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Review

Perilous paradigm of graphene oxide and its derivatives in biomedical applications: Insight to immunocompatibility

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ABSTRACT

With advancements in nanotechnology and innovative materials, Graphene Oxide nanoparticles (GONP) have attracted lots of attention among the diverse types of nanomaterials owing to their distinctive physicochemical characteristics. However, the usage at scientific and industrial level has also raised concern to their toxicological interaction with biological system. Understanding these interactions is crucial for developing guidelines and recommendations for applications of GONP in various sectors, like biomedicine and environmental technologies. This review offers crucial insights and an in-depth analysis to the biological processes associated with GONP immunotoxicity with multiple cell lines including human whole blood cultures, dendritic cells, macrophages, and multiple cancer cell lines. The complicated interactions between graphene oxide nanoparticles and the immune system, are highlighted in this work, which reveals a range of immunotoxic consequences like inflammation, immunosuppression, immunostimulation, hypersensitivity, autoimmunity, and cellular malfunction. Moreover, the immunotoxic effects are also highlighted with respect to *in vivo* models like mice and zebrafish, insighting GO Nanoparticles' cytotoxicity. The study provides invaluable review for researchers, policymakers, and industrialist to understand and exploit the beneficial applications of GONP with a controlled measure to human health and the environment.

1. Introduction

Graphene oxide (GO), a carbon-based nanomaterial derived from graphene, consists of a monolayer of sp2-hybridized carbon atoms arranged in a hexagonal honeycomb lattice (Fig. 1). This arrangement imparts unique electrical and conducting features to graphene. Graphene nanomaterials are studied for many biomedical applications, like bio-imaging, biosensors, gene and drug delivery, and scaffolds for cell culture. Graphene-based nanomaterials are recognized for high mechanical strength property and can be easily functionalized for different applications [1]. Another aspect of peculiar properties of Graphene oxide nanoparticles (GONPs) and their derivatives is the ability to generate reactive oxygen species (ROS), which make them a promising biomedical material for diseases treatment like cancer. Graphene oxide and its derivatives possess a plethora of biomedical properties that make them promising candidates for various applications in the field of medicine. Firstly, their excellent biocompatibility ensures minimal toxicity when interfacing with biological systems, which is crucial for biomedical applications. Moreover, their high surface area facilitates efficient drug delivery by enabling high drug loading capacity and controlled release kinetics. Additionally, graphene oxide-based materials exhibit remarkable photothermal properties, allowing for targeted

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cancer therapy through photothermal ablation of tumors. Furthermore, their unique physicochemical properties enable them to serve as versatile platforms for biosensing, bioimaging, and tissue engineering applications. Overall, the biomedical properties of graphene oxide and its derivatives hold great potential for revolutionizing diagnostics, therapeutics, and regenerative medicine.

Graphene oxide is synthesized by modifying graphite materials with oxidation reactions, typically the modified Hummers method, which involves oxidizing graphite powder in a concentrated solution of sulfuric acid and potassium permanganate [2]. The physical, biochemical and biological properties of nanoparticles like solubility, toxicity and reactivity can be fabricated by tailoring their structural and chemical properties during the synthesis process. The various factors in the synthesis process of graphene oxide like reagent quality and quantity determines their physicochemical characteristics like lattice defects, types of functional groups at the edges, and aggregating tendency. These factors tend to influence their utility in biomedical application. Though, the physiochemical structure determines the utility of biomedical applications of graphene oxide nanomaterials and its derivative, the most important factor that is considered for their usage is the biocompatibility with the physiological and biological components like cells, tissue etc.

Graphene oxide nanomaterials and their derivatives exhibit remarkable biocompatibility, a critical feature for their application in biomedical fields. Their biocompatibility stems from several factors, including their structural similarity to biological molecules, such as DNA and proteins, which reduces immune responses. Moreover, their surface can be functionalized with various biomolecules, enhancing their compatibility with biological systems and enabling targeted interactions with specific cells or tissues [3]. Additionally, graphene oxide nanomaterials demonstrate low cytotoxicity and minimal adverse effects on cellular functions, ensuring their safe use in biological environments. Furthermore, their high surface area facilitates efficient adsorption of biomolecules, making them promising candidates for drug delivery systems and biosensors. Numerous studies have determined the biocompatibility of GO and its derivatives focusing on different factors that plays important role in the determination of their usage as biomedical material. Chng and Pumera demonstrated a strong, dose-dependent cytotoxic response of four GO nanomaterials in lung epithelial cells, suggesting a correlation between GO oxygen content/functional groups and toxicological behavior[4]. The biocompatibility and bio-distribution of GO may be changed significantly by modification in their structure through conjugation and coating process using other materials like biopolymer and surfactant. However, the modification requires careful attention in maintaining GO's favorable properties required for biomedical application like drug delivery vehicle.

Graphene oxide and its derivatives have garnered significant interest for their potential interactions with immune cells, which play a crucial role in both innate and adaptive immune responses. Studies have shown that graphene oxide nanomaterials can modulate the activity of immune cells such as macrophages, dendritic cells, and lymphocytes. These interactions are highly dependent on various factors including size, shape, surface charge, and functionalization of the nanomaterials. Graphene oxide's unique physicochemical properties allow it to influence immune cell functions in diverse ways, including promoting cell proliferation, cytokine secretion, and phagocytosis. However, it's important to note that the immune response to graphene oxide can be complex and context-dependent, with potential for both immunostimulatory and immunosuppressive effects. Understanding the precise mechanisms underlying the interaction between graphene oxide and immune cells is crucial for harnessing its therapeutic potential and ensuring safe biomedical applications. A critical first step in assessing nanoparticle toxicity for its *in vivo* use is knowing how NP-protein corona formation relates to the surface features of the nanoparticles and its interaction with immune system molecules in the body. Graphene oxide (GO) can elicit both innate and adaptive immune responses, resulting in a significant recruitment of immune cells, which has the potential to revolutionize nano-based immunotherapeutic strategies[5].

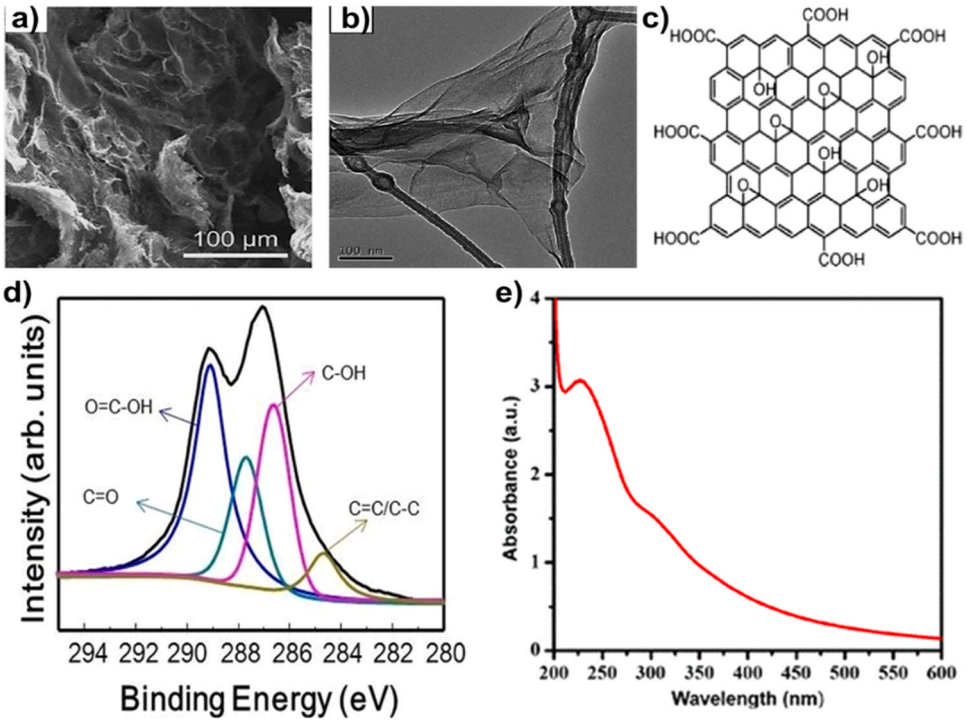

Fig. 1. Characterization of GO. (a) SEM of GO. Reproduced with permission [6]. (b) TEM of GO. (c) Structure of GO. Reproduced with permission[8]. (d) XPS spectra in the C1s of GO. (e) UV-Vis spectra of GO. Reproduced with permission [10].
(a) Reproduced from with permission [7]. (b) Reproduced from with permission[9].

2. Properties of graphene oxide

Graphene Oxide (GO) is often considered a promising material for various sectors due to its excellent water processability, amphiphilicity, and both covalent and non-covalent surface functionalization. Owing to the partial coverage of elemental planes and edges by diverse functional groups, primarily hydroxyl, epoxy, ketone, ester, organosulfur, and lactol structures, GO exhibits high chemical reactivity. In polar solvents like water, GO may hydrolyze to form carboxylic acid or sulfate groups, undergoing significant changes when combined with different compounds [11].

The presence of hydrophilic oxygenated functional groups, such as hydroxyl, ketones, epoxides, and carboxylic acids, on the surface of GO nanoparticles facilitates their interaction with proteins, DNA, and antibodies by enabling reactions across the GO plane and edges [12]. Graphene oxide features a two-dimensional aromatic surface with π-π stacking interactions, forming stable complexes with anticancer drugs, making it an ideal substrate for high drug loading. Consequently, GO nanoparticles are extensively studied as carriers for cancer drug delivery. A Chitosan-functionalized GO (CS-GO) complex was synthesized and employed for efficient delivery of anticancer drugs and plasmid DNA, loaded through π bonds and electrostatic interactions [13].

Controlling the characteristics of GO is a challenging task. The materials used for GO particle synthesis, such as graphite rods, graphite foils, or expanded graphite, vary in density, flake size, and stacking. The quality, amount, process temperatures, and the precision of post-reaction mixture purification have a crucial impact on the final product's characteristics. Contamination necessitates post-product synthesis treatment. During the sonication process, side dimensions of the flakes may change, or a rupturing of the C-C bond in the carbon network structure can occur, affecting its functionality[14]. GO features and their direct or indirect effects on toxicity may depend on lattice flaws, the quality of functional groups at the flake's edges, and the potential for aggregation. High-molecular-weight proteins are preferentially adsorbed by reduced graphene oxide nanomaterials through hydrophobic contact, as indicated by the study of the protein corona on reduced GO. Substantial aggregation of the reduced material is governed by its surface functionality[15].

3. Interaction of GO-based formulations with innate and adaptive immune systems

Graphene Oxide (GO) and GO-based formulations interact with the metabolic system of a living body through natural ingestion, inhalation, and dermal contact. Toxicological investigations of nanoparticles are conducted using animal models through direct exposure instigation. Other methods, such as intravenous, intraperitoneal, and subcutaneous injections, are employed to administer GO. Inhaled GO nanosheets reveal potential cellular toxicity by disrupting the pulmonary surfactant ultrastructure and biophysical characteristics, which serve as the host's first line of defense. GO is considered an excellent medication delivery system, often added to anticancer medications to enhance oral bioavailability. Graphene nanosheets have been specifically detected in the gut and intestines of mice in studies involving oral administration. It is noteworthy that the absorption of nanoparticles in the intestines following oral administration is limited, and they are safely excreted. In contrast, alternative research indicates that when administered intraperitoneal, the majority of GO remains localized near the injection site. An investigation into the placental barrier reveals that it does not effectively impede the dispersion of nanoparticles within the fetus or their transmission to the fetus[16]. The interactions between nanomaterials and immune cells within the human body are of significant interest due to the vital role played by the immune system in eliminating foreign substances from the bloodstream. The human immune system comprises two fundamental components: the innate immune system and the adaptive immune system. The innate immune system serves as the body's initial defense mechanism against invaders, swiftly eliminating contaminated cells or foreign substances through a rapid, non-specific immunological response. In contrast, the adaptive immune system, which developed later in evolutionary history, provides more precise, albeit slower, protective responses. This adaptation is particularly advantageous for long-lived and migratory species that do not consistently inhabit the same environment. The adaptive immune system employs specialized cells, such as T and B cells, to execute its functions. The innate immune system leverages the capabilities of the adaptive immune responses, as they evolve automatically and without conscious control. Innate immunity represents the body's first line of defense against invaders, comprising various elements like serum proteins, cells, and physical barriers. It relies on pattern recognition receptors (PRPs) to identify large molecular patterns found in pathogens and pathogen-associated molecular patterns (PAMPs) present in invaders. The inherent processes of the immune system, along with its defense mechanisms against external intruders, make it particularly receptive to the uptake of phagocytic cells[17].

Adaptive immunity encompasses humoral immunity and cell-based immunity as distinct categories. Cell-based immunity is responsible for the killing of affected endogenous cells by cytotoxic T cells, a process, that is antigen-specific. On the other hand, humoral immunity is responsible for antibody-mediated reactions that identify and neutralize their specific targets. When pattern recognition receptors simultaneously stimulate the recognition of pathogen-associated molecular patterns (PAMPs) along with the presentation of matching peptide-major histocompatibility complex (MHC), it encourages the production of co-stimulatory factors, which serve as positive indicators for antigen-specific T cells. Class II molecules, crucial for humoral immunity and T-helper cell proliferation, can present antigens, only found in specialized antigen-presenting cells (APC), while class I major histocompatibility complex molecules (MHC-I) can be found in every cell. This distinction is essential to the immune response. It is crucial to recognize that the interaction of nanoparticles (NPs) with the immune system is a multifaceted process. Key factors influencing this interaction include the size, shape, and surface properties of NPs, encompassing composition, structure, charge, and hydrophilicity. The presence of bioactive components within the sample or the promotion of chemical reactions that trigger immune activation plays a pivotal role. Modifying the size of NPs can impact their targeting, in vivo cellular uptake, intracellular distribution, and permeability across biological barriers [18]. The EPR (enhanced permeation and retention) effect, which takes advantage of the improved ability of nanoparticles (NPs) to penetrate defenestrated tumor vasculature, is effectively utilized with NPs ranging in size from 10 to 200 nm. NPs that can readily traverse biological barriers, typically fall within the size range of 10–500 nm. For many years, scientists have been aware that NPs in the size range of 5–10 nm are eliminated through the kidneys, while NPs sized 200 nm or larger are cleared by the mononuclear phagocyte system (MPS). However, smaller NPs face challenges in this regard, as their higher surface area to volume ratio increases the potential for particle toxicity due to an elevated risk of opsonization, which can lead to adverse reactions. To counter this, one approach involves coating the NP surface with natural membranes like those derived from red and white blood cells, extracellular vesicles (such as exosomes), or extracellular vesicles to prevent immune recognition. Another commonly used coating method employs Polyethylene glycol (PEG), which reduces hydrophilicity and neutralizes surface charge, thus prolonging NP circulation in the bloodstream. In addition to size, the shape of NPs significantly influences the response of the immune system. Just as non-spherical bacteria or viruses can evade immune recognition, non-spherical particles like rods, disks, and those resembling unidentified flying objects (UFOs) can also avoid recognition by the MPS. The 'invisibility' of elongated nano-objects is believed, to be a result of either (i) a faster flow rate due to fluid dynamics or (ii) the local geometry preventing internalization. With the capability to systematically study the significance of shape in NP interactions with

biological systems, materials of any conceivable shape can now be engineered[19].

