

Faculdade de Engenharia da Universidade do Porto



**Immunomodulatory Impact on Macrophages and
Dendritic Cells of UV Phototherapy Using
Nanomedicines**

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Resumo

As doenças de pele são doenças globais muito comuns que afetam mais de 30 % da população mundial. Incluem várias patologias, desde o cancro de pele a condições crónicas, como a psoríase, dermatite atópica e vitiligo. Os custos associados aos tratamentos são muito elevados. Apesar de não haver mortalidade associada, o tratamento de doenças crónicas da pele é geralmente caro e tem um grande impacto na qualidade de vida dos doentes. Os efeitos secundários dos tratamentos comuns da psoríase e dermatite atópica incluem hepatotoxicidade e náuseas, causadas pelos fármacos e/ou pela administração de irradiação. Dado que a atividade imunológica está frequentemente implicada, não só na patogénese destas doenças, mas também no seu tratamento, a imunoterapia revela-se como um novo potencial tratamento. Tais terapias podem reduzir a necessidade de doses elevadas de medicamentos e radiação, evitando os efeitos secundários acima mencionados. Foram alcançados avanços notáveis no campo da nanomedicina e da imunoterapia desde a descoberta do efeito imunomodulador exercida por vários nanomateriais sobre o sistema imunitário. O principal objetivo deste trabalho de dissertação foi estudar o efeito imunomodulador dos materiais à base de grafeno (GBM) combinados com fototerapia UV sobre as células imunitárias, nomeadamente macrófagos e células dendríticas (DC). O trabalho consistiu primeiramente na caracterização física e química do óxido de grafeno nanométrico (GOn) e do óxido de grafeno parcialmente reduzido nanométrico (p-rGOn), utilizando técnicas como por exemplo, Microscopia Eletrónica de Transmissão (TEM), Espectroscopia de Infravermelhos por transformada de Fourier (FTIR), Análise Termogravimétrica (TGA), espectroscopia UV-Visível, entre outras. O GOn e o p-rGOn apresentaram tamanhos médios de $284,2 \pm 38,4$ nm e $218,5 \pm 25,7$ nm, cargas de superfície de $-44,5 \pm 4,7$ mV e $-37,9 \pm 2,6$ mV, e índices de polidispersão de $0,49 \pm 0,06$ e $0,431 \pm 0,061$, respetivamente, resultados obtidos por análise com um equipamento Zetasizer. Estes valores estão dentro da gama nanométrica que permite alcançar uma boa estabilidade em água, boa biocompatibilidade e também permeação através de tecidos biológicos. Notavelmente, ambas as dispersões aquosas de GBM mantiveram-se estáveis por um tempo de pelo menos 4 meses, o que é um bom indicador relativamente à futura preservação para administração em fototerapia. Quanto à caracterização química, esta foi feita para confirmar a oxidação bem sucedida do GOn e a eficácia da redução parcial através do uso de radiação UV, apresentando o p-rGOn uma diminuição dos grupos funcionais com oxigénio. Além disso, o p-rGOn apresentou um aumento de

aproximadamente 2 vezes em comparação com o GOn na absorção UV, no comprimento de onda (270 nm) utilizado para fototerapia no presente estudo.

Subsequentemente, o trabalho centrou-se na compreensão dos mecanismos imuno-terapêuticos relacionados com o efeito dos GBM, especificamente na sua incorporação por parte de macrófagos e DC e, consequentemente, na sua ativação. Os ensaios de viabilidade não mostraram toxicidade consistente, para ambos os GBM, na concentração de $100 \mu\text{g mL}^{-1}$, sendo esta concentração escolhida para as experiências seguintes. A análise morfológica em imagens de microscopia de fluorescência mostrou algumas anomalias celulares que foram confirmadas após realização de TEM, incluindo a perda e degradação de alguns organelos, particularmente na região peri-membranar e também a desintegração de mitocôndrias em macrófagos incubados com ambos os GBM.

Em seguida, a polarização dos macrófagos foi avaliada pela expressão de marcadores fenotípicos específicos e citocinas, combinando citometria de fluxo, expressão genética e ELISA. Ambos os GBM, com e sem irradiação, induziram um fenótipo mais pró-inflamatório, com aumento da expressão de IL-6 e IL-1 β , e reduzindo IL-10. Finalmente, a produção de espécies reativas de oxigénio (ROS) foi aumentada em presença de GOn e reduzida com p-rGOn, o que indica uma potencial capacidade de remoção de ROS por parte do último nanomaterial.

Relativamente à ativação das DC, foi realizada uma reação linfocitária mista (MLR), mostrando que ambos os nanomateriais, quando em condições pró-inflamatórias, aumentam a capacidade das DC para promover a proliferação de células T, em comparação com os controlos. A análise das citocinas secretadas revelou um aumento significativo na relação IFN- γ /IL-4, em MLR no que diz respeito a DC ativadas com TNF- α e expostas a p-rGOn, indicando que este GBM tem uma propensão para potenciar a reação inflamatória (polarização Th1).

Em resumo, os resultados obtidos suportam um papel pro-inflamatório dos GBM, sozinhos ou como adjuvantes de outras formas de estimulação. Para concluir, seria importante repetir alguns dos ensaios com maior número de dadores, para fortalecer os resultados obtidos e realizar um estudo detalhado de concentrações de GBM e diferentes condições de irradiação, para determinar melhor quais as doses terapêuticas não tóxicas e eficientes na imunomodulação.

Abstract

Skin diseases are very common global illnesses that affect more than 30 % of the population worldwide. They include several pathologies, from skin cancer to chronic conditions, like psoriasis, atopic dermatitis, and vitiligo. The costs associated to the treatments are very high. Despite no associated mortality, chronic skin diseases treatment is usually expensive and impact on patients' quality of life. Side effects of common treatments for psoriasis and atopic dermatitis include hepatotoxicity and nausea, caused by the drugs and/or irradiation administration. Given that immunological actions are often implicated, not only in the pathogenesis of these diseases, but also in their treatment, immunotherapy appears as a new potential treatment. Such therapies can reduce the need for high doses of medicines and radiation, avoiding the aforementioned side effects. Remarkable advances have been achieved in the field of nanomedicine and immunotherapy since the discovery of the immunomodulatory action exerted by several nanomaterials over the immune system. The main objective of this dissertation work is to study the immunomodulatory effect of graphene-based materials (GBM) combined with ultraviolet (UV) phototherapy over immune cells, namely macrophages and dendritic cells (DC). The work was first based on the physical and chemical characterization of nanographene oxide (GOn) and partially-reduced nanographene oxide (p-rGOn), using techniques such as Transmission Electron Microscopy (TEM), Fourier Transform Infra-Red Spectroscopy (FTIR), Thermogravimetric Analysis (TGA), UV-Visible spectroscopy, among others. GOn and p-rGOn presented average sizes of $284,2 \pm 38,4$ nm and $218,5 \pm 25,7$ nm, surface charges of $-44,5 \pm 4,7$ mV and $-37,9 \pm 2,6$ mV, and polydispersity indexes of $0,49 \pm 0,06$ and $0,431 \pm 0,061$, respectively, measured using a Zetasizer equipment. Those values are within the range of small nanometric dimensions required to achieve good water stability, biocompatibility, and penetration through biological tissues. Remarkably, both GBM aqueous dispersions were stable for up to 4 months of shelf-time, which is a good indicator regarding future preservation for administration in phototherapy. Chemical characterization, allowed to confirm GOn successful oxidation and the effectiveness of p-rGOn partial UV-reduction, which presented decreased oxygen-containing functional groups. Also, p-rGOn presented approximately a 2-fold increase in comparison to GOn in UV absorbance, at the wavelength (270 nm) used for phototherapy in the present study.

Subsequently, the work focused on the understanding of immunotherapeutic mechanisms related to the effect of GBM, specifically in their uptake by macrophages and DC and consequently, their activation. Viability assays showed no consistent toxicity, for both GBM, at the concentration of $100 \mu\text{g mL}^{-1}$, being this concentration chosen for the following experiments. The morphology analysis from fluorescence microscopy images showed some cell abnormalities that were confirmed by TEM analysis, including loss and degradation of some organelles, particularly in the peri-membrane region and also the disintegration of mitochondria in macrophages incubated with both GBM.

Next, macrophage polarisation was assessed by expression of specific phenotypic markers and cytokines, combining flow cytometry, gene expression and ELISA. Both GBM, with and without irradiation, induced a more pro-inflammatory phenotype, upregulating IL-6, and IL-1 β , and reducing IL-10. Finally, ROS production was increased by GOn and reduced in presence of rGOn, which indicates a possible scavenging capability of the latter nanomaterial.

Regarding DC activation, a mixed lymphocyte reaction was performed, showing that both nanomaterials, when in pro-inflammatory conditions, increase DC capacity to promote T cell proliferation, in comparison to controls. The analysis of secreted cytokines revealed a significative increase in the IFN- γ /IL-4 ratio, for MLR involving DC-activated with TNF- α and exposed to rGOn, indicating that this GBM has a propensity to potentiate the inflammatory reaction (Th1 polarization).

In summary, the results obtained support a pro-inflammatory role of GBM, alone or as adjuvants of other pro-inflammatory stimuli. To conclude, in the future it would be important to repeat some of the assays with a larger number of donors to strengthen the results obtained, and to perform a detailed study of GBM concentrations and different conditions of irradiation to determine the most adequate therapeutic doses that are non-toxic and immunomodulatory.

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Abbreviations, Acronyms e Symbols

BSA	Bovine Serum Albumin
CFSE	Carboxyfluorescein succinimidyl ester
CNM	Carbon Nanomaterial
CNT	Carbon Nanotube
DAPI	4',6'-diamino-2-fenil-indol
DC	Dendritic Cell
DLS	Dynamic Light Scattering
ELISA	Enzyme-Linked Immunosorbent Assay
FBS	Fetal Bovine Serum
FITC	Fluorescein isothiocyanate
FTIR	Fourier Transform Infrared Spectroscopy
GBM	Graphene-based Materials
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GO	Graphene Oxide
GOn	Nano graphene oxide
GQD	Graphene Quantum Dot
IL	Interleukin
INF- γ	Interferon Gamma
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharides
M-CSF	Macrophage-colony stimulating factor
MLR	Mixed Lymphocyte Reaction
MWCNT	Multi-walled Carbon Nanotubes
ND	Nanodiamond
NIR	Near-infrared radiation
NK	Natural Killer Cell
PBMCs	Peripheral Blood Mononuclear Cells

PBS	Phosphate-buffered saline
PEG	Polyethylene glycol
PFA	Paraformaldehyde
PHA	phytohaemmagglutinin
PI	Propidium iodide
p-rGOn	partially-reduced nanographene oxide
qPCR	Quantitative Real-Time Polymerase Chain Reaction
rGO	Reduced Graphene Oxide
ROS	Reactive Oxygen Species
TEM	Transmission Electron Microscopy
TGA	Thermogravimetric Analysis
TLR	Toll-like receptor
TNF- α	Tumor Necrosis Factor Alpha
UV	Ultraviolet

