closed-loop systems. These methodologies have already demonstrated their utility in evaluating the immunotoxicity of various therapies, showcasing their potential as reliable substitutes in the assessment of immune-related safety.

Validation of these alternative approaches is a crucial step toward their broader acceptance and application in regulatory settings. As these models continue to evolve, they offer an invaluable opportunity to enhance our understanding of immune-modulating mechanisms, thus facilitating the development of safer and more effective therapies. By combining these innovative tools with ongoing research, the scientific community can refine therapeutic designs, evaluate safety with greater precision, and reduce the incidence of adverse immune-related effects.

Moreover, integrating these advanced systems into the preclinical and clinical workflow aligns with the global push for ethical, innovative, and scientifically robust methods of drug evaluation. This shift not only improves the predictive accuracy of immune responses but also supports the reduction of animal testing in therapeutic development, contributing to a more humane approach. As these systems become increasingly sophisticated and validated, they will pave the way for a more efficient, reliable, and ethical path forward in therapeutic evaluation, leading to better outcomes for both patients and society.

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Declaration of competing interest

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Data availability

Data will be made available on request.

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