Numerous factors, including administration routes, physicochemical properties, particle aggregation, and surface coatings, have the potential to influence the biodistribution, biotransformation, and excretion of GO. When administered intraperitoneally, modified GO derivatives with polyethylene glycol (GO-PEG) are primarily retained within the reticuloendothelial system, encompassing the liver and spleen. However, when taken orally, these GO-PEG compounds do not exhibit tissue absorption. GO can undergo substantial biotransformation due to its increased chemical reactivity. In contrast, modifications involving amphiphilic polymers enhance the adherence of large sheet-like GO aggregates to the cell membrane without cellular uptake. Research has demonstrated that the toxicity of GO is influenced by its biotransformation in blood plasma, resulting in the development of a biological corona on biodegraded GO nanosheets by components in human blood plasma and free radicals. This biotransformation affects the interactions between GO and cells, causing damage to the cell's ultrastructure and reducing the levels of reactive oxygen species. However, no degradation or structural alterations are observed when GO is orally absorbed, suggesting that oral absorption does not lead to GO biotransformation. GO is particularly challenging to eliminate from the lungs, potentially leading to granuloma formation, inflammation, cell infiltration, and pulmonary edema. GO nanoparticles can be removed from the liver via the hepatobiliary pathway, following the bile duct into the duodenum. Additionally, functional GO-PEG derivatives primarily accumulate in the liver and can be gradually eliminated through the kidneys and feces. The spleen acts as a physical filter, trapping GO particles larger than 200 nm[17]. The fate of graphene-based materials (GBMs) inside an organism exposed to them, is determined by a combination of their extrinsic and intrinsic characteristics. Extrinsic features, shaped by interactions with the biological environment and primarily governed by the corona, play a significant role. Intrinsic physicochemical traits, such as thickness, the carbon-to-oxygen (C/O) ratio, and functionalization, are also key factors. These intrinsic characteristics of GBMs influence their accumulation, degradation, clearance, biodistribution, and potential translocation to secondary organs. However, the adsorption of proteins and other biomolecules from the surrounding biological milieu can modify these properties. Moreover, local ion concentrations close to nanomaterials like GBMs can also have an impact. It is important to note that the intrinsic material properties of GBMs can undergo alterations due to processes like immunological cell degradation or other forms of biotransformation. This underscores the importance of thoroughly characterizing the material, both in its pristine state and in situ, during or after exposure[20].

Presently, conflicting data exists regarding the impact of graphene oxide on various types of immune system cells. Some studies indicate that GO nanoparticles damage cell membranes, increase the production of reactive oxygen species (ROS) in neutrophils, and the proliferation of T lymphocytes is impeded by unaltered GO, and activated T helper cell viability is decreased[21]. Other studies show that ROS production decreases in monocytes[22]. Furthermore, evidence suggests that graphene quantum dots may have a dendritic cell-mediated effect on T-cell response[23]. Binding to proteins and reacting with phosphatidylcholine, which creates ROS. Large GOs can damage cell membranes. Increasing the dose and duration of exposure to GO also induces a steady drop in SOD and GSH activity. Stimulation of these signaling pathways results in the activation of two pro-apoptotic Bcl-2 protein family members, Bim and Bax. The caspase-3 pathway activation results in DNA damage, which triggers inflammatory reactions, apoptosis, and necrosis. The generation of ROS is the main factor that activates the TGIF-β and MAPK signaling pathways in cells treated with GO[24].

The notable immune cells -monocytes and macrophages, are found in the bloodstream and in different tissues. They play a crucial part in the innate immune response because their primary tasks include phagocytosis, antigen processing and presentation (APCs), and the production of cytokines. A study by Wibroe et al. demonstrated the activation of a complement cascade due to the oxygen content of GO[25]. Alveolar macrophages interact with Graphene-based nanomaterials like graphene oxide after pulmonary exposure. Weimann et al. used the rat NR8383 alveolar macrophage cells to investigate on the toxicity of eighteen distinct inorganic nanomaterials, including graphite nanoplatelets [26]. Upon pulmonary exposure to GBMs, neutrophils are mobilized in the airways. According to research by Mukherjee et al., The interaction between GO sheets and isolated human neutrophils causes a dose-dependent loss of cell viability and the size-dependent formation of neutrophil extracellular traps (NETs)[27]. Neutrophils use NETs, which are nuclear chromatin structures, alongside neutrophil elastase (NE) and myeloperoxidase (MPO) to destroy pathogens, extracellularly. The study emphasizes the significance of nanomaterials' direct contact and suggests that immune cells may react to such materials in a way that is similar to how they react to bacteria and fungi. In previous research, Orecchioni et al. used a variety of assays, including whole-genome micro-array analysis, to investigate the effects of two types of GO sheets, with different lateral sizes [100–500 nm small(GO-S) and 1–10 μm large(GO-L)] on human immune cells[5]. This work verified that the number of transcripts that graphene changed were strongly impacted by the functionalization of GO. GO-S sheets were shown to have a greater influence on donor peripheral blood cells than GO-L sheets, as evidenced by the activation of the important genes linked to immune responses and the production of the pro-inflammatory cytokines IL-1 and TNF-α. Microarray analyses of THP-1 monocytic cells (innate immune system) and Jurkat T cells (adaptive immune system) demonstrated that T cell migration was associated with the activation of many important immunological pathways, leukocyte chemotaxis, and T cell chemotaxis regulation. The two GO molecules' surface functionalization with amino groups (GO-NH2) had a more targeted impact on the production of cytokines in particular cell subpopulations[20].

Recently Zamorina *et al.* examined *in vitro*, how PEGylated graphene oxide (GO-PEG) nanoparticles affected activated Th17 [22]. The quantity and viability of T-helper cultures did not change when GO-PEG was present at high quantities (25 g/mL). Yet, in these cultures, the proportion of proliferating T-helpers has decreased. GO nanoparticles treated with linear PEG boosted the proportion of Th17/22 cells and the production of IFN-γ, while those treated with branched PEG reduced the production of IL-17. The study also found that BP-GO nanoparticles showed cytotoxic and/or apoptotic effects towards the most proliferating population of Th17, leading to a decrease in IL-17 production. The study also found that NK cells were apoptotic when exposed to high GO-PEG concentrations. The apoptosis-inducing qualities of nanoparticles may rise because of the reduction in protein adsorption at the BP-GO surface. The findings suggest that GO-PEG nanoparticles could have therapeutic potential in modulating the immune response in autoimmune diseases, however, detailed research is needed to understand their effects on different immune cells. A study by Gao *et al.* explores the organ-specific response to GO exposure on immune responses and hematopoiesis in mice [28]. The results show that GO exposure causes alterations in immune components in the liver and spleen at different time points, affecting populations of macrophages, increasing inflammation, and altering cytokines transcription. The study also found that GO disrupts hematopoiesis in the spleen, leading to fluctuations in circulating platelet counts. This suggests that GO exposure could lead to systemic immune responses and hematopoietic disturbances, which could be potentially harmful.

In the examination of GO effects on the dendritic cell line DC2.4, the different forms of GO enhanced TNF-α production, while IL-6 production remained unaffected. Single-layer GO increased the release of TNF-α and IL-6, while multi-layer GO pretreatment resulted in a decreased IL-6 response in cells. Notably, single-layer GO induced cell aggregation without significantly increasing cell mortality, whereas multi-layer GO exhibited higher cytotoxicity and elevated levels of ROS[29]. Zhou et al. investigated the impact of lateral GO size by exposing mouse DC to

micro- or nano-sized GO [30]. Their findings revealed that smaller GO was primarily internalized, while larger GO caused cytoskeleton remodeling leading to ICAM1 translocation. The formation of large clusters of DC-GO-T-cells and the subsequent T-cell activation were confirmed through additional T-cell culture. Similarly, large GO promoted DC generated from human monocytes by increasing the expression of costimulatory molecules CD80 and CD83. In contrast, PEGylated GO with a size less than 200 nm reduced CD83 expression in human DC. These findings underscore the significance of GO size and emphasize the relationship between GO surface and cytoplasmic membrane[31].

4. Immunotoxic effects of graphene oxide nanoparticles

The immune system consists of intricate molecular and cellular networks that defend our bodies against foreign particles while maintaining tolerance towards internal substances. The literature contains numerous reports describing both the negative and positive impacts of GO nanoparticles on various cells in different organisms. Immune-competent cells are employed in research to regulate endotoxin content in cells.

4.1. Inflammation

Inflammation is a pivotal mechanism in immunotoxic effects, often triggered by exposure to nanomaterials resulting from oxidative stress, pollutants, and mechanical damage[32]. Macrophages play a crucial role in providing a robust barrier to Graphene Oxide Nanoparticles (GO NPs) through phagocytosis. However, before reaching the target site, GO NPs face a high likelihood of being eliminated by macrophages or inducing an inflammatory response. In a study by Yue et al.[33], using phagocytes and non-phagocytes as test cells, the cellular reactions of 2 μm GO and 350 nm GO were examined. Both sizes of GO sheets induced similar amounts of macrophage cellular uptake, but micro-sized GO triggered more potent inflammatory responses, evident by significant increases in several cytokine levels. The Fc receptor-mediated phagocytosis route allowed macrophages to surpass the strong electrostatic pull between the negatively charged cell surface and GO, showing a higher absorption capacity than non-phagocytes. The author's hypothesis suggested that the pronounced immunological response observed in macrophages in response to micro-sized GO was likely attributed to steric effects imposed by the larger GO particles. Subsequent research supported these findings, indicating that larger graphene oxide (GO) particles led to increased inflammation due to their stronger interaction with Toll-like receptors (TLRs). To mitigate macrophage recognition of nanomaterials, researchers maintained a particle size of approximately 150 nm and introduced hydrophilic surfaces to reduce opsonin-protein interactions[34]. Graphene has potential in cancer treatment by stimulating macrophages and dendritic cells. In a study by Feito et al.[35], investigating the impact of GO nanosheets designed for hyperthermia cancer therapy on macrophage and lymphocyte function, 6-armed GO increased the secretion of TNF-α by macrophages, while 1-GOs and 6-GOs led to dose-dependent cell proliferation and reduced IL-6 levels in primary splenocytes. These results suggest that GOs possess relatively mild inflammatory properties, making them favorable for hyperthermia cancer therapy.

In a study by Chen et al.[36], GO-induced autophagosome formation was modulated by activation of TLR4 and TLR9, leading to a pro-inflammatory response. Increasing the concentration of GO resulted in increased cell death. Another study with mouse macrophages demonstrated that high accumulation of GO caused oxidative stress, the release of TNF-α, and TLR4-dependent necrotic death [37]. In a contrasting experiment with human macrophages, endotoxin-free GO caused no inflammatory response. In primed macrophages, GO caused inflammasome activation due to lysosomal damage, probably induced by mechanical stress [38]. Another study by Mukherjee et al. [27] demonstrated that the interaction of nano-sized GO with the lipid cell membrane caused disruptions leading to the formation of neutrophil extracellular traps (NETs). The Graphene Flagship work has established a Hummers' method for endotoxin-free GO production [39]. Using this protocol, a study was conducted with primary human macrophages, where small and large GO sheets (50–300 nm, 1–2 nm thickness) did not trigger pro-inflammatory TNF-α production and were phagocytosed by macrophages [40]. However, it was also discovered that GO caused LPS-primed macrophages to produce caspase-dependent IL-1, a sign of inflammasome activation. Additionally, it was shown that the inflammasome sensor NLRP3 has a particular function in GO-induced IL-1 production [41]. These differences could be attributed to the use of different cell models. An in vivo study on mice found evidence of

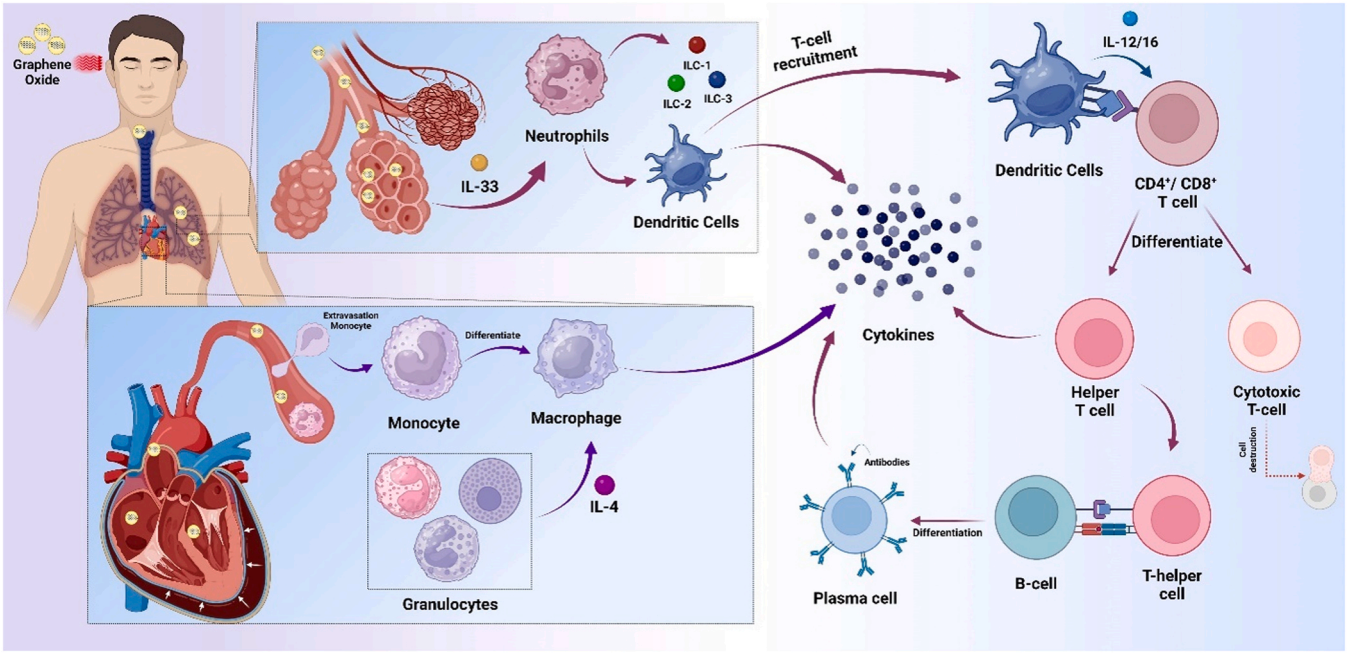

Fig. 2. Interaction of GONP with adaptive immune cells and innate immune cells.

size-dependent effects of GO, with large GO showing considerably greater inflammatory reactions than tiny GO. Greater activation of the NF-κB pathway and enhanced TLR-TLR interactions were correlated with large GO. Additionally, it improved M1 polarization, connected to higher inflammatory cytokine production and immune cell recruitment [42].