Chapter 1 : Introduction

1.1. Context

Skin disease is a very common global illness that affects more than 30 % of the population worldwide. Its main causes include aging, genetic reasons, and environmental conditions. The costs associated with treatments were close to 80 billion dollars in 2013 in the US alone, similar to cardiovascular diseases. Mortality was seen in several skin disease types, including skin cancer (around 60 %) and wounds and burns (around 15 %). [1, 2] Chronic skin diseases, like psoriasis, atopic dermatitis, and vitiligo, despite no associated mortality, are substantially aggravated by psychological issues, namely anxiety and depression. The quality of life of patients is reduced due to the symptoms associated with disease and treatments. [3-5]

Side effects resulting from existent therapies have been the major challenge to overcome. UV irradiation, apart from all advantages, can cause erythema and skin inflammation, and when in high quantities, can provoke skin necrosis. [6] Also, the use of drugs, like topical corticosteroids, can disturb skin barrier. [7] Immunotherapy has been offering new approaches for the treatment of chronic skin pathologies and for the development of new platforms of immunomodulation through antibody targeting and drug delivery systems, reducing the side effects. [8]

Nanoparticles have been used for various health applications, like diagnostics, imaging, and in drug delivery systems. Due to their high surface area, they can extensively interact with other molecules, which can be used in some cases to induce immunomodulatory actions, for pro- or anti-inflammatory purposes. [9] In 2004, the first graphene layer was obtained, and its unique optical, mechanical, and thermal properties have particularly fascinated the scientific field. Graphene-based materials (GBM), such as graphene oxide (GO) and reduced graphene oxide (rGO), have been increasingly used for biomedical applications. [10] GO, which consists of one-atom thick layer of carbon atoms arranged in a 2D honeycomb lattice, comprises oxygenated groups at its surface, which improves the dispersion of GO in aqueous solutions and enables surface functionalization. [11] GO is commonly used as a molecular sensor or as drug delivery system because of its large surface area that can load high quantities of immune stimulators and antigens. [12, 13] Reduced graphene oxide (rGO) is a result from the extraction of oxygenated groups from GO by

electrochemical, chemical, or thermal reduction. rGO is both conductive and chemically active which makes it a potential material to be functionalized with several biomolecules. [14] GO and rGO have been shown to be capable of adsorbing inflammatory cytokines. [13, 15]

1.2. Motivation

The objective of this dissertation work is to evaluate the response of immune cells, namely macrophages and dendritic cells (DC), to GBM in the presence or absence of UV irradiation. This approach will serve as the basis to create new phototherapy platforms using innovative nanoparticles that can simultaneously increase the efficacy of phototherapy and act as drug delivery systems for the treatment of chronic skin diseases. By understanding the immune mechanisms associated with phototherapy combined with GBM/drug conjugates, it can be possible to control skin disease-related inflammation, induce immunosuppression or immunostimulation targeting cancer therapy, providing innovative treatments that increase the quality of life of many people who suffer from such diseases.

Nanographene Oxide (GOn) and partially-reduced nanographene oxide (p-rGOn) were chosen for the development of the dissertation work since their use for UV photoimmunotherapy is a complete novelty. State-of-the-art literature points out graphene-based materials potential to be used in immunotherapy of skin disease. A recent study by Han *et al.* demonstrated that GO (with lateral dimensions around 200 nm) have the capacity to reduce immune cell proliferation and decrease reactive oxygen species (ROS) levels. This inhibited the release of pro-inflammatory cytokines from macrophages and lymphocytes, attenuating the inflammatory reaction. [16] Also, Tkach *et al.* found that, comparing to fullerenes, GO was capable of suppressing antigen-specific T cell response and inhibiting antigen presentation in DC. [17] These anti-inflammatory reactions bring new prospects for the appliance of these nanomaterials in chronic skin diseases treatment. Also, Li *et al.* studied the development of a hydrogel composed by cyclosporine loaded Pluronic® F127 and rGO to improve drug permeation and retention on tissues affected by psoriasis. Results demonstrated high quantities of drug trapping on the damaged tissue in *ex vivo* assays and psoriasis damage was reduced in mice. [18] These results open the possibility of using both GO and rGO as efficient drug delivery systems with immunosuppressive characteristics, for application on the above referred chronic skin diseases. It has also been described in the literature, by Deng *et al.*, Xu *et al.* and Wang *et al.*, that GBM can induce an immunostimulatory effect, that is not favourable for chronic skin diseases, but can be potentially used in cancer treatment. [19-21]

All above mentioned subjects will be further developed in the next sections of this thesis.

1.3. Goals

The main goal of this dissertation work was to open new prospects to a future use of innovative pharmaceutical formulations based on GBM in combination with phototherapy and understand its effects in the immune system, in order to achieve immunostimulation or immunosuppression

effects, to develop a selective treatment for skin disease or/and cancer, minimizing side effects resultant from current offered therapies. Specific objectives included:

- Collection of information and preparation of a literature review regarding state-of-the-art studies using carbon nanomaterials for application in immunotherapy;
- Development of GBM with adequate physico-chemical and photoproperties for photo-immunotherapy: production and characterization of GBM regarding surface chemistry, morphology, deagglomeration state, particle size, and phototherapeutic properties;
- Understanding the effect of GBM in immune cell response, biocompatibility, and pro/anti-inflammatory activation, using DC and macrophages, in the presence/absence of UV irradiation.

1.4. Contributions

- Bringing to light how the immune system (macrophages and dendritic cells) responds to UV phototherapy, combined with the immune dynamics of GBM (GOn and p-rGOn), with and without UV irradiation (phototherapy);
- Identification of possible synergistic actions of functionalized GBM, namely a targeted pro/anti-inflammatory action, to maximize phototherapeutic activity, in the field of phototherapy for chronic skin diseases;
- Future publication of a review article regarding the novelty of using CNM in immunotherapy (cancer and immunomodulation).

1.5. Structure

This dissertation presents the following structure: the first Chapter, introduces key concepts regarding the immune system and the use of (carbon nanomaterials) CNM in immunotherapy, including a description of various CNM's properties. Chapter 2 will present CNM applications in immunotherapy, namely on cancer treatment and immunomodulation. It also comprises an overview about challenges that still remain, and clinical issues related to immunotherapy using CNM. Chapter 3 and 4, reports the materials and methods, and the results obtained during the dissertation work. Chapters 5 and 6, consist on the results discussion and conclusions with drawn from the work developed, being future perspectives also pointed out.

Chapter 2 : Carbon nanomaterials in Immunotherapy

2.1. Background: Immune System and Immunotherapy

The ability to activate or suppress an immune response, generally designated as immunomodulation, is important for different areas of medicine, such as cancer, allergy, autoimmune diseases, transplantation, and infectious diseases. [22-26] The immune system identifies and neutralizes foreign pathogens and materials, that may threaten our health, and then induces a response to allow returning to homeostasis. However, the immune system can be disturbed in several ways, resulting in undesired suppression and inability to fight pathogens or cancer; or overstimulation, potentially leading to chronic activation and tissue damage. There is increasing biomedical research about different immunomodulatory strategies to better understand the mechanisms underlying immune system responses caused by specific compounds and take profits from that modulation to promote immunity (*e.g.*, to fight cancer) or tolerance (*e.g.*, to resolve autoimmunity). [27, 28] One of those strategies is using nanomaterials with capacity to stimulate or inhibit an inflammatory response. The natural reaction of the innate immune system to the direct recognition of a foreign body is a non-specific inflammation. Macrophages and dendritic cells (DC) are cells of the innate immune system that can phagocyte pathogens and eliminate them, but also release mediators, like cytokines, to activate other immune cells. Macrophages are very important in the inflammatory process, since they phagocyte dead cells, pathogens, and debris (and also nanoparticles), secrete a plethora of pro- and anti-inflammatory mediators that can initiate the inflammatory cascade. They are also able to present antigens to T cells, whereas DC main function is to survey the periphery and present antigens to T cells, coordinating the communication between innate and adaptive immune systems. [29] Lymphocytes (Natural Killer (NK), T and B cells) may be recruited to fight against threats, including pathogens or tumour cells. In cancer, for instance, cytotoxic T and NK lymphocytes, release cytokines, blockers of CTLA-4 (cytotoxic T-lymphocyte-associated protein 4) and PD-1 (programmed cell death protein 1)/PD-L1 (programmed death-ligand 1) inhibitors, to directly kill tumour cells. [30,

31] On the other hand, prolonged or uncontrolled immunostimulation, can lead to or exacerbate chronic inflammation, autoimmunity and allergic reactions. [32, 33]

Over the last few years, evidence has been mounting supporting the potential of nanomaterials to modulate the immune response. Biomaterials alone, or conjugated with specific biomolecules, can be used to directly modulate the immune system, consequently offering new approaches for the enhancement of immune-based therapies against inflammatory and autoimmune diseases, as well as cancer. [34-36] Nanoparticles have been used for various health applications, like in diagnostics, imaging, and as drug delivery systems. Due to their high surface area they can extensively interact with other molecules, which can be used to modulate immune cells towards pro or anti-inflammatory purposes. [9] Since they are foreign to the host organism, several nanoparticles induce an immune system response and have been increasingly explored for applications in immunotherapy. Nanoparticles can present anti-tumour properties or ideal characteristics to be used as therapeutic agents or vaccine adjuvants. Their size, surface charge, functionalization, hydrophobicity or hydrophilicity, and capability to bind to diverse biomolecules are critical factors that define the biocompatibility between the nanoparticle and the target immune cells. [37] Nanomaterials can be designed to imitate specific foreign signals and consequently manipulate the adaptive immune response, providing new biological systems to be used in the field of immunotherapy. [38, 39] However, it has also been shown that there are characteristics of the nanoparticles that can have relevant immunotoxic effects, such as activation of stress-related genes, membrane disruption, and release of pro-inflammatory cytokines. [40, 41] Therefore, a thoughtful approach is needed to select and design materials with desired properties for immunotherapy.

Nanoparticles modified by adsorption or cross-linking of biomolecules or antibodies can identify and target specific cell types, including tumour cells. Silica particles or metal-derived nanoparticles have been traditionally used, since they can directly or indirectly, through antibodies or drugs attached to them, induce a desired immune response. However, metal nanoparticles, such as gold nanoparticles, have been shown to be nondegradable, ultimately leading to bioaccumulation. Silica nanoparticles have been reported to require surface functionalization toward improving their biocompatibility, being their effect on immune cells dependent on their size, structure and other physical properties. [42] On the other hand, carbon nanomaterials (CNM) can be biocompatible even without functionalization, easily degradable or excreted from the human body. Additionally, CNM can provide a multiversity of possibilities for surface functionalization, when comparing with other nanomaterials. [43-45] CNM embrace a large variety of versatile carbon atom structures, that can be easily tuned for immunotherapy by varying their physico-chemical properties, such as size, degree of oxidation, and surface functional groups. Carbon nanotubes (CNT), graphene-based materials, fullerenes, and others, when alone or combined with therapeutic agents, can induce immune responses, useful for the treatment or prevention of several diseases. [12, 46-48]

Recently, CNM have gained huge attention in the biomedical scientific community, and the results obtained regarding immune system modulation are increasingly satisfactory. Due to their

exceptional properties and capacity to present variable sizes, shapes, surface chemistry, low cytotoxicity, and ability to attach different biomolecules, drugs or antibodies, the use of these nanomaterials became very promising. The main mechanisms of immunomodulation (immunosuppression and immunostimulation) that can be elicited by CNM are presented in Figure 1, with particular focus on the response mediated by macrophages and dendritic cells.