4.2. Immunosuppression and immunostimulation

4.2.1. Immunotoxic effects of graphene oxide nanoparticles (GONP) on immunosuppression

Graphene Oxide Nanoparticles (GONPs) have been reported to exhibit immunotoxic effects, leading to immunological suppression within the immune system. Cytotoxicity of GONPs was observed at a dosage of 500 μg/mL in experiments conducted on murine macrophages and human whole blood cell cultures. These findings suggest that GONPs might have detrimental effects on immune cells. Furthermore, research has shown that GONPs triggered an inflammatory response in both murine whole blood cell cultures and macrophages, even without the presence of lipopolysaccharide (LPS). This finding suggests that GONPs can stimulate the immune system and initiate a series of inflammatory responses. Another noteworthy discovery is that the application of GONPs inhibited the synthesis of interferon-gamma (IFN-γ) in phytohaemagglutinin (PHA)-stimulated cells and the creation of interleukin 6 (IL-6) in lipopolysaccharide (LPS)-stimulated cultures. IL-6 and IFN-γ are crucial elements of the immune response. Suppressing these cytokines can significantly impair immunological function. GONPs stimulated the synthesis of interleukin 10 (IL-10) in whole blood cell cultures, even without the presence of mitogens. IL-10 is a cytokine that possesses anti-inflammatory characteristics and has a vital function in controlling the immune response. Although this observation suggests a potential effort to mitigate the inflammatory impact of GONPs, more investigation is required to comprehensively comprehend the broader immunomodulatory consequences [43].

4.2.2. Immunotoxic effects of graphene oxide nanoparticles (GONP) on immunostimulation

Graphene Oxide Nanoparticles (GONPs) have been observed to elicit immunostimulatory effects. In the performed research, it was revealed that GONPs induced an inflammatory response in both murine human whole blood cell cultures and macrophages. This finding implies that GONPs can stimulate the immune system, hence initiating an immunological response. GONPs were shown to stimulate the synthesis of interleukin 10 (IL-10) by whole blood cell cultures without the presence of mitogens. IL-10 is a kind of protein called a cytokine that can reduce inflammation. It is important in controlling how the immune system responds to different stimuli. The observed increase in IL-10 production suggests that GONPs can regulate biomarkers of the immune system. Nevertheless, GONPs demonstrated a dose-dependent suppression of the production of IL-6 stimulated by LPS and interferon-gamma (IFNγ) produced by phytohaemagglutinin (PHA). Both IFNγ and IL-6 are key components of the immune response, and inhibiting their activity implies a possible compromise in immunological activation. The precise processes by which GONPs elicit immunostimulation remain incompletely comprehended, necessitating more investigation to unveil the involved fundamental molecular pathways [44].

4.2.3. Mechanisms of immune suppression induced by graphene oxide nanoparticles (GONP)

Graphene Oxide Nanoparticles (GONP) can elicit immunological suppression via several pathways. One of the primary processes involves the initiation of an inflammatory response, which can result in the synthesis of pro-inflammatory cytokines, subsequently inhibiting the functionality of immune cells. Moreover, ROS generation by GONP might result in oxidative stress. Oxidative stress has the potential to exacerbate immunological dysfunction and induce immune suppression. The phenomenon of oxidative stress can harm immune cells, compromising their regular physiological processes and resulting in a state of immunosuppression. Furthermore, it is noteworthy that GONP can engage in direct interactions with immune cells, namely dendritic cells, which play a crucial role in immunological responses. These interactions have the potential to disturb the regular operation of immune cells, thereby resulting in immune suppression. The immunological suppression caused by Graphene Oxide Nanoparticles (GONP) is attributed to the combined effects of these mechanisms [45].

4.2.4. Mechanisms of immune stimulation induced by graphene oxide nanoparticles (GONP)

The precise mechanisms behind the immune-stimulating effects of Graphene Oxide Nanoparticles (GONP) remain incompletely elucidated. Research findings have demonstrated that GONP, although passivated on its surface, is capable of inducing robust immunological reactions. The research indicates that peritoneal macrophages exhibit robust cytokine responses when exposed to PEGylated graphene oxide nanosheets (nGO-PEGs). Nevertheless, the precise molecular mechanisms responsible for the immunological activation generated by GONP have yet to be fully elucidated and are now the subject of ongoing research. Additional research is needed to comprehensively understand the fundamental processes and signaling channels by which GONP (graphene oxide nanoparticles) engage with immune cells and initiate an immunological response [46].

4.2.5. Recommended exposure limits for graphene oxide nanoparticles (GONP)

The determination of exposure limits for nanoparticles is subject to variation based on the regulatory criteria established in different countries. For the most precise and current information regarding suggested exposure limits for Graphene Oxide Nanoparticles (GONP), it is recommended to refer to certain regulatory bodies or organizations focused on occupational health and safety. Given the possible health hazards linked to exposure to GONPs, it is imperative to adhere to appropriate safety practices and recommendations to mitigate the risk of exposure to these nanoparticles [47].

4.3. Hypersensitivity

The variability in exposure limits for nanoparticles is contingent upon the regulatory rules established in different nations. For the most precise and current information regarding suggested exposure limits for Graphene Oxide Nanoparticles (GONP), it is recommended to refer to certain regulatory bodies or organizations focused on occupational health and safety. Given the possible health hazards linked to exposure to Graphene Oxide Nanoparticles (GONP), it is imperative to adhere to appropriate safety practices and recommendations to mitigate the risk of nanoparticle exposure[29,48,49].

Although the study is scarce on the immunotoxic impact of Graphene Oxide Nanoparticles (GONP) on hypersensitivity, existing studies indicate that Graphene Oxide Nanoparticles (GONP) can cause immunotoxic effects, hence potentially endangering people who come into contact with these nanoparticles. Additional investigation is required to comprehensively comprehend the underlying processes via which Graphene Oxide Nanoparticles (GONP) elicit hypersensitivity, as well as to evaluate the likelihood of hypersensitivity responses occurring in human subjects.

4.4. Autoimmunity

At present, there exists a dearth of comprehensive data about the direct correlation between autoimmunity and Graphene Oxide Nanoparticles (GONPs). While there has been research conducted on the immunotoxic effects of Graphene Oxide Nanoparticles (GONPs), there is a lack of studies specifically focusing on their influence on

autoimmunity. The immunotoxicological impacts of Graphene Oxide Nanoparticles (GONPs) have been investigated, revealing many observable effects such as the induction of an inflammatory response, alteration of cytokine levels, and cytotoxicity towards certain cell types. Gold nanoparticles (GONPs) have been seen to trigger an inflammatory response in macrophages derived from mice and in cultures of whole blood cells. Additionally, Graphene Oxide Nanoparticles (GONPs) have been shown to hinder the production of certain cytokines and to have cytotoxic effects at higher doses. Nevertheless, it is essential to acknowledge that the immunotoxic effects of Graphene Oxide Nanoparticles (GONPs) might vary depending on variables such as Graphene Oxide Nanoparticle (GONP) concentration, cell type, and experimental parameters. Moreover, the intricate interplay between nanoparticles and the immune system is multifaceted and may be modulated by several aspects, including nanoparticle size, surface curvature, and surface charge [50].

Previous research has demonstrated that graphene oxide has low toxicity and does not induce notable cytotoxicity or immunological dysfunction in dendritic cells [50]. However, the precise consequences of Graphene Oxide Nanoparticles (GONPs) related to autoimmunity have not been explored fully. Autoimmune disorders are characterized by the immune system's failure to maintain proper control, and there exists a potential for graphene oxide nanoparticles (GONPs) to intensify or initiate autoimmune reactions. Nevertheless, more research is needed to comprehensively comprehend the correlation between graphene oxide nanoparticles (GONPs) and autoimmunity, encompassing the fundamental processes and possible impacts on the emergence and advancement of autoimmune disorders.

4.4.1. Long term effect of immunotoxicity caused by GONP

The comprehensive investigation of the possible long-term consequences of immunotoxicity induced by Graphene Oxide Nanoparticles (GONPs) remains limited. Nevertheless, according to the existing body of research, it is postulated that Graphene oxide nanoparticles (GONPs) have the potential to elicit immunological dysfunction in dendritic cells and instigate ROS production. Although the acute toxicity of Graphene Oxide Nanoparticles (GONPs) has been assessed, there exists a dearth of chronic toxicological investigations that particularly address the long-term consequences associated with their exposure. It is imperative to acknowledge that the comprehensive understanding of the prolonged ramifications resulting from Graphene Oxide Nanoparticle (GONP)-induced immunotoxicity remains limited, necessitating more investigation to ascertain the precise long-term implications. However, it has been demonstrated that nanoparticles, such as Graphene Oxide Nanoparticles (GONPs), possess immunotoxic properties in a broad sense. The potential consequences include modifications in immunological responses and the initiation of inflammation, both of which may have enduring ramifications for immune well-being [51].

While there is a scarcity of specific research investigating the prolonged consequences of immunotoxicity induced by Graphene Oxide Nanoparticles (GONPs), existing studies on the immunotoxicity of nanoparticles in a broader context indicate that detrimental impacts on the developing immune system, potentially stemming from nanoparticle exposure, could result in enduring effects on immune functionality and elevated susceptibility to non-communicable diseases. It is vital to undertake more research that precisely examines the prolonged impacts of Graphene Oxide Nanoparticle (GONP)-induced immunotoxicity on human health to enhance our comprehension of its possible enduring ramifications.

4.5. Protein corona-NP effect

Nanoparticles actively absorb biomolecules like proteins and lipids onto their surface when they come into contact with biological fluids, creating a 'corona' of these molecules. This intricate structure known as "bio-corona" determines the interaction and reactions of host immune cells react to nanoparticles. Studies have shown that the stimulation or suppression of the immune responses actively depends on protein corona formation. The protein corona has been classified as "hard corona" and "soft corona" as the two variant of protein corona formations. The highly affinity proteins that are strongly attached to the nanoparticle (NP) surface and are attached for a longer period are known as hard corona. While the weakly bound proteins over short periods (seconds to minutes) on an NP surface are known as soft corona. Long-term exposure of Graphene Oxide (GO) to plasma creates hard corona, which reduces the generation of reactive oxygen species (ROS) and decreases its immunotoxicity, [52].

In a study conducted by Xu et al., they compared pristine Graphene Oxide (GO) toxicity with a series of graphene oxide derivatives. In the result, polyacrylic acid-functionalized Graphene Oxide (GO-PAA) was found to be least toxic followed by poly (ethylene glycol)-functionalized Graphene Oxide (GO-PEG) [53]. They concluded that the different protein corona-NP compositions dictate pro-inflammatory reactions at the surface level. However, another research reported the stimulation of cytokine response by small-PEGylated Graphene Oxide (GO) flakes of 200 nm. It was suggested that integrin αvβ8 signaling resulted in the binding of PEGylated Graphene Oxide (GO) to lipid cell membranes leading to macrophage activation by surface receptors [54]. A recent study of cationic lipid-coated graphene oxide nanoflakes (GOCL NPs) interacting with human plasma revealed that a high-protein corona, reduces the *in vivo* transfection efficiency of graph lipoplexes in cancer cells and increases the likelihood of DNA payload degradation at the intracellular level. Conversely, pre-coating graph lipoplexes with artificial coronas formed at low protein concentrations may result in high transfection efficiency and low cytotoxicity. To study the impact of surface chemistry on macrophage absorption, the surface of carboxylated Graphene Oxide (GO) nano-complexes was coated with polyethylene glycol (PEG), bovine serum albumin (BSA), or polyetherimide (PEI). The endocytosis by macrophages of the surface-coated Graphene Oxide (GO)-NPs was prevented by Graphene Oxide (GO)-PEG (most effective) and Graphene Oxide (GO)-BSA nano-complexes. On the other hand, Graphene Oxide (GO)-PEI produced a two-dimensional surface with a positive zeta potential that aided to endocytosis. The electrostatic interaction of nGO-PEI with the mitochondria within the macrophages caused a reduction in macrophage vitality following their absorption at the surface. The mitochondrial release of reactive oxygen species (ROS) and cytochrome C stimulated the caspase cascade, culminating in macrophage death. The findings suggest that surface functionalization can be used to adjust interaction of Graphene Oxide (GO) with macrophages for reducing the chances of their elimination by these phagocytic cells [55]. Complement factor H coating provided nearly total inhibition (>90% decrease) of complement activation, providing even better protection against complement activation than coating with serum albumins [56].