This first chapter presents a review that congregates and discusses, the most relevant information available presently, regarding the use of different types of CNM in immunotherapy. The main characteristics of CNM and their effects in the immune system will be presented. The first part will comprise studies regarding cancer immunotherapy. The second part will focus on immunomodulation therapies, for diseases other than cancer. It is divided in immunostimulation and immunosuppression. (Figure 2.1). In the third and final part, some challenges and questions that still remain related to the side effects of proposed immunotherapies will be highlighted, together with future prospects.

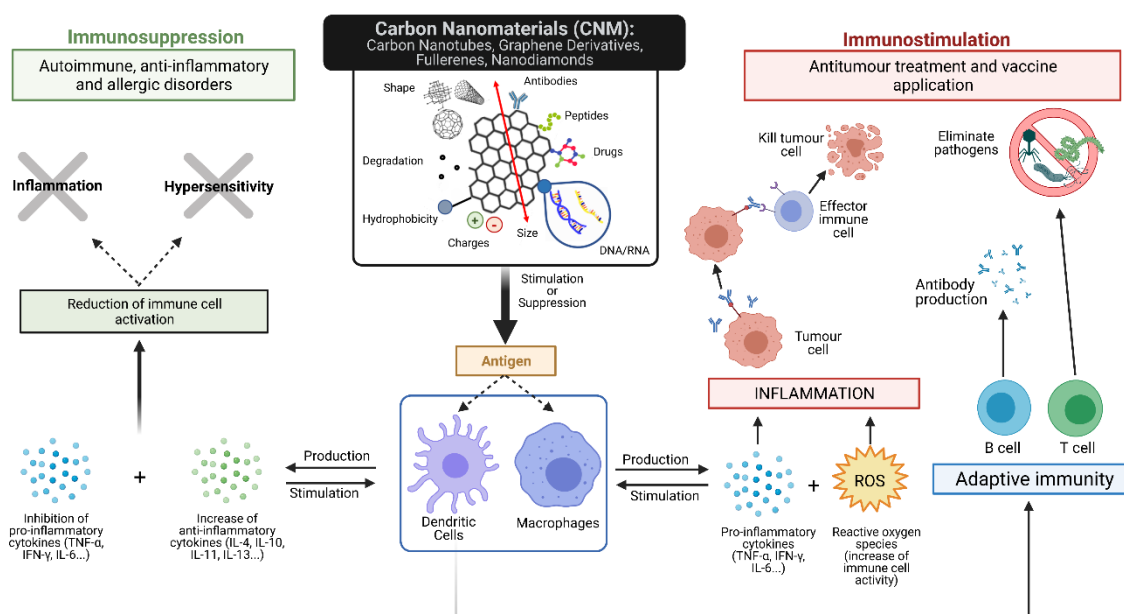


Figure 2.1. CNM most relevant properties and modifications and their effect on dendritic cells and macrophages. CNM with different structures, surface chemistry, sizes and shapes can be functionalized with various biomolecules (drugs, peptides, antibodies, DNA/RNA, and others). These structural differences provide specificity to the nanomaterials and can be tailored according to the desired immunomodulatory effect, towards immunosuppression or immunostimulation. Immunosuppression will lead to increased anti-inflammatory cytokines production and/or inhibition of pro-inflammatory cytokines, resulting in a reduction of immune cell activation, which is advantageous for the treatment of autoimmune, autoinflammatory and allergic diseases. Immunostimulation leads to the production of pro-inflammatory cytokines and reactive oxygen species (ROS) and/or to activation of the adaptive immunity (B and T cells) by CNM, resulting in antibody production and specific killing of tumour cells or pathogens. Created with [BioRender.com](https://www.biorender.com/).

2.2. Carbon Nanomaterials: Classification and Properties

Carbon atoms contain six electrons, arranged in the $1s^2 2s^2 2p^2$ electronic configuration. Their unique properties and high plasticity allow them to form different covalent bonds with other carbon atoms or elements which empower their presence from small organic molecules to complex structures such as CNM with distinct and remarkable properties. Figure 2.2, depicts the classification of CNM according to their dimensions, hybridization and crystallinity. CNM can be divided into 0D nanomaterials, which include graphene quantum dots (GQD), nanodiamonds (ND) and fullerenes, 1D nanomaterials comprising different types of carbon nanotubes (CNT), and 2D nanomaterials, like graphene (G) and its derivatives. Also, CNM can present variable amounts of sp^2 and sp^3 carbon bonds, diverse ranges of crystallinity, and can be chemically modified in numerous ways, making them outstanding tuneable platforms for immunotherapy. [49] This section provides background on the different CNM types and on their most relevant properties. Functionalization strategies and immunotherapeutic approaches will be addressed more detailedly in the following sections.

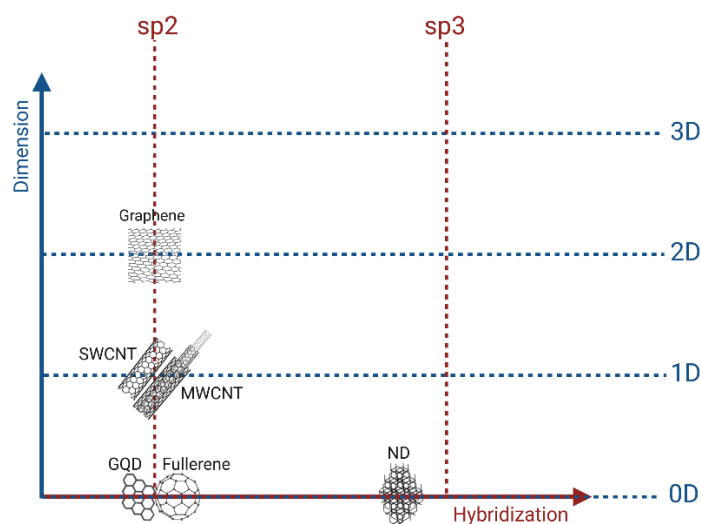


Figure 2.2. Representation of the structural characteristics of different CNM - Graphene, Multi-walled Carbon Nanotubes (MWCNT), Single-walled Carbon Nanotubes (SWCNT), Graphene Quantum Dots (GQD), Nanodiamonds (ND), Fullerenes, and classification according to their dimensions and carbon hybridization. Dimension was categorized considering their size or grain size, being 0D the nanomaterials with all dimensions < 100 nm, 1D the nanomaterials with 1 dimension > 100 nm and 2D the nanomaterials with 2 dimensions > 100 nm. Created with [BioRender.com](https://www.biorender.com) and adapted from [50].

2.2.1. Carbon Nanotubes

Carbon nanotubes (CNT) are 1D materials with a hexagonal lattice crystalline form of carbon mostly constituted by sp^2 hybridized atoms. CNT can be single-walled (SWCNT), having a single layer of carbon atoms in the wall, double-walled (DWCNT), or multiwalled (MWCNT), as they possess two or more layers, respectively. CNT can be produced by laser ablation, electric arc discharge, catalytic chemical vapour deposition (CVD), catalytic plasma-enhanced CVD, or template-based CVD. They present a very high aspect ratio, usually having lengths that can achieve several micrometres, while presenting diameters below 100 nm. CNT are mechanically strong,

electrically, and thermally conductive, having unique optical properties. These characteristics can vary according to their dimensions, chirality, and number of walls. [31] Due to their hydrophobicity, CNT are usually modified to improve dispersibility in the body and to improve their biological properties. Decreasing CNT size usually improves their biocompatibility, potentiating their interaction with other molecules, and increasing the chance of cell uptake. [32-34] Most CNT that are functionalized seem to avoid toxicity *in vivo*, since chemical functionalization enhances biocompatibility, biodistribution, biodegradation and elimination. However, it is very important to clarify which characteristics assure biocompatibility for each CNT type, in order to guarantee its safe use. [35-37] A long and rigid specific type of CNT, MWCNT-7, was considered carcinogenic after studies in mice, although several other types lack this negative effect. [38, 39]

2.2.2. Graphene and Graphene-based materials

Graphene (G) is a two-dimensional material with a crystalline form of carbon containing sp^2 hybridized atoms. It is the basic building block of hexagonally bonded carbon materials, with a 6-ring honeycomb lattice structure where each atom is bonded to three others. G presents high specific surface area, outstanding electrical, mechanical, and optical properties, which make it an important tool in nanotechnology. G can be produced by top-down approaches, using graphite as raw material, and/or bottom-up approaches, using alternative carbon sources. Graphene-based materials (GBM) are a broad class of materials that involve single-layer G, few-layer G (FLG, 2-5 G layers packed together), and multilayer G (MLG), also called graphene nanoplatelets (GNP) (2-10 layers). GBM can be oxidized, usually by chemical methods, being their most common form graphene oxide (GO). GBM oxidation disrupts the aromatic system, leading to a decrease in conductivity, which can be partially re-established by chemical, thermal, or electrochemical methods, resulting in reduced GO (rGO) when GO is used. [11, 12, 50-54] GO presents a higher amount of oxygen-containing functional groups, having higher water dispersibility and being more versatile for surface functionalization. However, both GO and rGO present a large surface area and oxygen groups, that allow conjugation with peptides, targeting ligands and/or drugs. GBM physicochemical properties, such as lateral dimensions and surface chemistry have been shown to impact their biocompatibility and immunological effects, therefore those can be adjusted according to desired effects. [55-58]

Graphene quantum dots (GQD) are 0D materials (2-20 nm) with a crystalline form of carbon containing sp^2 hybridized atoms. These materials can be produced by top-down techniques, applying a cutting process to graphite, carbon black, GO, and CNT, or by bottom-up techniques, through the assembly of large aromatic molecules. [50] Due to their small size, GQD are water dispersible and are usually easily eliminated and biodegraded, presenting good biocompatibility. Their large surface area and functional groups (e.g., containing O, N) allow efficient drug loading and/or functionalization with targeting moieties. [59-61] GQD optoelectronic and electronic properties are highly dependent on their lateral size, with smaller particles having nonzero band gaps, exhibiting outstanding properties. Their quantum confinement and edge properties originate fluorescence, which makes GQD a very stable and robust tool to be used in theranostics. [50, 59]

2.2.3. Nanodiamonds

Nanodiamonds (ND) are 0D materials with a crystalline form of carbon containing sp^3 hybridized atoms. These nanoparticles have a diameter range from 1 to 20 nm. ND can be produced by several different methods, which have high influence on their properties, such as size, shape, and quality. Detonation nanodiamonds are produced by the detonation of an explosive mixture of carbon-containing compounds (e.g., trinitrotoluene), with particles with sizes around 5 nm being usually obtained. The main disadvantage of this procedure is the formation of poorly dispersible aggregates coated with a graphitic layer. However, ND with higher purity and with less defects can be obtained by grinding larger diamonds. These ND have a flake-like shape and different sizes that can be selected by centrifugation. [50] ND derived from detonation present a very small size and large surface area, enabling extensive drug loading, holding potential to be used as drug delivery systems. [62] ND have been shown to cause low cytotoxicity in many cell lines, however, some studies revealed genotoxicity towards *in vivo* models, which demands caution when considering biomedical uses. [63-65] Fluorescent nanodiamonds are produced by irradiation with a high-energy ion beam and consequent thermal annealing that causes the emission of fluorescence at the nitrogen-point defects. Such diamonds are highly photostable and emit fluorescence in the near-infrared region. Recently, they have been explored for cancer immunotherapy, drug targeting on the body after administration and other biomedical applications. [66, 67]

2.2.4. Fullerenes

Fullerenes are 0D materials with a crystalline form of carbon containing sp^2 hybridized atoms. These materials are presented as closed hollow cages with icosahedral symmetry made of 12 pentagon rings and a number of hexagons dependent on the total number of carbon atoms ($20 + 2n$, where n is the number of hexagons). [50] Fullerenes are spheres with approximately 1 nm in diameter and, through the benzene rings on their surface, they can absorb electrons, which enables them to function as ROS scavengers. They exhibit high polarizability and hydrophobicity. C_{60} is the smallest carbon nanostructure and also, the most abundant and most studied fullerene. Aqueous suspensions of C_{60} have been shown to be stable and nontoxic to several human cell lines, such as PBMC, HeLa S3 and CEM cells. [68-70] However, fullerenes water stability is low, requiring further surface modification. For this reason, many types of functionalized fullerenes have been produced according to desired applications. [68] Due to the pyramidalization of the carbon spherical form, fullerenes present a high reactivity. Their structural cage is stable, but readily functionalizable, allowing drug loading, and furthermore, the use of endohedral metallofullerenes with encapsulated metals (e.g., Gd) allows applications in bioimaging. [71, 72] C_{60} and C_{70} types of present potential to be used in DNA cleavage and photodynamic therapy since they can absorb in the UV and visible light region and generate oxygen reactive species. C_{60} can accept up to six electrons, which allows the intermolecular electron transport. Also, the fullerene C_{60} is a specie with a large surface which allows the attachment of hydrophilic molecules. [69, 73] Fullerenes are

molecules that are completely formed by carbon and they have strong antioxidant, since they can react with many superoxides without being consumed, and antiviral activity, since they can fit inside of HIV hydrophobic cavity of proteases, for instance, due their unique molecular architecture. [74]

2.3. Cancer Immunotherapy

Oncological malignancies affect worldwide population with a yearly incidence of around 18 million new cancer cases and almost 10 million cancer deaths each year, making it the second leading cause of death globally. [75] Early detection and selective treatment strategies are paramount to fight this disease effectively. The most common treatments include surgical tumour excision chemo and radiotherapy, or combinations thereof. Tumour removal is invasive, leaving potentially large defects in the tissue, and some cancer cells can still remain leading to recurrence, often requiring a combination with chemo, radio, or immunotherapies. Systemic chemotherapy has severe side effects, affecting healthy cells, debilitating the entire organism and potentially leading to the development of resistance. Radiotherapy is often performed after surgical tumour removal, but its side effects include cardiovascular complications, especially when used to treat thoracic cancers (e.g., lung, breast). On the other hand, immunotherapy approaches stem from the perception that while immune cells can kill abnormal tumour cells, tumours can turn off immune responses and promote the development of tolerogenic immune cells that favour tumour growth and metastasis. [76-79] Figure 3, represents the main CNM interactions with tumor cells. In general, anti-cancer immunotherapy consists of a stimulation of the immune response against the tumour. This was initially achieved by the administration of cytokines like tumour necrosis factor (TNF)- α and interleukin (IL)-2, that are naturally produced in response to pathogens and can activate several immune cell populations, like macrophages, T and NK lymphocytes. [80] However, their short half-life and the need for repeated high doses can cause severe secondary effects. Antibodies that target checkpoint inhibitors, like cytotoxic T lymphocyte antigen 4 (CTLA4) and programmed cell death 1 (PD-1) or its ligand PD-L1, are still widely researched for cancer therapy. [31] They allow for T cells to remain active, recognize and kill tumour cells. The engineering of autologous T cells to express chimeric antigen receptor (CAR) that are tumour-specific has also been approved for clinical use, although currently it is only used for melanoma. These engineered T cells are then re-administered to the patient, where they recognize and kill tumour cells. [81] Although patients are reported to achieve remission and improved survival, their long-term effects are yet unknown, and particularly in solid tumours, combination therapies are required to improve the survival of CAR T cells. [82] NK cells can target tumour cells and release granules and granzymes that cause tumour cells lysis. As NK cells do not often infiltrate solid tumours, their use in cancer immunotherapies has been mainly focused on hematopoietic malignancies. [83, 84] The adaptive immune response against tumours can be triggered by activated antigen presenting cells (APC), like macrophages and DC. Autologous DC are the most

widely researched for anti-cancer vaccines, whether by exposure to cancer antigens or engineering them to express those antigens. [85] These cells are then re-injected into the patient and can specifically activate T cells against the tumour. [86] Cancer vaccines based on the delivery of exogenous nucleic acids, RNA or DNA to APC, toward triggering an immune response against tumours, are also being tested in several clinical trials. [87, 88] The success of immunotherapies still depends on several factors, like the tumour immunogenicity as well as, the choosing of the best pathway, bearing in mind that it should be the one that most benefits the interaction between, cells, proteins and antibodies, in order to more efficiently fight against the tumour and avoiding side effects. [89, 90]

Over the last years, the number of studies in cancer immunotherapy field involving the use of CNM has been considerably increasing. Diverse strategies have been reported, including modulation of the immune system due to their intrinsic characteristics and/or functionalization, targeted drug delivery to enhance the efficacy of chemotherapy, and their functionalization with antibodies to allow a selective accumulation in tumours, which might be followed by hyperthermia therapy using near infrared (NIR) radiation, owing to the intrinsic high NIR absorption exhibited by some CNM, like CNT and GBM. [91, 92] CNM can be administered inside the tumours or be targeted by surface modification, localizing the heating effect in cancer cells, and decreasing damage to surrounding tissues. Also, drug release can be triggered by NIR irradiation of CNM/drug conjugates, increasing treatment specificity and efficacy. [93, 94] Some mild hyperthermia strategies with low energy irradiation, using light emitting diodes (LED) or low power lasers, have been shown to increase treatment selectivity even further, when compared with thermal ablation strategies, being an alternative to more invasive cancer phototherapies. [95-97] CNM specific functionalization and sizes have been reported to influence immune cells, inducing specific activation of antigen presenting cells, regulating the immune response against tumours through the stimulation of pro-inflammatory cytokines secretion. To improve targeting, those nanomaterials can be functionalized with drugs or antibodies, which can be combined with polymers, like polyethylene glycol (PEG) that adds stability to the nanomaterials in dispersion, improves treatment specificity and potentiates the anti-tumour immune response. [98] PEG has also been shown to provide efficient targeting and protective coating on particles for immune evasion. [99, 100] The conjugation of anti-cancer drugs with CNM allows preferential accumulation and delivery at the tumour site, due to the enhanced permeability and retention (EPR) effect, reducing the required doses, thus minimizing side effects. CNM size and surface properties can be adjusted to maximize cell uptake in a targeted way, this can be achieved as for example by varying the molecules attached at their surface. [99, 101-104]

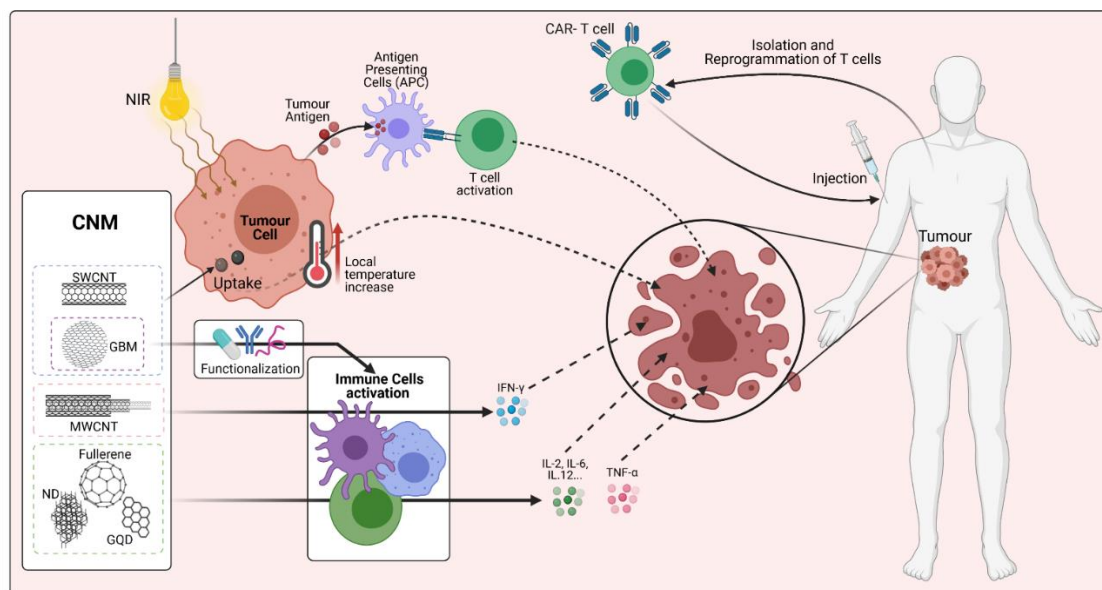


Figure 2.3. CNM interactions with tumor cells. SWCNT and GBM combined with NIR irradiation can target and kill tumour cells through the local increase in temperature. [91, 92] Also, some GBM, like graphene oxide, functionalized with anti-cancer drugs or other biomolecules can activate the immune cells (e.g., macrophages, dendritic cells, T cells) to kill tumour cells. [98] The release of tumour antigens, induce APCs (Antigen Presenting Cells) to process and present antigens that will be recognized by certain lymphocytes, such as T cells, leading to their immunoactivation. Also, autologous T cells can be engineered and reprogrammed (CAR-T cells) to improve their function and destroy the cancer cells. Both therapies can be administered through injection (Cancer vaccines). [82, 85] CNM: Carbon Nanomaterials; GBM: Graphene Based Materials; GQD: Graphene Quantum Dot; MWCNT: Multi-Walled Carbon Nanotube; ND: Nanodiamond; NIR: Near Infrared; SWCNT: Single-Walled Carbon Nanotubes; Cytokines: IFN- γ , TNF- α , IL-2, IL-6 and IL-12. Created with [BioRender.com](https://www.biorender.com).

CNM can also boost vaccines efficacy, through their functionalization, enhancing the anti-cancer immune response, by promoting biosafety and by optimizing immunization routes. They can deliver the totality or a part of the tumor cell which provides tumor antigens as targets, triggering cell death by priming antitumor immune actions. [105, 106] Table 2.1, condenses studies where CNM, with or without functionalization, were applied in cancer immunotherapy field and anti-cancer vaccination.

Table 2.1. Applications of carbon nanomaterials in cancer therapy and immunotherapy.