5. Cellular dysfunction caused by GO Nanoparticles

Nanotechnology has witnessed a surge in the utilization of engineered nanoparticles, with Graphene Oxide (GO) nanoparticles standing out for their unique properties. However, concerns have arisen about their effects on human health, particularly their potential to disrupt cellular function, especially in scenarios involving blood cultures and different cell lines. The impact of GO nanoparticles on cancer cell lines has attracted significant interest due to their potential applications in cancer therapy **(**Fig. 3**)**. Leukemia, lung cancer, breast cancer, and colorectal cancer are among the most prevalent and deadly forms of cancer. Fig. 4 represents the effects of Graphene Oxide (GO) in some of the cell lines. Understanding how Graphene Oxide (GO) nanoparticles affect the growth, proliferation, and survival of these cancer cell lines is essential for assessing their therapeutic potential and potential risks. This detailed review of the impact of Graphene Oxide (GO) nanoparticles on cellular dysfunction spans a wide range of scientific

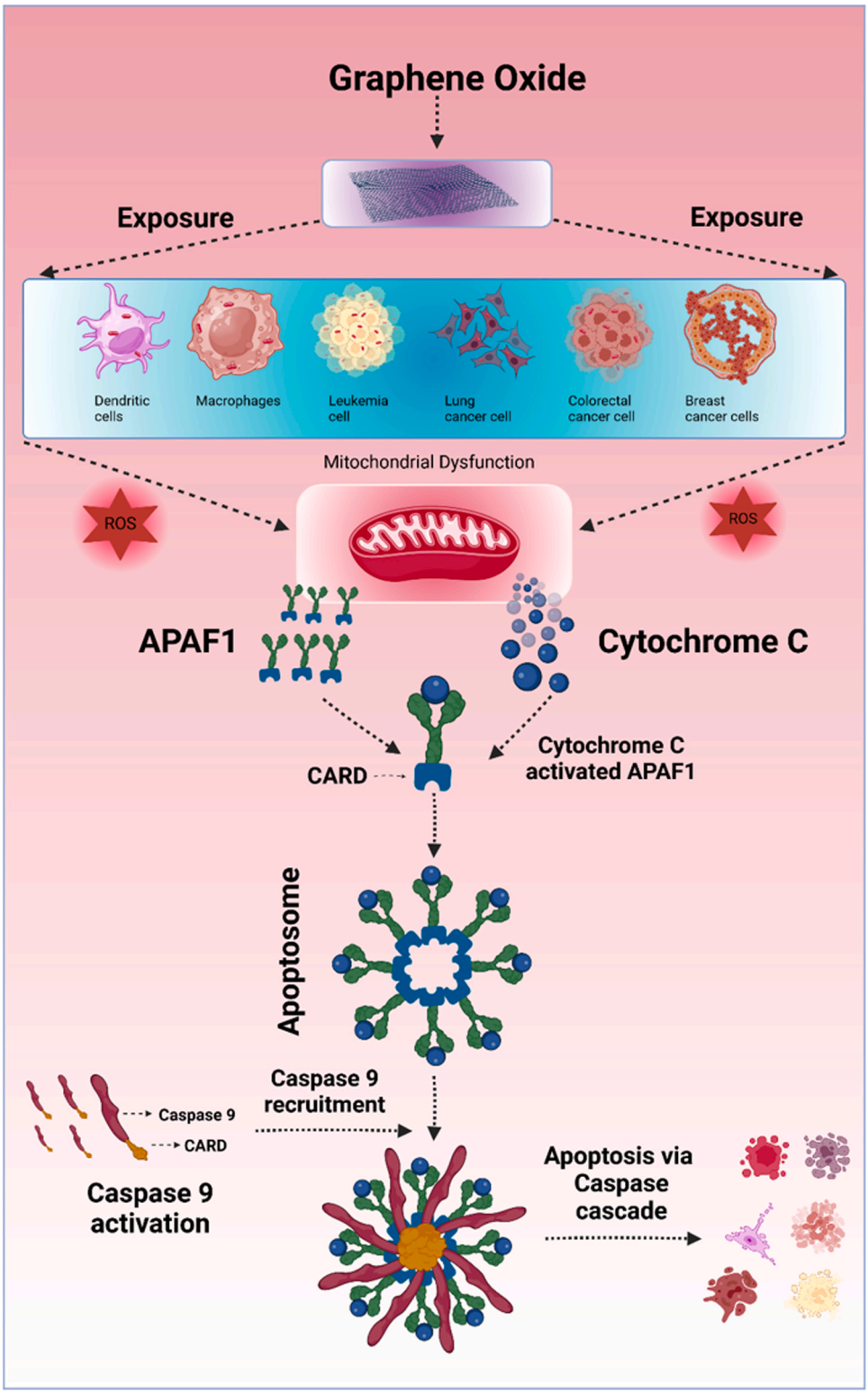

Fig. 3. The interaction of Graphene Oxide (GO) with various cells, including dendritic cells, macrophages, leukemia cells, lung cancer cells, breast cancer cells, and colorectal cells, leads to cellular dysfunction. In various cellular pathways, reactive oxygen species (ROS) function as signaling molecules. Excessive ROS production by mitochondria in the cells induces mitochondrial dysfunction and triggers apoptosis, causing changes in mitochondrial DNA and the mitochondrial membrane. As a result of ROS production, cytochrome c (a heme-containing protein acting as a pro-apoptotic factor) is released into the plasma. Together with APAF1 (Apoptotic Protease-Activating Factor 1), it forms an apoptosome. The CARD (Caspase Activation and Recruitment Domain) protein in APAF1 and initiator caspases facilitates the recruitment of caspase 9, and the apoptosome activates caspase 9, initiating a cascade of events via downstream effector caspases that lead to the apoptosis of the cell.

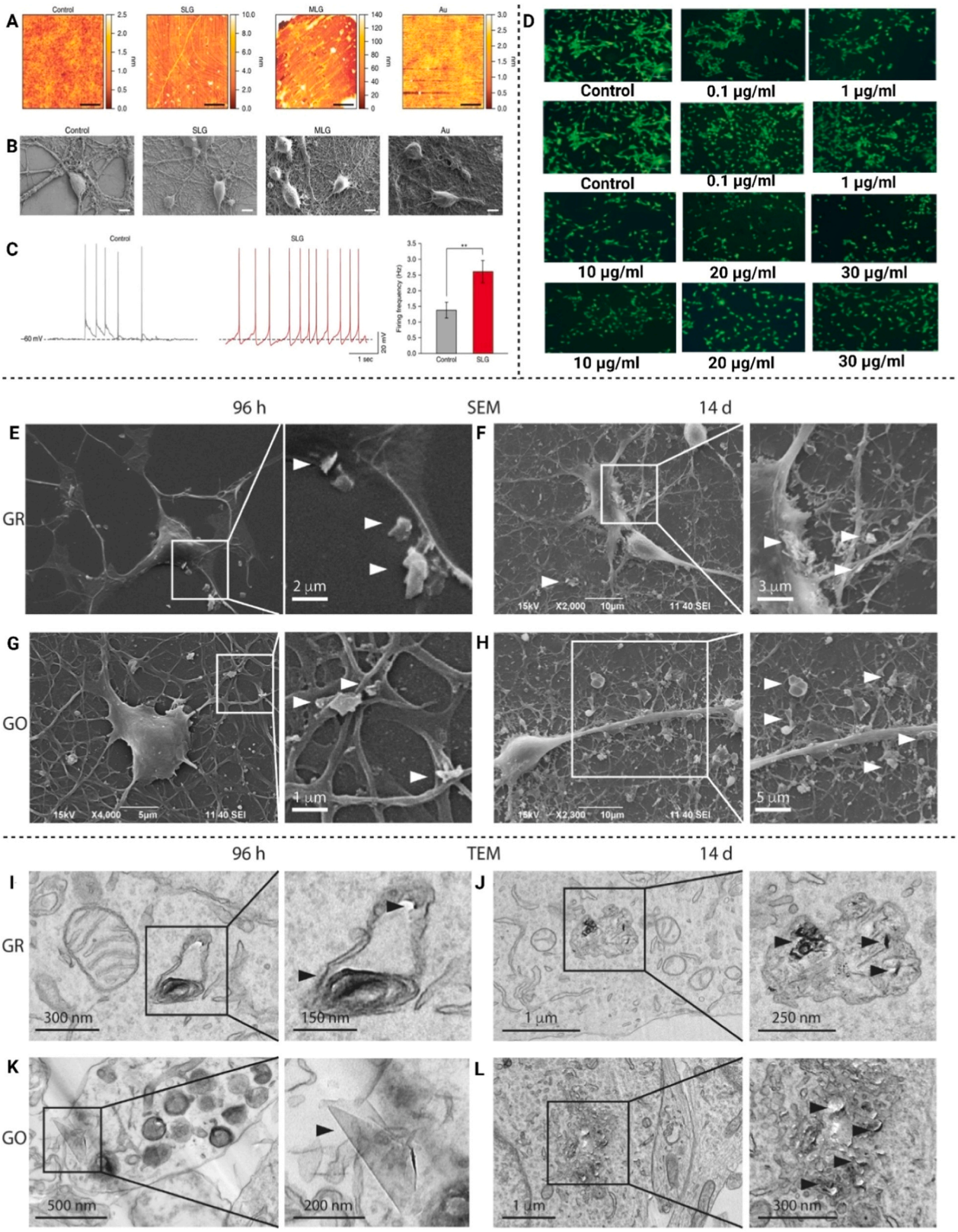

Fig. 4. A. AFM topography of glass (control), Single layer graphene, multilayer graphene and gold-plated graphene in neural communication. **B.** SEM image of Hippocampal neuron. **C.** Single-cell intrinsic excitability is promoted by SLG. **(E-L).** SEM and TEM images of the interactions of GO with primary rat cortical neurons which were exposed to FLG for 96 hrs and 14 days, **(E-H).** The cell membrane was seen to have attached a large number of flakes (white arrowheads), **(I-J)** Intracellular localization of GO and FLG was studied with TEM.
(a) Adapted from [57] **D.** Quantitative analysis of Pristine and aminated GO in colon cancer cells after incubation with 26 cells for 24 hours. (b) Adapted from [58]. (c) Adapted with permission from [59].

specialties, from cancer and nanotoxicology to immunology. In addition to addressing possible risks associated with their broad usage, researchers can pave the path for safer and more tailored nanoparticle-based therapeutics by unraveling the complex mechanisms underlying the interactions between Graphene Oxide (GO) nanoparticles and various biological systems. Table 1 compiles the effect of graphene oxide and its derivatives on various organs.

5.1. Human whole blood culture

Even though graphene oxide is composed of the carbon atoms similar to human cells, its bi-dimensional structure results in peculiar interactions with blood proteins, potentially causing serious repercussions such as thrombogenicity and immune cell activation. Albumin, the most abundant protein in human plasma, efficiently adsorbs onto large graphene oxide (GO) sheets, with minimal alterations to its secondary structure. However, its functionality is diminished, likely due to epoxy residues on GO crosslinking with albumin's surface and potentially obstructing bilirubin binding sites. In a subsequent study, the albumin activity of the group was kept intact by oxidizing GO into GO-COOH with sodium chloroacetate, resulting in carboxyl groups masking epoxy groups on the GO surface[60,61]. Gamma globulin (g-globulin) remains stable after interacting with graphene oxide (GO), and its adsorption is relatively insensitive to GO sheet size. Fibrinogen undergoes denaturation on GO. At lower concentrations (0.5–2 mg mL^{-1}), fibrinogen adsorbs equally well on all GO nanosheet samples, regardless of lateral size distribution. However, at higher concentrations, fibrinogen adsorption increases as GO nanosheet lateral size decreases [62]. Chong et al. reported the adsorption capacities of four highly abundant blood proteins on GO and rGO nanosheets in the order of Fibrinogen > Immunoglobulin > Transferrin > Albumin. Also, upon comparing the cytotoxicity of SWNTs and GO, they experimentally concluded that the fast and greater adsorption of protein on GO nanosheets hinders its cellular uptake and cell membrane adhesion, leading to reduced cytotoxicity [63].

An investigation was conducted to understand the interaction of red blood cells (RBCs) with graphene oxide (GO) and reduced graphene oxide (rGO). Red blood cells (RBCs) treated with graphene oxide (GO) exhibited minimal hemolysis, except at the highest concentration of 200 μg/mL. Additionally, even at this concentration, the inclusion of rGOs did not disrupt the clotting potential of the blood. Furthermore, experimental evaluations of the interaction between rGOs and skin fibroblasts demonstrated the biocompatibility of rGO-1 and rGO-10, which were also found to be hemocompatible with RBCs. The implications are promising, indicating the potential application of rGO-1 or rGO-10 as nanofillers in biomaterials for tissue engineering or as platforms for targeted drug delivery [64]. In a separate study, the synthesis of functionalized reduced graphene oxide (heparin-rGO) was showcased for enhanced stability in aqueous environments along with impressive anticoagulant activity and biocompatibility. Notably, heparin served as both a reducing agent and stabilizer in this context. The mechanism involved the heparin-mediated reducing of graphene oxide (GO) to yield reduced GO (rGO). Heparin molecules were then incorporated onto rGO surfaces through hydrogen bonding and hydrophobic interactions. This novel configuration demonstrated the ability to extend both intrinsic and extrinsic blood coagulation pathways. This contrasts with GO and hydrazine-rGO, which predominantly prolonged the intrinsic pathway, underscoring the specificity of heparin-rGO's anticoagulant action [65].

Table 1
Effects of Graphene Oxide in various organisms.