Nanomaterial	Dimensions	Functionalization	Molecules Delivered	<i>In vitro</i> model	<i>In vivo</i> model	Results	Ref. No.
	d = 1.4 nm l = 0.5-1 μ m	w/ PEG, HER2 and IGF1R specific antibodies	-	Human BT474 and MCF7 breast cancer cells	-	100 % of MCF7 cells died after NIR irradiation	[92]
	d = 1.17 $\approx$ 0.28 nm l = 88.00 $\approx$ 43.68 nm	Carboxylation and conjugation w/ anti-HER2	-	Human breast carcinoma cell lines SK-BR-3 and MCF-7	-	$\approx$ 97.7 % of SK-BR-3 cells died after NIR irradiation	[107]
	l = 0.2-1.4 μ m	w/ DSPE-PEG-biotin	-	Human PBMCs and Burkitt's lymphoma cell line (Daudi)	-	> 90 % of Daudi cells and PBMCs died after NIR irradiation	[108, 109]

	l = 100-1000 nm d = 0.8-1.2 nm	NH ₂	WT1 peptide 427 (Wilm's tumour)	Human CD4 T-Cells	BALB/c mice injected w/ WT1Pep427 alone and conjugated to SWNTs	<i>In vitro</i> : no relevant data (SWCNT maintained the CD4 T cell response) <i>In vivo</i> : > 70 % ↑ Peptide-Specific IgG Response with SWCNT + TiterMax [110]
	l = 200-400 nm	w/ PEG	CpG	Murine GL261 glioma cell lines and primary bone marrow-derived monocytes	C57BL/6 or CX3CR1 ^{GFP} mice	<i>In vitro</i> : 5 % ↑ of CpG uptake; IL-12 (>100 pg mL ⁻¹) and TNF-α (> 250 pg. mL ⁻¹) production <i>In vivo</i> : 50 % of elimination of intracranial GL261 gliomas in mice with > 3 months tumour-free remission [111]
	sa = 845 m ² g ⁻¹	3 M HNO ₃ and 3 M HNO ₃ / LiBH ₄	-	B3Z T cell hybridoma line	-	> 20% ↑ T cell stimulation with 3 M HNO ₃ / LiBH ₄ SWCNT and 3 M HNO ₃ SWCNT [112]
	d = 200 nm	3 M HNO ₃ / LiBH ₄	-	Primary splenocytes from C57BL/6 mouse	-	Secretion of IFNγ (≈ 2500 pg mL ⁻¹ at 96 h) with the f-bSWCNT; 30 % ↑ proliferation of T cells (peak population of T cells at 72 h) with f-bSWCNT+Ab [113]
	l = ~100 nm	3 M HNO ₃ / LiBH ₄	SIIN peptide	OT-1 mice splenocytes	-	^N CNT + MHC-I/SIIN enhanced T cell response with IFN- γ values around 3500 pg mL ⁻¹ [114]
MWCNT	d = 20-30 nm l = 500-800	Carboxylation	H22 tumour lysate protein	Murine H22 hepatocellular carcinoma cells	BALB/c mice inoculated with of H22 cancer cells	<i>In vitro</i> : > 200 H22 target cells (number) died comparing to another kind of cancer cell (tumour specific dead) <i>In vivo</i> : > 15 % improvement of the cure rate of H22 liver cancer in mice comparing to common tumour cell vaccines [115]
	d = 20-30 nm l = 50 μm	-	-	-	BALB/c mice inoculated w/ H22 cancer cells	40 % ↑ of inhibition tumour growth rate comparing with the control group; > 80 % ↑ in IFNγ and some ILs production [116]
	d = 3-5 nm l = 10-50 μm	-	-	Murine MC38 colon cancer cells	C57 BL/6 mice inoculated w/ MC38 (ATCC) colon cancer cells	<i>In vitro</i> : no relevant data <i>In vivo</i> : ~80 % ↓ in tumour weight and volume with ESC + MWCNT; ↑ of the percentage of CD4+ (>20 %) and CD8+ (>40 %) lymphocytes; ↑ expression of IL-2 and IFNγ in mice injected with ESC + MWCNT [117]
	d = 20-30 nm l = 0.5-2 μm	Oxidation	OVA, CpG and anti-CD40 Ig	B16F10 murine melanoma cells	C57BL/6 mice inoculated w/ OVA-B16F10-Luc cancer cells	<i>In vitro</i> : ↑ IFNγ production in treatment with S ^{-/+} (OVA-CpG) for CD4+ (>800 pg. mL ⁻¹) and CD8+ (>1500 pg. mL ⁻¹) <i>In vivo</i> : >40 % ↑ antigen-specific death w/ S ^{-/+} (OVA-CpG) [85]
	d=20-30 nm l=5-200 μm length	H ₂ SO ₄ and HNO ₃	7-TOG	Human monocytes	-	No significant MoDC maturation induction and cytokine production [118]

GO	d = 300-500 nm	PEG	-	Murine RAW264.7 and human osteosarcoma cell line HOS	BALB/C mice injected with human osteosarcoma cells	<i>In vitro</i> : ~16 % ↑ levels of CD206 in the IL-4 group (M2 polarization of RAW264.2) and ~11 % ↓ in the GO-PEG+IL-4 group (reduced M2 polarization) <i>In vivo</i> : >50% ↓ in tumour weight/volume with GO-PEG + NIR comparing to control [19]
	50 nm	PEG-PEI	Urease B (specific antigen for gastric cancer)	Bone marrow-derived dendritic cells from mice	BALB/c mice	<i>In vitro</i> : 8 % ↑ in the activation of TLR2 and TLR4 signalling pathways in DC and ↑ of the delivery of Ure B from ≈ 40% to ≈ 90%, both in the presence of GO-PEG-PEI; <i>In vivo</i> : ≈ 40% ↑ of GO-PEG-PEI-Ure B enhancement of T cell proliferation comparing to free Ure B and Alum-Ure B; [20]
	318.36 nm	Aluminium oxyhydroxide (Al (OH))	OVA	Murine E.G7 thymic lymphoma and B16 melanoma cells	C57BL/6 mice with induced lymph nodes	<i>In vitro</i> : >50% ↑ in OVA uptake with GO-Al; ↑ in the expression of CD86 (≈60%), MHC-I (≈20%), and MHC-II (≈70%); <i>In vivo</i> : >70 % ↓ in tumour weight; >10 % ↑ in CD3+ CD8+ T lymphocyte responses with GO-Al (OH) [21]
	2 μm	-	OVA	Murine BMDC	C57BL/6 mice injected with E.G7 lymphoma cells	<i>In vitro</i> : 60 % ↑ OVA uptake attached to GO by BMDC at 12 h; >20 % ↑ in the expression of CD86, MHC I and II with OVA+GO; <i>In vivo</i> : ↓ in tumour volume (< 3000 mm ²) with OVA+GO [119]
GO and rGO	GO:100 ≈ 10 nm; V-rGO: 110 ≈ 20 nm	Vanillin (only rGO)	-	Human Leukaemia THP-1 cells	-	At 100 μg.mL ⁻¹ : ↓ cell viability with V-rGO (≈ 20 %) and GO (≈ 40 %); ↑ LDA leakage (almost 200 % with V-rGO); ↑ > 30% in the production of IL1-B, TNF-α, GM-CSF, IL-6, IL-8, and MCP-1 [120]
rGO	> 30 nm	PEI and FA	DOX and EA	CBRH7919 cancer cells	-	Drug loading of > 45 % of EA and > 28 % of DOX with rGO-PEI-FA; > 70 % of cancer cells arrested to the G2 phase, (phase where those are more vulnerable to damage), causing their apoptosis [121]
GQDs	h = 2.0 nm	-	-	Peripheral blood cells	-	> 60% necrosis in PBMC for 750 μg of GQD (after 48 h); No toxicity for concentrations around 200 μg mL ⁻¹ and stimulation of IL-10, IL-4, and IL-5 [122]
	d = 3.03 nm	-	-	THP-1 monocyte cell line	-	20% ↑ in autophagy in 48h and with the increase of GQDs concentration (200 μg mL ⁻¹); > 60% ↑ apoptosis; > 500 pg mL ⁻¹ ↑ of the expression of TNF-α and IL-8 (with 10 μg mL ⁻¹ of GQDs) [123]

ND	d = 4-5nm	w/ Polyglycerol, L-arginine, glycine, n and L-aspartic acid	Doxorubicin	Mouse THP-1-derived DC, bone marrow-derived DC and spleen derived LC	Athymic BALB/c mice	<i>In vitro</i> : >300 pg mL ⁻¹ release of IFN γ with GC/mDC/ND; >30% $\uparrow$ up-regulation of CD69 by CD4 ⁺ CD8 ⁺ <i>In vivo</i> : CFSE-derived fluorescence in the brains of all tumour-bearing mice (48 h); staining of caspase-3 (apoptosis) in all groups [124]
	70 $\approx$ 28 nm	-	-	Murine RAW264.7, NK and NK cells and monocytes	BALB/c mice	<i>In vitro</i> : Fluorescent ND uptake in RAW264.7, NK, NKL, and monocytes; 10-fold change $\uparrow$ in CD69 expression with IL-2+FND <i>In vivo</i> : Clear visualization of fluorescent NDs in mice [125]
Fullerenes	d $\approx$ 80 nm	-	-	Mouse peritoneal macrophages	H22-tumour bearing mice	<i>In vitro</i> : $\uparrow$ phagocytic rate from 26 $\approx$ 4 to 34 $\approx$ 4 in the presence of C ₆₀ (OH) _x 2.0 <i>In vivo</i> : $\uparrow$ activity of arginase (> 130 $\times$ 10 ³ U) and acid phosphatase (> 300 U) = macrophage activation [126]
	d = 22 nm	w/ Gadolinium	-	Human A549 Adenocarcinoma cells or rBCECs	C57BL/6 mice injected with Lewis lung carcinoma cells	<i>In vitro</i> : > 60 % dehydrogenase activity in A549 and rBCE cells with 66.8 μ g/m of fullerol specie <i>In vivo</i> : Growth inhibition of malignant MCF-7 solid tumour (maintenance in 50 mg) [127]

Ab: Antibody; BMDC: Bone Marrow-derived Dendritic Cells; CFSE: 5(6)-carboxyfluorescein diacetate succinimidyl ester; d: diameter; DOX: Doxorubicin; EA: Elnochrome A; ESC: Embryonic Stem Cells; FA: Folic Acid; GC: Gioblastom Cells; h: height; f-bSWCNT: Functionalized Bundled Single-Walled CNT; l: length; mDC: Mouse bone marrow-derived DC; MoDC: Human Monocyte-derived Dendritic Cells; OVA: Ovalbumin; PBMCs: peripheral blood mononuclear cell; PEG: polyethylene glycol; PEI: Poly ethyleneimine; rBCECs: Rat brain capillary endothelial cells; S^{-/+}: pristine-MWNTs functionalized with an amine-terminated spacer; sa: surface area; TC: TAT-chitosan; Ure B: Urease B.

Shao *et al.* attached 1-pyrenebutanoyl succinimide non-covalently to the surface of functionalized SWCNT, where fluorescent antibodies were linked by reaction with succinimide. [92] Anti-HER2 and anti-IGF1R were selected because they can recognize two receptors, human endothelial receptor 2 (HER2) and insulin-like growth factor 1 receptor (IGF1R), which are commonly overexpressed in several cancer cells, enabling to specifically target those cells. The SWCNT-antibodies complex (1 mg mL⁻¹) was incubated for 48 h with MCF-7 breast cancer cells. Effective targeting and internalization were observed, which resulted in 100 % death of breast cancer cells after 3 min irradiation with NIR light (808 nm, $\sim$ 0.8 W cm⁻²). [92] A similar result was obtained by Xiao *et al.* in a study reporting the use of oxidized SWCNT activated by the introduction of carboxylic groups and further covalently functionalized with anti-HER2 antibody. [128] SK-BR-3 breast carcinoma cells were cultured in the presence of the complex SWCNT-antibody for 24 h. In this case, CNT were not found inside the cells but attached to the cellular membrane. After irradiation with NIR light (808 nm, 5 W cm⁻²) for 2 min, cells treated with the SWCNT-antibody complex presented a viability decrease of around 98 %, while controls revealed no changes (around 100 % viability). [107] Marches *et al.* studied the functionalization of SWCNT with 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[biotinyl(polyethylene glycol) 2000] (DSPE-PEG (2000)-biotin). Initially, avidin was activated by two specific IgGs, RFB4 (mouse IgGs anti-human CD22) and RFT5 (mouse IgGs anti-human CD25). Then, biotin-functionalization of SWCNT allowed their specific

linking to avidin-derivatized antibodies targeting tumour cells. The complexes (0.06 mg mL^{-1}) were incubated with Daudi cells and peripheral blood mononuclear cells and irradiated with NIR light (805-811 nm, 9.5 W cm^{-2}) for 4 min, to induce cell death by thermal ablation. It was observed over than 80% viability decrease in the RFB4-CNT-treated Daudi cells and RFB5-CNT-treated PBMCs after NIR light exposure. CD22⁺CD25⁻ activated Daudi cells, bounded to CNT coupled to the anti-CD22 antibodies, while CD22⁻CD25⁺ activated peripheral blood mononuclear cells bounded to CNT coupled to the anti-CD25 antibodies. The results showed that the conjugation of CNT with antibodies is a promising way to target cancer cells. [108, 109] Villa *et al.* covalently attached WT1Pep427 on solubilized SWCNT scaffolds, in order to improve the immune response against tumour antigens. The internalization, in a first phase, occurred through their uptake in peripheral vesicles and then, transportation to a perinuclear area. Although no differences were found between the peptide-CNT conjugates and the peptide alone *in vitro*, conjugates were internalized by human dendritic cells *in vivo*. Additionally, in the presence of an immunological adjuvant (Titer Max), it induced over 70% of peptide-specific IgG response, comparing with the peptide alone. [110] Zhao *et al.* conjugated functionalized SWCNT with CpG oligodeoxynucleotides and verified SWCNT uptake *in vitro* and *in vivo*, in intracranial GL261 gliomas. The CpG delivery mediated by mouse primary monocytes was potentiated by SWCNT and resulted in the production of pro-inflammatory cytokines, such TNF- α and IL-12. Intracranial GL261 gliomas elimination was verified in 50 % of the mice treated with SWCNT-CpG low doses ($2.5 \text{ } \mu\text{g CNT}/5 \text{ } \mu\text{g CpG}/10 \text{ } \mu\text{L PBS}$) by intracranial injection, while free CpG and free SWCNT did not present any effect. Additionally, animals that survived to tumour implantation, did not present tumour recurrence after treatment with SWCNT-CpG in a time superior to 3 months, which can indicate long-term immunity. [111] Fadel *et al.* showed that (3 Molar $\text{HNO}_3/\text{LiBH}_4$)-functionalized SWCNT conjugated with anti-CD3 increased the stimulation of T cells *in vitro*. Cell response occurred in a concentration-dependent manner, increasing as higher amounts of $\text{HNO}_3/\text{LiBH}_4$ functionalized-SWCNT were administered, which constituted an innovative strategy for stimulation of lymphocytes. Such an approach can be used in situations that require a strong stimulus for an effective expansion of T cells outside the body for several clinical applications, for instance, cancer treatment. [112] Fadel *et al.*, in 2010, studied the effect of the (3 Molar $\text{HNO}_3/\text{LiBH}_4$)-SWCNT loaded with anti-CD3 antibodies in the enhancement of T cell stimulation in comparison with free antibodies. They verified increased IL-2 and IFN- γ production by T cells, comparing to free antibodies, suggesting that SWCNT can act as an efficient platform to be adopted in cancer immunotherapy. [113] Some years later, in consequence of the results previously obtained, the use of CNT as a platform for antigen-specific response to a multimeric T cell stimulus with MHC-I protein has been demonstrated. Results showed an enhancement of T cell response through the increase of IFN- γ levels and an active way for presenting MHC-I, proving that engineered complexes with CNT can be adapted to efficiently respond to immunomodulatory needs. [114]

Meng *et al.* produced MWCNT/H22P tumour lysate protein conjugates, being the later a cocktail of tumour proteins alternative to tumour-specific antigens. MWCNT/H22P influence on the immune system toward developing a tumour-cell vaccine to hepatocarcinoma was evaluated.

An enhancement on the antitumour cell response and a 55 % improvement of the cure rate of H22 liver cancer were observed in mice (Figure 4.A). [115] Some years later, they validated the results on the immune response of the use of MWCNT alone on an H22 tumour-bearing mouse model through subcutaneous injection. Results showed more than 80 % improvement in the production of inflammatory cytokines, namely interferon- γ (IFN- γ) and some ILs and an increase of more than 40 % on tumour rate inhibition with the complex (0.2 mg mL^{-1}). [116] Also, Mocan *et al.* used MWCNT (0.1 mg mL^{-1}) combined with Embryonic Stem Cells (ESC) to induce anticancer effects in a mouse model. An enhanced antitumour response was verified with increased CD4+ and CD8+ lymphocytes percentages, and higher expression of IL-2 and IFN γ in the ESC-MWCNT treated group. Tumour weight and volume decreased over 80%, comparing to results obtained when only ESC, MWCNT or none of them were administered. [117] Hassan *et al.* studied the possibility of modulating the immune response against tumour cells. They used MWCNT functionalized with an amine-terminated spacer, $S^{-/+}$, to deliver immunoadjuvants, namely CpG and anti-CD40 Ig (α CD40), in an Ovalbumin (OVA) antigen model. *In vitro* studies showed increased IFN γ production by CD4+ and CD8+ T cells, with $S^{-/+}$ + OVA-CpG- α CD40 treatment, comparing to the complex OVA-CpG- α CD40 without $S^{-/+}$ + MWCNT. *In vivo* studies showed a significant potentiation of antigen-specific killing with $S^{-/+}$ + OVA-CpG- α CD40 incorporated on MWCNT and, consequently, the inhibition of the OVA expressing melanoma cells. [85] Čolić *et al.* conjugated a selective endosomal Toll-like receptor (TLR) 7, 7-TOG, to oxidized MWCNT and analyzed their ability to activate monocyte-derived cells (MoDC). The nanocomplex 7-TOG+MWCNT upregulated CD86 expression and enhanced T helper polarizing capability. Authors reported a partially mature phenotype of MoDC and attributed this effect to the cellular uptake of 7-TOG+MWCNT complex, which led to intracellular accumulation of 7-TOG. A similar effect was obtained with 10-fold higher amounts of soluble 7-TOG, suggesting a role for MWCNT functionalized with TLR7 agonists in improving immunostimulatory approaches using DC-based vaccines. [118]

Deng *et al.* studied the combination of GO-PEG with NIR irradiation *in vitro* using human osteosarcoma (HOS) and macrophage cell lines, as well as *in vivo* for applications in PTT for osteosarcoma treatment through the inhibition of M2 polarization as an anti-tumour therapeutic target. Herein, authors explored PTT potential to modulate tumour-associated macrophages and demonstrated that NIR irradiation (808 nm, 0.7 W cm^{-2} for 2 min) of RAW264.2 cell line in the presence of GO-PEG resulted in decreased expression of CD206, suggesting an impairment of M2 polarization of macrophages. Afterwards, macrophage conditioned media were collected and used to evaluate the migration and invasion capacity of HOS cells. Strikingly, HOS cultured with macrophage conditioned media collected upon treatment with GO-PEG and NIR irradiation were unable to repair the scratch in a wound healing *in vitro* assay after 24 hours, as opposed to controls. Similar results were obtained using a transwell invasion assay, demonstrating that macrophages treated with GO-PEG and NIR may have an altered secretome, which inhibits tumour cell invasion and migration. *In vivo* studies demonstrated tumour weight/volume decreased by more than 50 % in the presence of GO-PEG (0.05 mg mL^{-1}) when NIR irradiated (808 nm; 0.7 W cm^{-2}) for 90 s (Figure 4, B). Immunohistological analysis revealed that CD206 expression was reduced

in tumours treated with GO-PEG and NIR irradiation, whereas higher levels of arginase-1 were detected, supporting preferential macrophage differentiation toward M1 phenotype. [19] Xu *et al.* idealized a highly effective GO-based vaccine nano-adjuvant, using the urease B model antigen. GO was single (GO-PEG and GO-PEI) and double (GO-PEG-PEI) coated with PEG and polyethyleneimine (PEI) polymers to enhance its surface properties. *In vitro* studies showed that GO-PEG-PEI induced the activation of TLR2 and TLR4 signalling pathways in DC and the delivery of Ure B with low toxicity. *In vivo* experiments showed the potential of this complex to be used as vaccine adjuvant, since GO-PEG-PEI-Ure B improved T cell proliferation comparing to free Ure B (Figure 4, C). [20] Also, Wang *et al.* engineered a GO nano-based system (GO-AlO(OH)), by adding aluminium sulfate through chemical precipitation onto GO nanosheets (2 mg mL^{-1}), in order to deliver OVA and elicit an immune response. *In vitro* studies showed a 50 % increased OVA uptake with the GO-Al complex, together with an increase of CD86, MHC-I and MHC-II expression in murine bone marrow cells, indicating immune cell activation. Furthermore, *in vivo* assays showed an increase of T lymphocyte response and more than 70 % decrease in tumour weight. [21]

A similar approach was tested by Yue *et al.*, that attached OVA to GO and assessed the effects in bone marrow DC. An increase in OVA uptake was observed after 12h and also, the expression of co-stimulatory CD86, and MHC molecules, MHC-I and MHC-II, were enhanced with the complex OVA+GO. The tests in E.G7 tumor-bearing mice showed a relevant decrease ($> 2000 \text{ mm}^2$) in tumour volume. [119] Gurunathan *et al.* conducted a study to evaluate GO and vanillin-reduced GO (V-rGO) toxicity towards THP-1, a leukemia-derived monocyte/macrophage cell line. Both GO and V-rGO at a concentration of $100 \mu\text{g mL}^{-1}$ induced a decrease in THP-1 cells viability of 40 % and 20 %, respectively. These materials induced oxidative stress in THP-1 cells, as demonstrated by a redox imbalance with increased levels of malondialdehyde and decreased levels of glutathione, glutathione peroxidase, superoxide dismutase and catalase, thereby accelerating apoptosis observed through up-regulation of apoptosis-related genes (p53, p21, Bax, caspase-9, and caspase-3). Furthermore, GO and V-rGO induced the production of several cytokines and/or chemokines, such as IL1- β , TNF- α , GM-CSF, IL-6, IL-8, and MCP-1, which was more pronounced in cells cultured with V-rGO, as compared to GO. The activation of pro-inflammatory pathways (cytokines release and ROS generation), which are potentiated with the vanillin functionalization and the oxygen groups reduction, could help to fight leukaemia. [120] However, unfortunately the authors did not explore the impact of the materials in healthy immune cells, which would be needed to further clarify GBM immunotoxicity, to highlight the eventual role of surface chemistry on the activation of pro-inflammatory responses.