Graphene Oxide's Immuntoxic effects	Effects	Implications	Target Organ	Applications
Inflammation	In Vitro Studies In Vivo Studies Biocompatibility	Inflammatory Response Oxidative Stress Immune Cell Interaction Nanoparticle Distribution	Lungs Liver Spleen	Immunotoxicity and Inflammation Anti-Inflammatory Effects In Vitro Studies. In Vivo Studies
Immunosuppression and Immunostimulation	Immunosuppression Cytokine Dysregulation Inhibition of Immune Cell Activation Immune Stimulation: Activation of APCs Enhanced Immune Cell Activity	Immunosuppression: Some studies have suggested that exposure to graphene oxide might lead to immunosuppressive effects. Immunostimulation: On the other hand, there have been reports of immunostimulatory effects of graphene oxide as well.	Spleen Thymus Lymph node Bone marrow	Immunosuppression: Organ Transplantation Autoimmune Disease Treatment Immune Stimulation: Cancer Immunotherapy Vaccine Adjuvants
Hypersensitivity	Inflammation and Immune Response Allergic Reactions	Allergic Responses and Hypersensitivity Reactions Inhalation Hazards	Respiratory System Skin Immune System	Hypersensitivity Assessment in Immunotoxicity Testing Allergen Delivery and Immunomodulation Immunomodulatory Therapies for Hypersensitivity Disorders
Autoimmunity	Inflammatory Response Autoimmune Trigger	Inflammatory Response and Immune Dysregulation Antigen Presentation and Autoantibody Production Barrier Disruption and Tissue Permeability	Lungs and Respiratory System Immune System Dysregulation Blood-Brain Barrier and Central Nervous System	Immunomodulation Targeted Drug Delivery Tolerogenic Nanomaterials
Protein Corona NP Effect	Inflammatory Response Cellular Uptake and Internalization Antigen-Presenting Cells	Immunotoxic Implications of Graphene Oxide Interaction with Proteins Nanoparticle Effects and Immune Response	Lung Tissue Liver and Spleen	Immunotoxicity of Graphene Oxide (GO) Protein Interactions with Graphene Oxide Graphene Oxide Nanoparticle Interactions

Investigation into the hemolytic properties of graphene-based nanomaterials (GBNs) revealed their dose-dependent effects. Graphene oxide (GO) sheets, particularly when sonicated to smaller dimensions, evoked greater hemolysis compared to larger untreated GO sheets. The prothrombotic nature of GO was evident by platelet aggregation mediated by intracellular calcium release and the activation of non-receptor protein tyrosine kinases. This thrombogenic tendency translated into extensive pulmonary thromboembolism upon intravenous administration of GO in mice. Notably, rGO demonstrated significantly reduced platelet aggregation, stressing the influence of surface charge distribution on platelet activation and thrombotic response [66]. Exploration into the hemolytic behavior of graphene oxide (GO) and graphene sheets unveiled a size-dependent and exfoliation-related trend. Sonicated GO sheets, which are smaller and have a higher surface oxygen density, displayed enhanced hemolytic activity compared to larger untreated GO sheets and aggregated graphene sheets. Chitosan coating ameliorated the hemolytic activity of GO sheets. The relationship between size, exfoliation, surface oxygen content, and hemolysis highlights the multifaceted determinants of GBN-induced erythrocyte damage [67].

Investigation into the interactions between graphene oxide nanoparticles (GO-NPs) and lipid membranes revealed a propensity for interaction with both neutral and negatively charged lipid bilayers. This interaction resulted in the rupture of neutral vesicles while leaving negatively charged counterparts unaffected. A strategic intervention to mitigate the hemolytic potential of GO-NPs involved coating them with lipid membranes, such as phosphatidylcholine [68].

Researchers have explored various covalent and non-covalent immobilization techniques to attach biomolecules (e.g., proteins, lipids, and saccharides) and functional groups to reduced graphene oxide (rGO), aiming to enhance its properties for applications such as drug delivery and toxicity reduction. Carboxylated GO-lanthanum complexes demonstrated minimal hemolysis, indicating improved biocompatibility. Mannosylation of GO resulted in reduced protein corona formation and approximately 75% lower GO-induced hemolysis, possibly attributed to reduced electrostatic charges [69]. Investigations into the hemotoxicity of graphene oxide (GO) nanostructures functionalized with arginine, lysine, and ginsenoside Rh2 revealed that pristine GO exhibited higher hemolysis, whereas Rh2-functionalized GO showed the lowest hemolysis and had the least impact on coagulation systems [70]. Graphene oxide nanoparticles (GONPs) were found to upregulate the pro-inflammatory cytokines IL-6 and MIP-1β in unstimulated whole blood cell cultures. This activation of the inflammatory response could be linked to complement system activation and subsequent production of proinflammatory mediators. GONPs exhibited a dose-dependent inhibition of inflammatory responses and influenced cytokines associated with adaptive immune responses. Under unstimulated conditions, GONPs specifically upregulated the humoral immune-regulating cytokine IL-10 but not the cell-mediated response cytokine IFNγ. However, under simulated infection conditions, both humoral and cell-mediated responses were downregulated, suggesting that GONP exposure during infections may induce immunosuppression [44].

5.2. Dendritic cells and macrophages

In an exploratory study, the DC2.4 cell line was employed to investigate the *in vitro* immunotoxicity of two distinct graphene oxide types: mono-layer graphene oxide (mono-GO) and multi-layer graphene oxide (multi-GO). Mono-GO had a milder impact on cell viability than multi-GO, but both variants of GO stimulated ROS production in DC2.4 cells. Additionally, Mono-GO induced DC2.4 cell aggregation, inducing significant morphological changes, a phenomenon not mirrored by multi-GO exposure. Furthermore, the study revealed the propensity of both mono-GO and multi-GO to enhance the release of tumor necrosis factor-alpha (TNF-α) from DC2.4 cells, both in the presence and absence of lipopolysaccharide (LPS) stimulation. While interleukin-6 (IL-6) remained unaffected by GO exposure, multi-GO demonstrated an enhancing effect on IL-6 secretion, whereas mono-GO led to the inhibition of IL-6 production upon LPS stimulation. Delving deeper into the molecular responses, the utilization of RNA sequencing (RNA-seq) revealed that both mono-GO and multi-GO elicited immune responses in DC2.4 cells, as evidenced by changes in their transcriptome profiles. Interestingly, mono-GO exhibited a greater propensity to induce altered gene expression compared to multi-GO [29]. Presenting an intriguing dual role, GO showcases potential both as a natural antioxidant and as a gene carrier for enhanced cardiac therapy. The study highlights GO-NP's capability to mitigate inflammation through the reduction of intracellular reactive oxygen species (ROS) levels in macrophages. This anti-inflammatory attribute aligns with its antioxidative ability. In addition to its immune-stimulatory effects, GO-NPs can also be used to deliver interleukin-4 plasmid DNA (IL-4 pDNA), promoting M2 macrophage polarization, a critical process in cardiac repair. The development of macrophage-targeting/polarizing GO complexes (MGCs) demonstrates the potential of GO to suppress ROS production in immune-stimulated macrophages. Leveraging DNA-functionalized MGC (MGC/IL-4 pDNA) further underlines GO-NP's potential to shift macrophages towards an M2 phenotype, consequently enhancing the secretion of cytokines conducive to cardiac repair. This innovative dual role highlights GO-NP's versatility in both attenuating inflammation and modulating macrophage polarization for cardiac therapeutic applications [71]. Another study demonstrates the immunomodulatory behavior of polyethylene glycol-functionalized (PEGylated) graphene oxide nanosheets (nGO-PEGs). Despite surface passivation, nGO-PEGs surprisingly induce robust cytokine responses in peritoneal macrophages, contrary to the notion of universally weakened immune reactions to passivated nanomaterials. Molecular dynamics simulations reveal that nGO-PEGs preferentially adhere to or partially insert into cell membranes, strengthening their interactions with stimulatory surface receptors. This distinctive property amplifies cytokine secretion by augmenting integrin b8-related signaling pathways. The findings extend beyond conventional understanding, presenting PEGylated nanomaterials as potential immune modulators [54].

Expanding the horizons of vaccine development, graphene oxide (GO) emerges as a promising platform for protein adsorption, epitomized by its complexation with the ovalbumin antigen (OVA) and dendritic cells (DCs). In a study, GO nanosheets were harnessed to adsorb proteins, forming complexes that are internalized by dendritic cells, effectively initiating antigen presentation. This positions GO as a potential nanocarrier in vaccine formulations, bearing the potential to enhance antigen uptake and presentation by dendritic cells, fostering the development of more effective and versatile vaccine strategies [72]. The intricate interplay between dendritic cells (DCs) and graphene oxide (GO) of varying lateral sizes has been meticulously explored. The internalization of GO by DCs unveiled minimal influence on viability, activation phenotype, or cytokine production. Capitalizing on this unique interaction, GO facilitated the uptake of antigen-loaded protein, influencing DC-mediated T-cell responses. Interestingly, diverse sizes of GO sheets accentuated distinct facets of adaptive immune responses. This complex interplay underscores the potential of GO as an immunomodulatory platform for finely tuned modulation of immune reactions [73]. Capitalizing on the unique properties of graphene oxide, researchers delved into the potential of graphene oxide (GO) loaded with the glioma antigen survivin to enhance dendritic cell (DC)-mediated anti-tumor immune responses. They found that GO-survivin elicited a robust anti-glioma immune response *in vitro*, using the T98G human glioma cell line as a model to demonstrate the potential of GO in harnessing dendritic cells for enhanced immune responses against tumors [74]. The complex immunomodulatory effects of graphene oxide (GO) on dendritic cells (DCs) were examined, revealing its dual influence on antigen presentation. Although GO suppressed LPS-induced expression of certain chemokines and cytokines, it induced caspase-dependent IL-1β expression, suggesting inflammasome activation. This multi-faceted impact extended to the modulation of anti-inflammatory

and pro-inflammatory mediators in macrophages, underlining the complexity of GO's immune interactions and potential consequences for immune surveillance and inflammation [75]. Elucidating the intricate relationship between graphene oxide (GO) sizes and their pro-inflammatory polarization effects, the study revealed that large GO (LGO) promoted a more pronounced pro-inflammatory polarization in murine macrophage cell lines and in vivo models. Conversely, small GO (sGO) induced a distinct activation pattern in human monocytes. This observation emphasizes the role of production methods and potential endotoxin contamination in generating conflicting reports on GO's inflammatory potential [40]. A study examined the impact of graphene oxide (GO) on dendritic cells (DCs), revealing that pre-exposure to GO significantly reduces DCs' ability to stimulate immune responses. This contrasts with the conventional belief that nanomaterials like GO enhance DCs' antigen presentation. The findings suggest a potential application of using DCs to treat autoimmune disorders by exploiting GO's inhibitory effect on DCs' antigen presentation. DCs play a key role in processing protein antigens through immunoproteasomes. Exposure to GO decreased the expression of a critical immunoproteasome subunit, LMP7, disrupting the protein antigen processing in DCs. This impairs DCs' ability to activate antigen-specific T lymphocytes, crucial for adaptive immune responses [75]. GO (graphene oxide) has been found to affect immune responses by altering cytokine and chemokine production. In the presence of lipopolysaccharide (LPS), GO suppresses the induction of various cytokines and chemokines, including the anti-inflammatory IL-10. Graphene oxide (GO) induces caspase-dependent expression of IL-1β in LPS-primed macrophages, suggesting inflammasome activation. GO also affects cytokine secretion in LPS-primed macrophages, with GO-S (small-sized GO) and GO-L (large-sized GO) curtailing the release of IL-10 and other cytokines, chemokines, and growth factors essential for immune regulation and response. Interestingly, GO promotes the production of significant levels of IL-1RA (IL-1 receptor antagonist) and limited amounts of IL-1β in naïve macrophages. However, exposure of LPS-primed macrophages to GO increases IL-1β levels while decreasing IL-1RA levels [40].

By utilizing GO's special qualities, such as its biocompatibility and immunomodulatory effects, DC vaccines may be modified to overcome the restrictions of the tumor microenvironment and obtain better results in cancer immunotherapy. Large-sized graphene oxide nanosheets (L-GO) have shown remarkable potential as adjuvants in DC vaccines targeting SARS-CoV-2. Their high binding affinity with DC membrane promotes the formation of DC-T cell clusters without interfering with DC-T cell interactions due to their low binding affinity with T cells. These clusters significantly enhance antigen-specific T-cell responses, surpassing conventional cytokine cocktail adjuvants. In mice infected with SARS-CoV-2, L-GO-adjuvant DCs trigger a robust cytotoxic T-cell immune response against the virus's spike protein, leading to an impressive >99.7% clearance of viral RNA from lung tissues. L-GO acts as a "nano zipper" to facilitate the aggregation of dendritic cell (DC)-T cell clusters, creating a stable microenvironment for T cell activation. This process relies on integrin ICAM-1 positioning through cytoskeleton-dependent membrane mechanisms. This innovative approach holds promise for enhancing DC vaccine efficacy against SARS-CoV-2 infections [30]. Graphene oxide (GO) modified with PEG and PEI (GO-PEG-PEI) emerges as a multifaceted adjuvant with the potential to enhance dendritic cell (DC) maturation, cytokine secretion, and T cell proliferation. Dual-polymer-modified GO exhibits positive modulation of DCs through the activation of multiple toll-like receptor (TLR) pathways, highlighting its role as an antigen carrier and vaccine adjuvant [76]. RNA-based antigens have shown promise in DC vaccine development, and using graphene oxide (GO)-bound total RNA from tumors could lead to more potent antitumor responses. GO can enhance RNA stability and protect against RNase degradation. This study explores the nano-biological effects of total RNA/GO interaction, revealing that total RNA can be effectively bound to the surface of GO. This binding creates a shield effect that significantly enhances the resistance of total RNA to degradation by RNases. This stability is maintained at room temperature for up to 4 days. These findings highlight the potential of GO as a next-generation nano inhibitor against RNase degradation, offering new possibilities for RNA-based DC vaccine strategies [77].Top of Form

5.3. Leukemia cell line

A comprehensive study investigated the cytotoxic effects of graphene oxide (GO) and vanillin-functionalized graphene oxide (V-rGO) on THP-1 cells, a human acute monocytic leukemia cell line. The study revealed dose-dependent effects, with V-rGO exhibiting more pronounced effects than GO, especially at higher concentrations. The critical mechanism underpinning cell death involves the disruption of mitochondrial membrane potential (MMP). Exposure to both GO and V-rGO led to heightened intracellular Reactive Oxygen Species (ROS) levels, triggering the loss of MMP. Subsequent cellular assays showed dose-dependent results, including cell survival, proliferation, ROS production, lipid peroxidation, LDH leakage, and MMP integrity. These findings highlight the potential of utilizing V-rGO and GO as agents to modulate immunomodulatory responses in human acute monocytic leukemia cells [77]. A novel assay, based on a complex termed Dual-Aptameric Functionalized Graphene Oxide (DAFGO), was ingeniously designed to identify Molt-4 cells, a type of human acute lymphoblastic leukemia T-cells. This pioneering assay relies on the internalization of the DAFGO complex exclusively into Molt-4 cells, facilitated by the Sgc8c aptamer molecular recognition probe. High concentrations of Adenosine Triphosphate (ATP) within lysosomes prompt the release of Fluorescein-labeled ATP aptamer from the complex, eliciting a robust fluorescence emission signal. The results underscore the efficient internalization of the DAFGO complex into target cells (Molt-4), culminating in pronounced fluorescence emission [78].