Wei *et al.* used rGO functionalized with p-aminobenzoic acid to engineer a drug delivery platform for cancer treatment. PEI was used to improve water dispersibility (rGO-PEI) and a covalent functionalization with folic acid (FA) was performed in order to target cancer cells. Doxorubicin (DOX) and Elnichrome A (EA) loading and release were analysed and it was observed that the conjugation of the drug with the rGO-PEI-FA system led to cell apoptosis and forced more than 70 % of the CBRH7919 cancer cells to the G2 phase, which is the phase where those are more vulnerable to damage, mainly induced by drugs or radiation. [121]

Sui *et al.* combined GQD with cisplatin (CDDP) to study its cellular and nuclear uptake aiming at increasing the efficacy of chemotherapy and overcome drug resistance. GQD improved the interaction of CDDP with DNA (CDDP normally forms a covalent adduct with DNA, that inhibits its replication and RNA transcription). Additionally, GQD increased DNA fragmentation, leading to an increase in cell death. However, the affinity of GQD to DNA requires further investigation, since the mechanisms that cause that interaction are not completely understood. [129] The use of GQD was also studied by Tomić *et al.* to induce autophagic-dependent suppression of pro-inflammatory T cells through the activation of tolerogenic DC. Non-toxic doses of GQD ($< 200 \mu\text{g mL}^{-1}$) inhibited the production of T helper cytokines and increased the stimulation of Th2 cytokines, such as IL-10, IL-4 and IL-5. [122] Likewise, Qin *et al.* investigated the effect of GQD in the immune response and found that GQD increased ROS production, autophagy, and the inflammatory response of activated macrophages. Also, in concentrations as low as $10 \mu\text{g mL}^{-1}$, GQD increased by more than 50 % the production of cytokines, such as TNF- α and IL-8. Higher concentrations (100 and $200 \mu\text{g mL}^{-1}$) induced opposite effects, reinforcing the importance of using the right concentration to elicit immunosuppressive or immunostimulatory responses. Also, macrophage apoptosis increased by more than 60 % at the concentration $200 \mu\text{g mL}^{-1}$. [123]

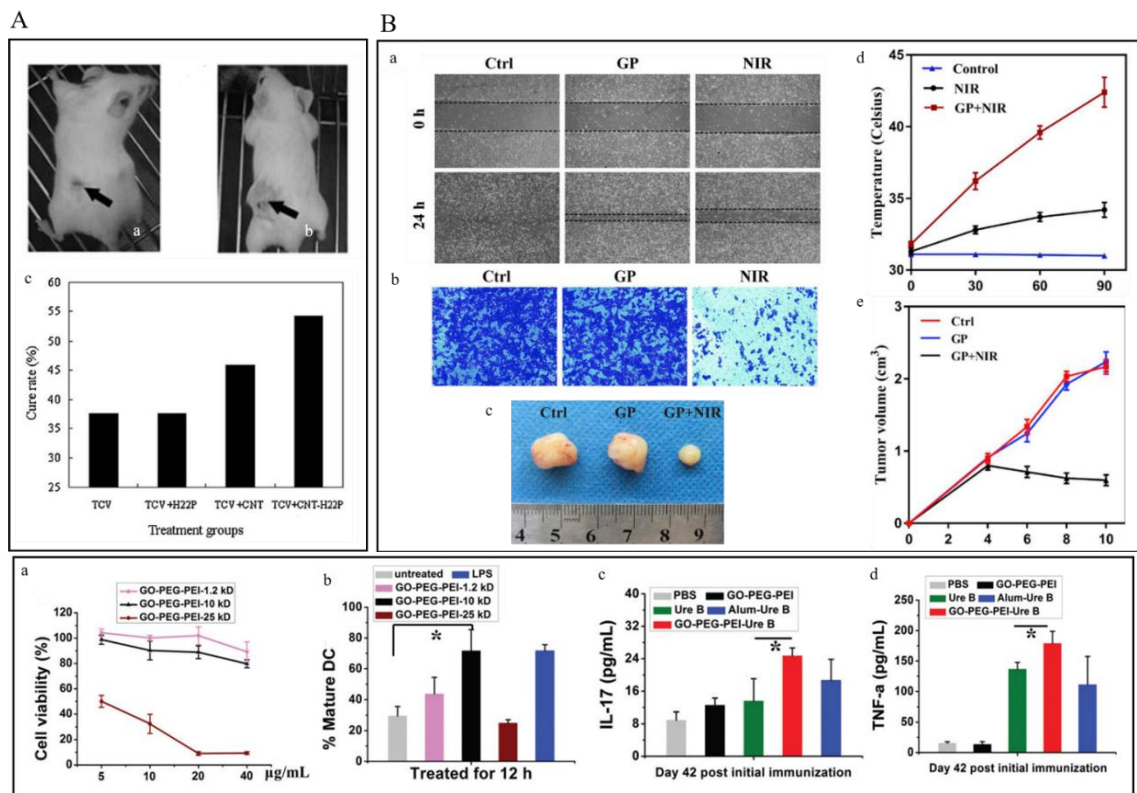


Figure 2.4. Examples of cancer immunotherapeutic modulation induced by the presence of CNM. (A) MWCNT conjugated to tumor lysate protein (H22P) were applied as tumor-cell vaccines (TCV) to treat liver cancer. Black arrow in (a) points to the place where a tumor used to grow but shrank to a minimal residue in TCV+CNT-H22P group, while the tumor in the control group without both CNT and H22P (arrow in b) expanded considerably; cure rates for the different conditions tested (c). [115] (B) Effects of GO-PEG (GP) combined with NIR on the induction of macrophage polarization on osteosarcoma growth and invasion. The inhibitory effect of GP + NIR on invasion and migration ability of HOS cells via macrophage polarization. (a,b); images of an explanted tumor 1 and 2 days after NIR photothermal therapy (PTT) using a laser (808 nm; 0.7 W cm^{-2}) for 90 s combined with GP (injected subcutaneously, 0.05 mg mL^{-1} of GO), and control tumours (c); temperature curve measured during PTT *in vivo* for the different conditions tested (d); tumour volume variation during the experiment (10 days, GP was added at day 4) (e) [19] (C) Dendritic cells (DC) viability

after being exposed to different concentrations of GO-PEG-PEI conjugates with variable PEI molecular weights (a) and their influence on DC maturation (b); Effect of intradermally injected GO-PEG-PEI (10 kDa), those modified with Urease B (Ure B), and controls, in mice sera levels of IL-17 and TNF- α . [20] Alum, Aluminum; CNT, Carbon Nanotube; GO, Graphene Oxide; PEG, Polyethylene glycol; PEI, polyethylenimine; TVC, Tumour Cell Vaccines.

Li *et al.* studied the delivery of doxorubicin conjugated with polyglycerol nanodiamonds (Nano-DOX) using dendritic cells as carriers (Nano-DOX-loaded DC) to treat glioblastoma. This complex (2 $\mu\text{g mL}^{-1}$) proved to maintain the viability of THP-1-derived DC *in vitro*, enhanced the release of IFN γ (from around 40 to 400 $\mu\text{g mL}^{-1}$) with the complex comparing to DC control and up-regulated CD69 expression in ~25 % by CD4 $^{+}$ CD8 $^{+}$ lymphocytes. The combination with carboxyfluorescein diacetate succinimidyl ester (CFSE) which provides fluorescence allowed labelling the glioblastoma cells in all tumour-bearing mice. The activation of mDC (mouse bone marrow-derived DC) and lymphocytes was observed, through the decay of CFSE fluorescence on the lymphocytes that proliferated (Mixed Lymphocyte Reaction), using flow cytometry. [124] In a similar approach, Suarez-Kelly *et al.* stimulated NK cells and monocytes with fluorescent ND (FND, 100 $\mu\text{g mL}^{-1}$), resulting in FND uptake and in an enhancement (more than 50 %) of CD69 expression comparing with the control without FND, an indicative of the activation of NK cells. In an animal model, FND were clearly visualized, allowing the targeting of carcionagenic cells since the FND were uptaken by them. [125]

Zhang *et al.* constructed a nanodiamond-based system to deliver paclitaxel (PTX-DNA@ND), with the objective of targeting breast cancer cells and improve treatment efficacy. PTX-DNA@ND conjugation with monoclonal antibodies (mAbs), allowed successful tumour cell targeting. Also, the uptake of the nanodiamond-base complex increased the fluorescent signal in MDA-MB-231 cells, comparing with free PTX and the complex without the antibodies. A significant (> 60 %) cell death was observed using low concentrations (17 nM for MCF7 cells and 43.8 nM for MDA-MB-231 cells) of PTX-DNA/mAb@ND. [130] Zhu *et al.* explored the possibility of using the C₆₀(OH)_x (average x = 24) fullerol as a new immunomodulatory approach in hepatocarcinoma. C₆₀(OH)_x enhanced the inherent immunity of tumour-bearing mice by potentiating peritoneal macrophages phagocytosis both *in vitro* and *in vivo*. Additionally, surrounding fibroblasts and lymphocytes activity was enhanced in consequence of TNF- α increase induced by C₆₀(OH)_x, which inhibited tumour growth to the neighbour tissues. [126] Similar effects have been observed by Liu *et al.*, with efficient anti-tumour activity *in vivo*. Increased pro-inflammatory cytokines production, TNF- α and INF- γ , by macrophages and lymphocytes treated with C₆₀(OH)₂₀, were verified *in vitro*, as well as an increased CD4 $^{+}$ /CD8 $^{+}$ ratio, demonstrating immune cells stimulation, which can lead to an efficient anti-tumour activity. [127]

Overall, CNM large surface area, small size, and chemical versatility has been shown to facilitate the conjugation with diverse molecules, including drugs, antibodies or molecules recognized by the target cells. Together with the enhanced permeability and retention effect that can be achieved for materials with adequate small sizes, this makes them some of the most advanced and promising platforms presently available for targeted immunotherapy. Furthermore,

CNM such as CNT and GBM present the ability to absorb radiation in the NIR region, resulting in localized temperature increase, which can lead to an increase in cancer cells membrane permeability. Incubating cancer cells with SWCNT in concentrations, generally inferior to 1 mg mL^{-1} for time periods around 24h and further NIR irradiation ($< 4 \text{ min}$, $0.7\text{-}9.5 \text{ W.cm}^{-2}$) resulted in 90-95 % cell death. [19, 107-109] MWCNT, in concentrations inferior to 1 mg mL^{-1} , revealed to increase IFN γ levels *in vitro* and to reduce hepatoma tumour volume *in vivo*. [85, 117] Due to its small size (from 50 to 2000 nm) and nanometric thickness, GO (in concentrations below 0.5 mg mL^{-1}) has been shown to facilitate cellular uptake of drugs loaded at its surface. Drug functionalization of GO with OVA and Ure B, for instance, allowed a better targeting comparing with non-functionalized GO as demonstrated by *in vitro* studies (less than 48 h of incubation). Graphene quantum dots, fullerenes and nanodiamonds, all at concentrations inferior to $200 \text{ }\mu\text{g mL}^{-1}$ and without any functionalization, have been shown to induce an immune response, mainly by inducing the production of ILs and TNF- α , since the immune system recognizes them as strange bodies. There is no data regarding tumour volume regression, for these nanomaterials, although $0.5 \text{ }\mu\text{mol kg}^{-1} \text{ day}^{-1}$ of $\text{C}_{60}(\text{OH})_{20}$, a fullerene, inhibited tumour development. In general, all carbon-derived materials referred above demonstrate to have the capacity to modulate and influence immune response directly or indirectly, through specific antibodies attached to them or drugs. Smaller nanomaterials, such as CNT and GO, have both shown elevated uptake by the tumour cells. Also, *in vivo* assays with CNM evaluated mainly PEG-functionalized graphene oxide, demonstrating a significant ($> 50 \%$) decrease in tumour weight and/or volume, while fullerenes and nanodiamonds only showed inhibition of tumour recurrence. In general, all the materials stimulate pro-inflammatory activation, through the production of IFN γ (mainly induced by MWCNT) and ILs (induced by GQD) in order to provoke a pro-inflammatory response to fight against cancer cells, being highlighted potential applications in lymphomas and carcinomas treatment. Such versatility and proven efficacy both in *in vitro* and *in vivo* tests, demonstrates CNM potential as new platforms for cancer treatment and immunotherapy.