The utilization of Graviola nanocomposite in leukemia treatment was investigated, particularly its influence on the efficacy of laser and ultrasound therapies. The presence of the Graviola nanocomposite exhibited an enhancement in both laser and US treatments. Pulsed/continuous ultrasound waves demonstrated superior efficiency compared to laser treatment when combined with the Graviola nanocomposite. This combined therapeutic approach showcased remarkable efficacy on human leukemia cell lines and *in vivo* models, potentially offering an integrated and more effective strategy compared to conventional individual treatments [79]. A study aimed to create an efficient targeted gene delivery system, utilizing Graphene Oxide (GO) conjugated onto Polyethyleneimine (PEI), followed by Folic Acid (FA) conjugation. The goal was to facilitate the expression of Interleukin-12 (IL-12) protein into cells overexpressing Folate Receptors, a characteristic often found in cancerous cells. The results showcased significantly enhanced transfection efficiency with GO-PEI polyplexes compared to simple PEI. The incorporation of FA-conjugated GO-PEI (GO-PEI-FA) amplified the potential for targeted delivery to cells with Folate Receptors. The ability of IL-12 to exert anti-angiogenic effects holds the potential to impede tumor formation and metastasis, underscoring the multifaceted therapeutic implications of this approach [77]. Graphene Oxide Nanoplatelets (GONps) with extremely sharp edges and minute lateral dimensions served as a pivotal element in a novel and highly sensitive leukemia detection methodology. The innovative technique harnessed the GONps to extract overexpressed guanine synthesized in the leukemia cell cytoplasm, subsequently employed for the electrochemical detection of leukemia cells. Leukemia cells could be detected quickly and with extreme sensitivity via the reduced graphene oxide nanowall (rGONW) electrodes, showcasing remarkable sensitivity at leukemia fractions (LFs) as low as 10–11. The five-order increase in sensitivity compared to contemporary technologies highlights the transformative potential of this integrated approach for heightened leukemia detection [80].

5.4. Lung cancer cell line

Reduced graphene oxide (rGO) has undergone extensive examination for its interactions with lung cancer cells (A549 and SKMES-1), encompassing both chemical and biological effects. A notable aspect of this study involves a direct comparison between two distinct lung cancer cell lines. The findings indicate that, at low concentrations, rGO induces significant late apoptosis and necrosis, suggesting a dose-dependent membrane disintegration phenomenon. Moreover, the study underscores that limited intact rGO distribution in lung cancer cells triggers late apoptosis and necrosis upon toxicity exposures. This revelation of molecular interactions provides insights into rGO's potential as a tool for targeted lung cancer interventions [81]. The utilization of carbon nanostructures, such as single-walled carbon nanotubes (SWNT) and graphene oxide (GO), holds promise for enhancing the therapeutic effectiveness of the bioactive compound paclitaxel (Tx) in the context of lung cancer treatment. Extensive studies have revealed a noteworthy synergy resulting from the combined approach involving SWNT/GO and Tx. This interaction is characterized by an intensified induction of cell death, highlighting the enhanced cytotoxic impact achieved through this combinational treatment strategy. This collaborative effect is found to be reactive oxygen species (ROS)-dependent, as evidenced by its reversal in the presence of an antioxidant, N-acetyl cysteine (NAC). Furthermore, the study unveils a novel pathway involving mitogen-associated protein kinase (MAPK) activation, modulated by ROS, because of CN treatment [82]. A significant scientific advancement has been achieved by employing thiourea as a dual-function agent for the reduction and stabilization of graphene oxide (GO), termed thiourea-reduced graphene oxide (T-rGO). Intriguingly, T-rGO demonstrates a significantly greater cytotoxic effect on lung cancer cells compared to its precursor GO form. This compelling finding leads to the identification of apoptosis as the primary mechanism behind T-rGO-induced cell death. Morphological transformations, particularly thinning and feebleness accompanied by elongated tips in treated cells, further delineate the disruptive influence of T-rGO. These effects remain markedly distinguishable from healthy, untreated cells. The study expands our knowledge of graphene oxide's versatile nature and its potential applications in the realm of lung cancer research, promising novel therapeutic approaches [83]. Using Pulicaria glutinosa extract for reduction, silver-doped significantly reduced graphene oxide nanocomposites (PGE-HRG-Ag) were created in a novel manner. These nanocomposites exhibit noteworthy anticancer properties against various human cancer cell lines, including A549 lung cancer cells, using tamoxifen as a standard drug. The anticancer activity of the resultant nanocomposites was shown to be substantially associated with the amount of silver nanoparticles on highly reduced graphene oxide (HRG), with larger concentrations of silver nanoparticles resulting in improved anticancer activity. The nanocomposite PGE-HRG-Ag-2 emerges as particularly potent in inducing cytotoxicity within A549 cells. A detailed analysis unravels G0/G1 phase cell cycle arrest and apoptosis induction as the mechanisms of action. This research unveils the apoptotic potency of highly reduced graphene oxide nanocomposites wrapped with silver, paving the way for innovative lung cancer therapies [84].

A study employed the SOLiD sequencing method to conduct an in-depth investigation into the molecular mechanisms underlying the toxicity of graphene oxide (GO) in GLC-82 lung adenocarcinoma cells through microRNAs (miRNAs). The analysis reveals that when exposed to GO at concentrations greater than 50 mg/L, cells exhibit substantial decreases in viability, the generation of reactive oxygen species (ROS), and apoptosis. Through extensive sequencing, 628 up-regulated and 25 down-regulated miRNAs are identified in GO-exposed cells. The interplay between dysregulated miRNAs and known apoptosis control pathways provides a molecular foundation for understanding GO toxicity. This comprehensive exploration of miRNA responses broadens our understanding of GO's intricate interactions with lung cancer cells and its potential implications as a biomarker [85]. The intriguing physicochemical transition of graphene oxide (GO) in Gamble's solution and artificial lysosomal fluid (ALF), two simulated human lung fluids, has been recently studied. It is important to note the significance of organic acids like citrate and acetate in these lung fluids because they facilitate the reduction of GO by converting epoxy and carbonyl groups into phenolic groups. This biotransformation significantly hinders GO's endocytosis by macrophages. Interestingly, the alterations induced by Gamble's solution result in GO aggregation, diminishing its interaction with cells, while changes in ALF foster edge-to-edge aggregation, increasing GO adhesion to cell membranes. The potential long-term consequences of biotransformed nanomaterials in accumulation sites are highlighted by the complex interaction mechanism that produces a range of proinflammatory responses [86]. Ammonia-modified graphene oxide (GO-NH2) and pure graphene oxide (GO) particles were tested for cytotoxicity against human lung cancer cells (A549). In cell cultures, GO-NH2 particles aggregated due to their higher thickness, positive surface charge, and reduced size. The GO-NH2 particles were more hazardous than the pristine GO. For a whole day, GO-NH2 at doses of 10, 20, and 50 μg/mL had an impact on the morphology of live embryonic stem cells, while A549 cells exhibited relative resistance. However, strong suppression of A549 cell growth occurs after a prolonged 48-hour treatment in a dose-dependent manner. A comparative analysis showcased varying sensitivity between tumor and non-tumor cells, reinforcing the cell-specific effects of GO-NH2 and pristine GO particles upon both short-term and long-term exposure. The selective induction of apoptosis in lung cancer cells underlines the potential of GO-NH2 as a targeted therapeutic agent [87].

A thorough examination of the exposure of lung cancer cell lines A549 and SKMES-1 to reduced graphene oxide (rGO) yielded intriguing insights. At low concentrations, rGO induces pronounced late apoptosis and necrosis, surpassing early apoptotic events in both cell lines. This unique pattern of cell death underscores rGO's capacity for dose-dependent cellular membrane disintegration. The study highlights the multifaceted nature of rGO's cytotoxic effects, attributable to factors such as sharp edges, functional groups, surface charge, and nanosheet structure [88]. A pioneering approach revolves around the synthesis of graphene oxide nanoplatelets (GONPs) from stacked graphite nanofibers, with a base size of 50 $\times$50 nm^2, for both toxicity assessments and drug potentiation studies. These GONPs exhibit dual roles—drug carriers and potentiation of cisplatin's anticancer effects in human lung cancer cells (A549). GONPs' ability to modulate cisplatin's uptake and release based on pH conditions emerges as a striking feature. Alkaline pH enhances cisplatin charging onto GO supports, while acidic conditions facilitate its release, catering to the hypoxic environment of tumor cells. This innovative study broadens horizons by demonstrating the potential of GONPs to amplify the anticancer efficacy of cisplatin, capitalizing on the tumor microenvironment [89]. Novel hybrid nanogels, incorporating doxorubicin and graphene oxide (GO), offer a transformative approach to lung cancer treatment. This strategy facilitates enhanced drug transport into human lung cancer cells (A549) and harnesses the thermal absorption properties of GO under near-infrared (NIR) laser irradiation (808 nm wavelength) for photothermal therapy. The combination of doxorubicin-loaded nanogels and NIR laser treatment yields amplified toxicity effects on A549 cancer cells, rooted in GO's photothermal capabilities. The synergistic therapeutic strategy optimizes doxorubicin uptake while harnessing photothermal effects to selectively induce cell death. This innovative study introduces a promising paradigm for lung cancer treatment that leverages nanotechnology and light-induced therapies [90].

5.5. Breast cancer cell line

Complexes of GO-curcumin and GQDs-curcumin were tested against MDA-MB-468 and MCF-7 breast cancer cell lines. Remarkably, cell viability exceeded 75% after 48 hours of exposure to these complexes, compared to curcumin alone, which resulted in around 40% cell

viability at the same concentration. Different ratios of these complexes induced cell death rates of approximately 60%, 80%, and 95% at a concentration of 100 μg/mL after 48 hours, suggesting their synergistic anti-cancer potential. The interaction of these complexes with cancer cells, facilitated by accumulation within cells, led to the continuous release of curcumin, aiding in cell destruction. This sustained release may be attributed to the formation of new bonds that facilitate controlled drug release upon accumulation in cells [91]. The development of nanosystems combining graphene oxide (GO) with zoledronic acid (ZOL) demonstrated potential for synergistic treatment of osteoporosis and breast cancer metastasis. This study focused on MCF-7 breast cancer cells and demonstrated that nanostructured ZOL-GO, synthesized using optimal concentrations of ZOL and GO, promoted mineralization and cellular cluster formation. This integration of GO and ZOL exhibited enhanced therapeutic potential against breast cancer by providing a suitable environment for drug delivery and cellular mineralization, offering a promising avenue for treating both osteoporosis and breast cancer [92]. A novel approach was employed to design a nanocarrier for combinational chemo-photodynamic breast cancer treatment. rGO was decorated with magnetic nanoparticles and camptothecin drug, linked via 4-hydroxy coumarin. This nanocarrier demonstrated notable cytotoxic effects against breast cancer cell types as well as pH-dependent release. Interestingly, free camptothecin exhibited higher toxicity to normal cells and DNA damage. The combination of magnetic nanoparticles, camptothecin, and reduced-GO induced apoptosis and showed promise as an effective therapy through the activation of apoptotic pathways and ROS production [93].

The fabrication of silver and gold nanoparticle-hybridized reduced GO nanocomposites, loaded with the flavone chrysin, demonstrated improved stability, bioactivity, and biocompatibility. These nanohybrids exhibited enhanced cytotoxic effects against breast carcinoma cell lines while being less toxic to normal cells. The presence of noble metal nanoparticles on the reduced GO surface promoted apoptosis by generating ROS [94]. The impact of reduced graphene oxide (rGO) on breast cancer was investigated, revealing its diverse effects on different breast cancer cell lines, including MDA-MB-231 and ZR-75–1. In these cancer cells, exposure to rGO caused autophagy, cellular cycle arrest, and apoptosis. Interestingly, the rGO-induced effects were limited in normal human fibroblasts. The mechanism of apoptosis was associated with mitochondrial membrane potential changes and the activation of caspase pathways. These findings suggest the potential of rGO to trigger apoptosis and autophagy in breast cancer cells [95]. Hydroxyl and carbonyl-functionalized reduced graphene oxide (RGO) synthesized using natural sources demonstrated significant cytotoxic activity against breast cancer cells, MCF-7 [96]. Graphene oxide flakes fortified liposome functionalized with folic acid (GOF-Lipo-FA) is a sustainable nanotheranostic system that showed impressive results in tumor diagnosis and photo-triggered tumor regression. The nanohybrid enhanced drug stability and promoted tumor regression in cellular and animal studies. The system demonstrated deep intracellular localization, tumor-binding ability, and promising biocompatibility [97]. It investigated how amygdalin and graphene oxide nanoparticles (GONPs) combined to inhibit the growth of breast cancer cells. Amygdalin and GONPs worked together to provide an integrated anti-proliferative impact on AMJ13 breast cancer cells, outperforming the individual agents [98]. Biologically synthesized reduced graphene oxide (rGO) prepared using different plant extracts exhibited cytotoxic activity against MCF-7 breast cancer cells. The synthesized rGO demonstrated significant anti-cancer potential, suggesting the feasibility of using biogenic rGO as an effective therapeutic agent against breast cancer [99]. A study investigated the cytotoxic and anti-proliferative effects of rGO on breast cancer cell lines (MDA-MB-231 and ZR-75–1) revealed time- and dose-dependent cytotoxicity in these cell lines, triggering apoptosis and necrosis. rGO-induced oxidative stress and structural changes contributed to these effects. The findings provided insights into the potential of rGO in inducing oxidative stress and apoptosis in breast cancer cells [100]. Graphene oxide nanocomposites, fabricated with chitosan (CS) or ethylene diamine tetra acetic acid (EDTA), displayed cytotoxic effects against the MCF-7 breast cancer cell line. Interestingly, GO-EDTA demonstrated the most potent cytotoxic effect [101]. Gene delivery-optimized homogenous nanoparticles with optimal physicochemical characteristics were produced by encapsulating graphene oxide nanoflakes with cationic lipids. Protein corona formation at high protein concentrations significantly influenced transfection efficiency and cellular interactions. These findings provide insight into how protein coronas affect the effectiveness of cell transfection reagents based on graphene oxide in terms of transfection efficiency, demonstrating the potential for improved transfection efficiency with low protein concentrations [102].

5.6. Colorectal cancer cell line

In a study by Lina et al., a composite material was synthesized by integrating curcumin-capped gold nanoparticles (CAG) with reduced graphene oxide (rGO). This composite was assessed for its dual properties as an antioxidant and anticancer agent. The synthesis involved the reduction of gold nanoparticles using the reducing capacity of curcumin, with simultaneous implication of conventional sodium citrate reduction. The composite's effectiveness was analyzed with two distinct human colon cancer cell types, HT-29 and SW948. Notably, cellular morphological changes and inhibition of proliferation were observed upon CAG treatment in a dose- and time-dependent manner. The composite's cytotoxicity, as evaluated through cell viability assays, indicated an IC50 of 100 μg/mL in both HT-29 and SW-948 cells, while normal colon cells (CCD-841) displayed lower susceptibility. Furthermore, the composite exhibited the ability to neutralize reactive oxygen species (ROS) and exert antioxidant effects, offering the potential to manage inflammatory intracellular tumor microenvironments [103]. The synergistic potential of graphene oxide (GO) combined with the anticancer drug doxorubicin (DOX) was explored in addressing colorectal cancer (CRC). Through the MTT assay, it was revealed that the GO-DOX combination led to apoptosis in HCT-116 human CRC cells. The combination exhibited apoptotic and autophagic effects, with increased expression of genes involved in these processes, including ATG5, caspase 3, and Bax. Enhanced apoptotic responses and cellular death were observed, attributed to the collaborative action of GO and DOX [104]. Amine-functionalized graphene oxide nanoparticles (GO-NH2) were investigated for their potential in colorectal cancer (CRC) therapy. Exposure of CRC cells to GO-NH2 led to heightened cytotoxicity after 24 hours, primarily through the generation of reactive oxygen species (ROS), consequent DNA damage, and apoptosis induction. The amine groups on GO-NH2's surface facilitated strong adherence to CRC cells, possibly due to their binding affinity with cellular membranes. The presence of amine groups also triggered signals, including apoptosis [105]. Graphene oxide's (GO) potential as a hyperthermia-inducing agent in anticancer therapies was explored. Its near-infrared (NIR) optical absorption property enables heat generation upon NIR irradiation, leading to localized hyperthermia. A study investigated the cell damage mechanism induced by GO-mediated hyperthermia. Results indicated that increased laser power was associated with elevated cell culture temperature and preferential necrosis, promoting cytokine release [105]. Hyperthermia not only directly induces tumor cell death but also enhances the cytotoxic impact of various anticancer drugs. This phenomenon has led to the investigation of innovative treatment approaches, such as the use of a reduced graphene oxide (rGO) nanosystem that contains biotin. This nanosystem combines targeted chemotherapy with heat to act as a multimodal anticancer drug. The integration of hyperthermia, achieved through rGO-based materials, with precise drug delivery strategies, holds promise for achieving synergistic effects in cancer treatment [106]. Graphene oxide (GO) nanogels loaded with irinotecan were developed, demonstrating the potential for synergistic hyperthermic/cytotoxic effects against colorectal cancer (CRC). The

nanogels exhibited notable uptake by CRC cells and, in combination with the antitumor drug irinotecan, demonstrated enhanced cytotoxic effects. The release of irinotecan was influenced by both external pH and near-infrared (NIR) irradiation [107].

Utilizing DNA aptamer and graphene oxide (GO), a novel method for detecting colorectal cancer (CRC) exosomes was developed. The approach involved fluorescence signal amplification through DNase I enzyme digestion of aptamers on exosome surfaces. This interaction facilitated increased binding with fluorescent aptamer probes, enabling sensitive CRC exosome detection with potential implications for cancer diagnostics [108]. A targeted theranostic approach was pursued using peptide-functionalized graphene oxide quantum dots (QD-P) for colorectal cancer (CRC) treatment. QD-P demonstrated improved targeting and absorption in CRC cells that were PLAC-1 antigen-expressing. QD-P treatment resulted in decreased PLAC-1 expression, decreased invasiveness, and enhanced cell cytotoxicity. This multifaceted nanocomposite shows promise as a potential therapeutic tool for CRC [109]. A graphene oxide (GO)-based nanocomposite loaded with the chemotherapeutic drug 5-fluorouracil (5-FU) and modified with EGFR-targeting ligand GE11 demonstrated effective 5-FU transfer into EGFR-overexpressing CRC cells. Irradiation accelerated glutathione oxidation in tumor cells, disrupting intracellular redox balance. The resulting nanocomposite exhibited synergistic anticancer effects through photothermal therapy (PTT) and chemotherapy, showcasing its potential in CRC treatment [110]. A nanocomposite comprised of curcumin-capped gold nanoparticles, reduced graphene oxide (rGO), and gold nanoparticles was studied for its effects on colon cancer cells. The nanocomposite exhibited cellular uptake, induced apoptosis, and triggered redox-related responses, aligning with the anticipated effects of curcumin [111]. A graphene oxide (GO)–based nano platform loaded with a photosensitizer was developed for enhanced photodynamic therapy (PDT) of colorectal cancer. This nano platform exhibited effective targeting of CD44-overexpressing cancer cells and NIR-induced photothermic conversion, leading to enhanced generation of singlet oxygen and subsequent ablation of target colorectal cancer cells [112]. Graphene oxide (GO) displayed selective toxicity towards human colorectal cancer (CRC) cells compared to normal cells. The mechanism of action involved disruption of microtubule assembly, causing cell cycle arrest at the S phase and inducing apoptosis via reactive oxygen species (ROS) generation. The structural impact of GO on cellular proteins, particularly tubulin, contributed to growth arrest and apoptosis in CRC cells [113].

6. *In vivo* cytotoxicity of GO nanoparticles

The research on the *in vivo* cytotoxicity of graphene oxide (GO) nanoparticles involves administering these nanoparticles to living organisms, often using murine models, to understand their potential deleterious effects. Cytotoxicity in GO nanoparticles can manifest through various mechanisms. One key mechanism is the physical contact between GO nanoparticles and biological components. The unique physicochemical characteristics of GO nanoparticles may lead to direct interaction with cell membranes, potentially causing cellular injury or disruption. Additionally, GONPs (Graphene Oxide Nanoparticles) have been shown to induce oxidative stress within cellular systems. They can generate reactive oxygen species (ROS), disrupting the balance between the production and elimination of these radicals. Elevated levels of ROS can result in oxidative damage at the cellular level, contributing to cytotoxicity. Moreover, GONPs have been found to elicit inflammatory reactions in biological systems. The introduction of GO nanoparticles can trigger the secretion of pro-inflammatory cytokines, initiating an inflammatory cascade that may harm surrounding tissues. It's important to note that the cytotoxicity of GO nanoparticles can be influenced by various parameters, including their dimensions, concentration, surface modifications, and the duration of exposure. Different studies may yield varying outcomes, emphasizing the need for further research to comprehensively understand the precise pathways underlying GO nanoparticle-induced cytotoxicity and its implications in vivo. In summary, the *in vivo* cytotoxicity of graphene oxide (GO) nanoparticles involves physical interactions with cellular entities, the initiation of oxidative stress, and the elicitation of inflammatory reactions. These processes contribute to our understanding of the potential detrimental effects of GO nanoparticles on biological entities.

6.1. Mice model

Research on the cytotoxic effects of Graphene Oxide (GO) nanoparticles in an *in vivo* setting using a mouse model has shown that GO nanoparticles exhibit cytotoxic properties, suggesting potential adverse impacts on living organisms (Fig. 5) [114]. The observed effects include detrimental impacts on the plasma membrane, leading to an increase in lactate dehydrogenase leakage. Additionally, GO has been shown to induce cellular apoptosis, as evidenced by a reduction in cell viability determined through trypan blue exclusion and XTT experiments [115]. Moreover, it has been observed that GONPs (Graphene Oxide Nanoparticles) have the potential to induce mutagenesis at the molecular level by disrupting DNA replication processes. These results imply that GO nanoparticles can induce substantial cytotoxicity in murine models. The specific strain of mice used in these experiments is not disclosed, but mice are commonly employed in biomedical research due to their anatomical, physiological, and genetic similarities to humans. Mice can be selectively bred or genetically modified to study specific human diseases, making them valuable tools for investigating complex diseases and genetic elements [116]. Numerous studies have highlighted the *in vivo* cytotoxicity of Graphene Oxide nanoparticles in mouse models, involving mechanisms such as plasma membrane perturbation, initiation of programmed cell death, and disruption of DNA replication. Further research is needed to comprehensively understand the extent of these cytotoxic effects and their implications [117].

6.1.1. Potential mechanisms of cytotoxicity of graphene oxide nanoparticles in mice

Numerous research studies have been conducted to explore the underlying processes of cytotoxicity induced by graphene oxide (GO) nanoparticles in live organisms, therefore elucidating the plausible adverse effects and interactions associated with GO nanoparticles. The aforementioned investigations have investigated the production, structure, and physicochemical characteristics of graphene oxide (GO), along with its potential toxicological impacts on murine subjects [118].

Scientists have employed many methods of charactersation to evaluate the harmful impacts of GONP on mice, both in laboratory settings (in vitro) and within living organisms (in vivo). Although graphene oxide (GO) nanoparticles have demonstrated the potential to augment cell adhesion and proliferation, hence suggesting favorable interactions and biocompatibility given that they have also been seen to induce lethal effects. The physical interaction between graphene nanoparticles and cell membranes is identified as an important factor in GO cytotoxicity. The toxicity of graphene oxide (GO) nanoparticles is subject to many parameters, including the density and lateral side of the functional groups present on the nanoparticles. Various studies have elucidated distinct harmful pathways associated with graphene oxide (GO) nanoparticles, encompassing the inflammatory response, DNA damage, apoptosis, autophagy, and other related processes [49].

Nevertheless, it is important to acknowledge that the specific processes behind the harmful impacts of GONP on mice have not been elucidated completely in the available search findings. Additional investigation is required to comprehensively comprehend the probable processes via which graphene oxide (GO) nanoparticles elicit harmful effects in mice.

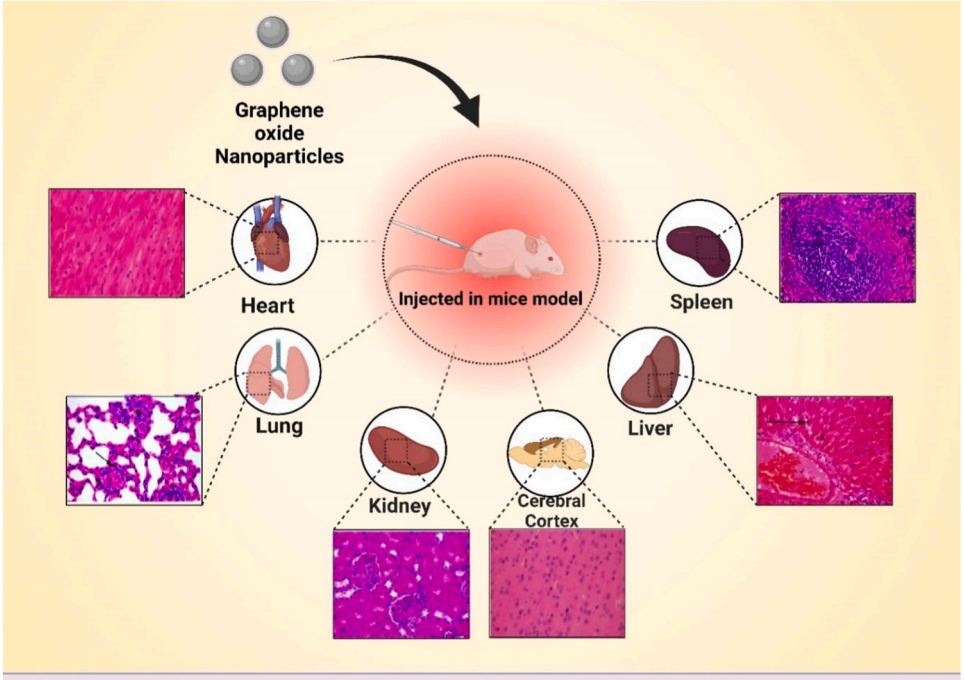

Fig. 5. Histological analysis of a rat treated with 10 mg/kg of GO using the Hematoxylin and Eosin (H&E) technique. No changes were observed in the cerebral cortex, heart, and kidney, and no accumulation of GO was detected in these organs. However, the administration of GO has the potential to elicit an inflammatory response in the liver, lungs, and spleen. Also, there was an accumulation of GO in the lungs and liver. Adapted from [119].

6.2. *Zebrafish*

The existing knowledge on the *in vivo* cytotoxicity of Graphene Oxide (GO) nanoparticles on zebrafish models suggests that GO nanoparticles may induce adverse effects on the health of these species. Various studies have demonstrated that GONPs (Graphene Oxide Nanoparticles) as well as other nanoparticles can induce oxidative stress, inflammation, and harm vital organs such as the liver, heart, and other anatomical structures in zebrafish [120,121]. In zebrafish embryos, exposure to graphene oxide (GO) nanoparticles led to a delay in hatching, as the particles adhered to the chorion and obstructed the pore canals of the chorionic membrane. GO successfully permeated the chorion and gained access to the embryos, distributing itself inside the yolk sac, heart, and eye areas[122]. This resulted in the excessive production of reactive oxygen species (ROS), heightened levels of oxidative stress, DNA degradation, apoptosis, and developmental abnormalities in the yolk sac, heart, and eyes. The impact on spinal cord/tail flexure and heart rate exhibited a pattern of initial escalation followed by subsequent attenuation with increasing concentrations of GO [121]. In adult zebrafish, exposure to GO led to an elevation in gill cell apoptosis and necrosis, accompanied by ROS production inside gill cells. Histopathological examination revealed several gill tissue lesions, such as dilated marginal channels, lamellar fusion, clubbed tips, and necrosis. Abnormalities were also observed in liver tissues, including nuclei positioned at the periphery, irregular shape of hepatocytes, and cell rupture [49]. A study compiling the toxicological impacts of graphene-family nanoparticles, including graphene oxide (GO), on various species found that zebrafish models exhibited toxicity when exposed to larger protein-coated graphene oxide nanoparticles (PCGO) [49]. The harmful effects caused by graphene oxide (GO) nanoparticles on zebrafish models involve multiple pathways, including oxidative stress, inflammation, DNA damage, mitochondrial malfunction, and dysregulation of autophagy. GO nanoparticles can induce the production of reactive oxygen species (ROS), initiate an inflammatory reaction, result in DNA damage within zebrafish cells, negatively impact mitochondrial activity, and disrupt the autophagy process, compromising cellular homeostasis and leading to harmful effects [123].

To investigate the *in vivo* cytotoxicity of Graphene Oxide (GO) nanoparticles on zebrafish models, various experimental techniques are employed. Zebrafish are typically exposed to GO nanoparticles through methods such as immersion in GO nanoparticle solutions or injection of GO nanoparticles into their circulation or specific anatomical areas. The assessment of toxicity involves the examination of alterations in behavior and morphology, quantification of oxidative stress parameters, histological study, and investigation of changes in gene expression or protein biomarkers [124]. Imaging modalities such as confocal microscopy and electron microscopy are used to visualize the interaction of GO nanoparticles with zebrafish tissues. Physiological functions of zebrafish are evaluated by monitoring measures such as heart rate, oxygen consumption, and swimming behavior to analyze the influence of GO nanoparticles [49]. Using zebrafish as a model organism, a study led by Bengt Fadeel investigated the connections between graphene oxide (GO), the gut microbiota, and the aryl hydrocarbon receptor (AhR). Zebrafish, both wild-type (WT) and AhR-deficient, were subjected to various GO concentrations over seven days. The findings revealed that GO significantly altered the gut microbiota in adult zebrafish, particularly in those with an AhR deficiency. Specific bacterial phyla showed changes in relative abundance due to GO exposure, with a more pronounced effect in AhR-deficient zebrafish. This indicates the involvement of AhR in modifying the microbiota in response to GO exposure. The study also explored the immunological reactions induced by GO in zebrafish larvae, showing AhR-dependent responses when combined with the short-chain fatty acid butyrate. This combination led to the activation of cyp1a and the homing of lck+ cells to the gut, exhibiting characteristics of regulatory cells, specifically ILC2-like cells with features of innate lymphoid cells (ILC). This suggests that the presence of GO, especially when combined with microbial butyrate, may induce specific immunological responses in zebrafish regulated by the AhR signaling system [125]. Research has indicated that GO attaches to and wraps around the chorion of zebrafish embryos, leading to delayed hatching and hypoxia. Aggregates of GO are found in various areas, including the embryos' eyes, hearts, yolk sacs, and tails. This results in

increased oxidative stress, DNA damage, excessive ROS production, and apoptosis in these tissues [126]. Another study reported that GO can cause cardiovascular abnormalities in developing zebrafish, with higher doses causing substantial abnormalities, increased apoptosis, delayed hatching, increased heart rate, and reduced hemoglobinization [127].

7. Overall impact

Graphene Oxide (GO) is extensively studied as a nanomaterial with diverse potential applications; however, its safety profile remains a critical concern. The unique physicochemical characteristics of GO, such as outstanding mechanical strength, a large surface area, and versatile functionalization abilities, have prompted extensive research into its uses. As the utilization of Graphene Oxide Nanoparticles (GONPs) increases, it becomes crucial to comprehend their full impact on both human health and the environment. A comprehensive investigation into how GONPs interact with living organisms at the nano-bio interface, encompassing cellular reactions, potential toxicity, and broader ecological effects, is essential. One notable property of GO is its ability to interact with biological systems, resulting in various outcomes, both advantageous and harmful. On the positive side, research has demonstrated that GO can effectively deliver medicines and other therapeutic substances to cells. GO may also find applications in the development of novel biosensors and scaffolds for tissue engineering. However, GO has also exhibited negative effects on biological systems. Notably, GO has been identified as toxic to the lungs in animal studies. Specifically, GO sheets have been shown to induce inflammation and lung damage in mice, with these sheets persisting in the lungs for up to 3 months [128]. In addition to its pulmonary effects, GO has demonstrated potential neurotoxic effects. A study exposing cultured rat hippocampal neurons to GO flakes revealed interference with synaptic signaling, particularly impairing excitatory synapses while sparing inhibitory synapses [129].

One primary mechanism through which Graphene Oxide Nanoparticles (GONPs) can induce pulmonary toxicity is by damaging the cell membranes of lung cells. Through interaction with the phospholipid bilayer, the fundamental component of cell membranes, GONPs compromise the integrity of the cell membrane, potentially leading to cell death [67]. Another avenue for GONP-induced pulmonary toxicity involves the production of reactive oxygen species (ROS) through the Fenton reaction. ROS generated can harm the cells lining the lungs and airways, contributing to pulmonary toxicity, fibrosis, edema, and inflammation [130]. In addition to the mentioned methods, GONPs have the potential to induce pulmonary damage through interactions with the immune system. White blood cells, such as neutrophils and macrophages implicated in inflammation, can be activated by GONPs, leading to the production of pro-inflammatory cytokines that encourage inflammation. Oxidative stress is identified as a factor causing liver damage through GONP exposure. This stress can damage hepatocytes and mitochondria, potentially leading to apoptosis and cell death. GONPs may also harm the liver by triggering hepatic stellate cell activation (HSCs), cells playing a role in liver fibrosis. Activated HSCs produce collagen, leading to scar tissue formation in the liver, posing potential problems [131]. Furthermore, GONPs can harm the liver by stimulating Kupffer cells, a type of phagocytic cells found in the liver. Activated Kupffer cells release pro-inflammatory cytokines, exacerbating inflammation and liver damage [132]. A study suggests that oral exposure to Graphene Oxide (GO) may alter the gut microbiome's composition, influencing immune responses. GO induces immunological responses dependent on the aryl hydrocarbon receptor (AhR) when coupled with the short-chain fatty acid butyrate. Characteristics of these responses include the rise of cyp1a expression, a sign of AhR activation, and the migration of lck+ cells to the gut. These CDK+ cells exhibit traits resembling innate lymphoid cells (ILCs), particularly ILC2-like cells with regulatory features. Notably, the presence of GO enclosed in a microbial butyrate "corona" is essential for these immunological reactions, working in conjunction with AhR to trigger responses [125]. In the majority of studies, the prevailing theory for GO cytotoxicity is ROS-mediated cellular damage. The physicochemical qualities, dosage, and administration methods directly impact the hazardous nature of GO. A comprehensive assessment of associated hazards and identification of molecular targets implicated in toxicity are imperative to minimize risks to human health and maximize the benefits of nanotechnologies.

8. Conclusion and future prospect

The immunotoxicity of graphene oxide (GO) nanoparticles is a complex issue influenced by various characteristics and interactions. GO, with its unique qualities like water processability, amphiphilicity, and surface functionalization, holds significant promise across diverse fields, particularly in medication administration. However, the synthesis, purification procedures, and impurities can considerably impact GO's characteristics and potential toxicity. Interactions with blood cells, macrophages, and other cell types have provided crucial insights into in vitro biocompatibility. Surface coatings, reducing toxicity to red blood cells, can enhance GO's hemocompatibility. Macrophages play a pivotal role in the immunological response to GO nanocarriers, potentially triggering inflammatory responses. Size and surface characteristics influence the cellular uptake, cytotoxicity, and immunological reactions of GO sheets. In conclusion, while graphene oxide nanoparticles exhibit promises across various applications, their immunotoxicity is influenced by a complex interplay of elements, necessitating thorough study for a comprehensive understanding of their behavior in vivo, long-term impacts, and ways to enhance biocompatibility in practical applications.

Several future research prospects for the immunotoxicity of graphene oxide nanoparticles exist. Studying dose-response relationships can provide information on safe exposure levels, aiding in the development of regulatory guidelines. Future research could delve into the long-term effects of graphene oxide nanoparticle exposure on the immune system, offering insights into potential chronic health risks. Understanding processes linking altered immune cell populations to the regulation and release of organ-specific cytokines is vital. Exploring unique surface coatings of GO with immune-modulating properties warrants investigation. The synergistic effects of graphene oxide nanoparticles with environmental pollutants or stresses, such as heavy metals or infectious agents, could reveal complex interactions and their impact on the immune system. Despite evidence of immunotoxicity, research should explore potential immunomodulatory effects of graphene oxide nanoparticles, especially in therapeutic applications for certain diseases. Human studies, including cohort studies of workers exposed or clinical trials of patients treated with graphene oxide-based therapies, are essential to determine the immunotoxicity of graphene oxide nanoparticles.

CRediT authorship contribution statement

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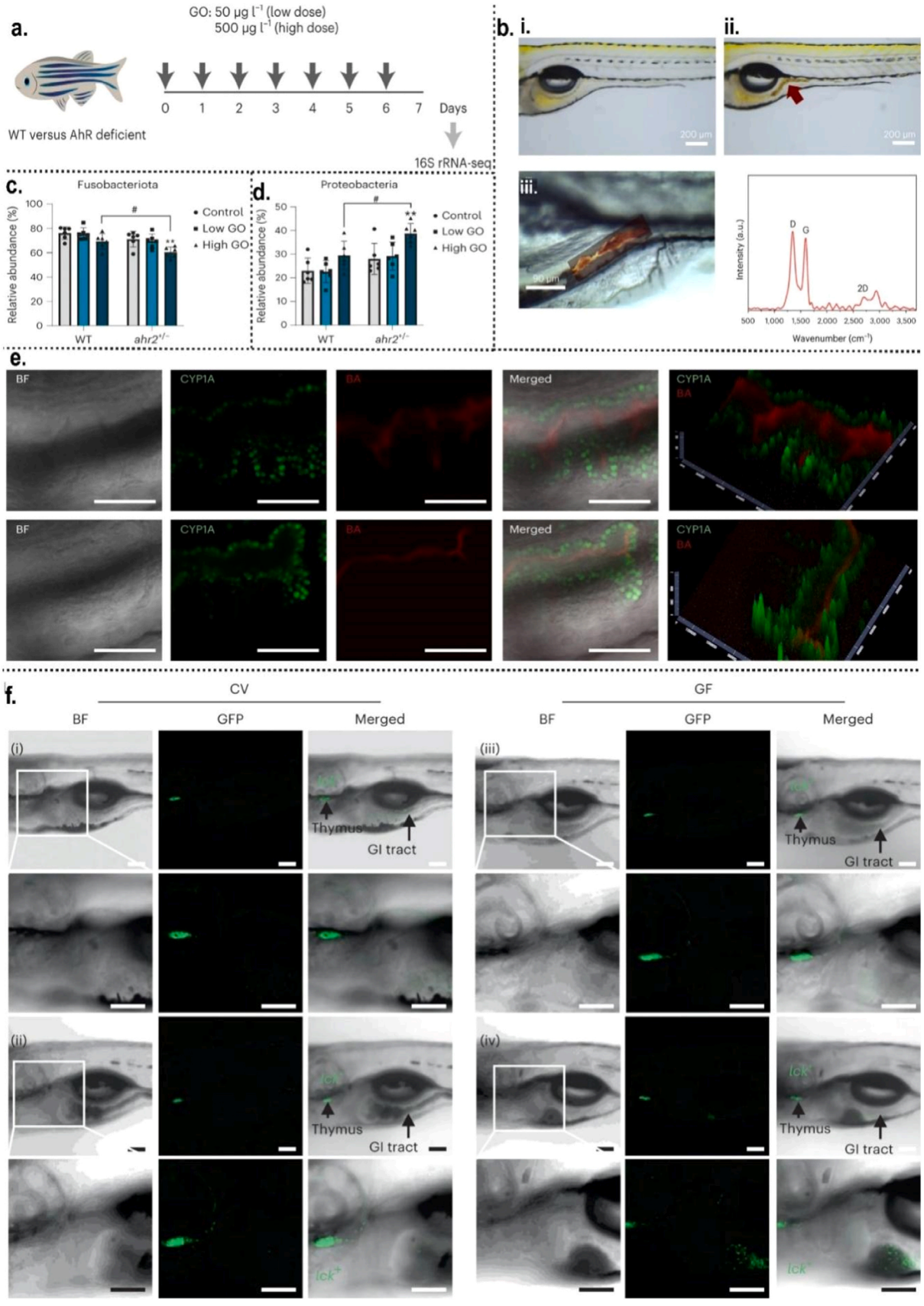

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Fig. 6. a. A seven-day exposure experimental setup was set up in adult zebrafish (WT and ahr2$^{+/=}$). **b**. (i and ii). Light microscopy, (iii). To identify the presence of GO Raman confocal mapping was. The treatment of 5 μg/mL was given on a 5 pdf zebrafish for 24 hrs. An average of 10,000 spectra is shown in the spectra graph. **c, d**. The phylum abundance of Fusobacteriota and Proteobacteria was compared between WT and ahr2$^{+/-}$ fish exposed to GO. **e.** The induction of cyp1a was visualized by employing zebrafish larvae with the Tg(cyp1a: GFP) transgene under germ-free conditions after being exposed to a combination of GO (30 μg mL–1) and resorufin butyrate (5 μM). Bile acid (BA) was discovered in the inside of the digestive tract, whereas the activation of cyp1a (cytochrome P450 1 A) was observed in the cells lining the gastrointestinal (GI) tract. **f.** lck^{+} cells were visualized using Tg(lck:GFP) zebrafish larvae. The larvae were treated to CV and GF conditions as described below: **(i)** Control group of fish with conventional diet (CV fish), **(ii)** Experimental group of fish with conventional diet supplemented with GO+BA (CV fish with GO+BA), **(iii)** Control group of fish with germ-free diet (GF fish), (iv) Experimental group of fish with germ-free diet supplemented with GO+BA (GF fish with GO+BA). Adapted with permission from[125].

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Declaration of Competing Interest

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

Data availability

All data included in this study are available upon request from the corresponding author.

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