2.4. Immunomodulation-based therapies

Immunomodulation comprises any modification in the immune response to a certain stimulus which can include an enhancement, an induction, or a suppression of any segment or of the totality of the immune response. Normally, this effect is generated by an immunomodulator, including biomolecules (e.g., proteins and amino acids), drugs (natural or synthetic) and others substances (e.g., nanomaterials), which can be either an immunostimulating agent or an immunosuppressor. [131, 132] Immunomodulatory therapies go much beyond cancer immunotherapy, which was discussed in the previous section. The use of CNM for immunomodulatory therapies targeted to other disorders, such as infectious, auto-inflammatory and autoimmune diseases, where immunoactivation or suppression are required, are discussed below.

2.4.1. Immunostimulatory therapies

Similar to anti-cancer vaccines discussed above, immunomodulatory agents can be used as vaccine adjuvants for the treatment of infectious diseases, like tuberculosis and malaria, and viral infections like foot-and-mouth disease. The attenuated pathogen, part of the pathogen, or its genetic material can be used as antigens to trigger an immune response, so that an eventual future infection by the live pathogen can be met with a strong and specific immune response. CNM structure and composition may be engineered to better stimulate the immune response. CNM are considered foreign substances to the host organism and are recognized by the immune system triggering a natural response to fight and eliminate them. CNM can induce cells to secrete ROS, consequently stimulating an inflammatory reaction. Due to their small size, CNM can also be phagocytosed, mainly by macrophages, and trigger ROS secretion, cell activation and cytokine production (TNF- α , IFN- γ , amongst others). Also, antibodies can be attached to the nanomaterials to target them towards antigen-presenting cells, like macrophages, dendritic cells, and B cells. Several studies have demonstrated that CNM, including CNT, GO, rGO, GQD, fullerenes and ND may have immunostimulatory capabilities, stimulate T-cell response as a consequence of surface functionalization and/or conjugation with drugs, acting also as delivery vehicles. [34, 133-135]

Table 2.2 summarizes the main results of the most relevant current studies addressing the effect of CNM as immunostimulatory agents, and Figure 2.5, summarizes the main immunostimulatory pathways triggered by CNM.

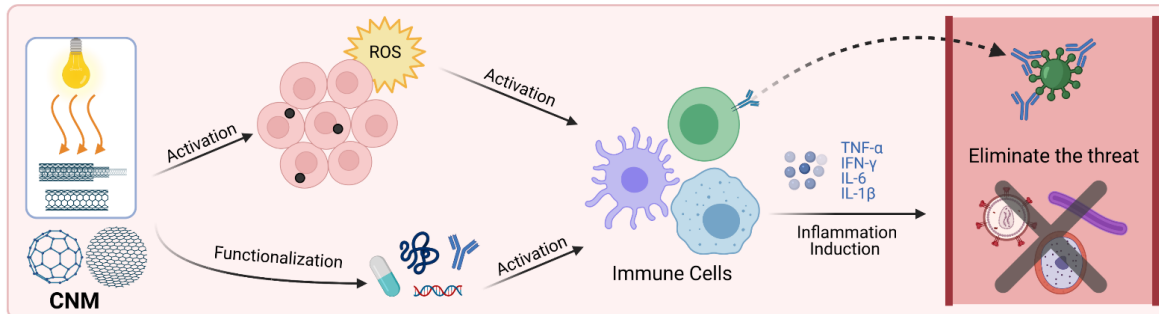


Figure 2.5. Immunostimulatory action induced by CNM, including immune cells activation and pro-inflammatory cytokine production. CNM can exert a direct effect on cells, inducing ROS generation and activating immune cells, triggering an inflammatory response, with increased pro-inflammatory cytokines (TNF- α , IFN- γ , IL-1 β , IL-6, among others) release. CNM can be further modified using specific drugs, polymers or antibodies which results in an indirect activation of antigen-presenting cells, also resulting in an inflammatory/immune response. [134, 135] Created with [BioRender.com](https://www.biorender.com). CNM, Carbon Nanomaterials; NIR, Near-Infra Red; ROS, Reactive Oxygen Species.

Table 2.2. Applications of carbon nanomaterials in immunostimulation therapies.

Nanomaterial	Dimensions	Functiona- lization	Target Disease	Molecules Delivered	<i>In vitro</i> model	<i>In vivo</i> model	Result	Ref. No.
SWCNT	d = 5-6 nm	PEG	Atheroscle- rosis	SHP1 inhibitor	Murine (RAW264.7) and human (THP-1) macrophages	Apolipo- protein-E- deficient Mice	<i>In vitro</i> : SWNT-SHP1i accumulate in murine macrophages (100% of cells) and >50 % ↑ apoptosis by macrophages, comparing to free SHP1i and SWNT-Cy5.5 controls <i>In vivo</i> : ↓ in the expression of genes related response to IL- 1 and IFN-γ	[136]
	d = 50 nm	Oxidation	TuberculosisPPD		Mice spleen monocyte / macrophage cells	BALB/c mice immuniz ed with PPD	<i>In vitro</i> : SWCNT (100 μg mL ⁻¹) induce a twofold ↑ in ROS level <i>In vivo</i> : IFNγ production (> 1600 pg mL ⁻¹) with SWCNT-PPD; IFN-γ/IL-5 ratio ↑ to 120 in the presence of SWCNT-PPD	[137]
	l = 0.145 nm	H ₂ SO ₄ and -		Ovalbumin	-	Inbred C57BL/6 mice	Uptake of ovalbumin +AF-SWCNT ↑ from 5% to 14 % in 20h of incubation; Antibody response with OVA-AF- SWCNT ↑ 20% comparing to OVA alone	[138]
MWCNT	d = 20-30 nm l=0.5-2 μm	1,3-dipolar cyclo- addition	Malaria	<i>Plasmodiu m vivax</i> apical membran e antigen- 1 peptide	-	BALB/c mice infected with <i>P. berghei</i>	delay in malarian infection and greater survival time in groups with peptide 21267 and CNT-21267 peptide (= 20 days)	[139]
	d = 9.5 nm l = 1.5 μm	Oxidation and 1,3- dipolar cycloadditi on	Immune system dysregulatio	LPS and concanav alin A	Human PBMCs and T lymphocytes	-	↑ % of CD25+ (12%) and CD69+ (80%) cells, and >50 % ↑ of TNF-α, IL- 10, IL-13, IFNγ and IL-2 production with ConA+Ox- MWCNT-NH ₃ ⁺ comparing with control groups	[140]
	d = 20-30 nm l = 0.5-2 μm; d = 9.5 nm l = 1.5 μm	Oxidation	-	-	Jurkat cells, THP1 cells and human primary monocytes	-	> 50 % ↑ of gene expression (CCL4L1, IL1B and TNFAIP6) and cytokine production (IL6, IL1B and TNF-α) with ox-MWCNT-NH ₃ ⁺ - 2 in both monocytes; ↑ IL10 with ox-MWCNT-NH ₃ ⁺ -1 in monocytes; ox-MWCNT- 2 did not present inflammatory reaction (smaller size)	[141]

GO	t = 1.3 nm	Chitosan	-	-	Murine RAW264.7 macrophages	-	>30% ↑ relative cell viability with GO-CS at 10 µg mL ⁻¹ comparing with GO; >50 % ↑ in IL-6 and TNF-α production with GO-CS	[142]
	Small (GO-S): 50-300 nm Large (GO-L): 10-40 µm	-	-	-	Human PBMCs and THP-1 monocytic cell lines	-	Time-dependent release of cathepsin B with GO-S and GO-L (> 40 %, after 24h); GO-S and GO-L ↓ in intracellular K ⁺ in HMDM at 24 h (-50% comparing to control); no IL-1β secretion in cells deficient for NLRP3, ASC, and caspase-1	[143]
	≈ 2.4 nm and ≈ 350 µm	None	-	-	RAW264.7 macrophage cell line	-	> 60 % ↑ of cells with LC3+ autophagosomes with GO (100 µg mL ⁻¹); ≈ 50 % ↑ in the production of IL-2, IL-10, IFNγ and TNF-α comparing with cells without GO treatment	[144]
Fullerenes	161 ± 19.2 nm	Aminoacid modification and pluhydroxilation	-	Tuftsins	Murine peritoneal macrophages	-	↑ phagocytosis comparing to control (20 µmol/L): tuftsins (30 %), NH ₂ -Tuftsins-C ₆₀ (35 %) and C ₆₀ -Tuftsins-COOH (> 40 %)	[145]
	41.6 nm and 44.4 nm (w/Env)	-	AIDS	Env peptide	HEK293 cells, splenocytes and (BM-DC)	BALB/c mice	<i>In vitro</i> : 20% proliferation of CD3 + T cells with Fullerenol-Env: 20-20 after 7 days <i>In vivo</i> : after immunizations, all of mice survived; ↑ of TNF- α and IFN- γ (50 pg mL ⁻¹) with Fullerenol-Env: 20-20	[146]
ND	80 ≈ 30 nm	Hyperbranched polyglycerol	-	Alhydrogel®	Human THP-1 and murine NSC-34 cell lines	C57BL/6 mice and CD1 mice injected w AluDia®	<i>In vitro</i> : Low toxicity of functionalized NDs (4.42 µg mL ⁻¹) comparing with free Aluminium oxyhydroxide; <i>In vivo</i> : 71% of granulomas disappeared (after 270 days); ↑ autophagy with AluDia®	[147]

AIDS: Acquired Immunodeficiency Syndrome; Alhydrogel®: Aluminium oxyhydroxide; AluDia®: Alhydrogel® + functionalized nanodiamonds; BAL: bronchoalveolar lavage; CS: Chitosan; d: diameter; HMDM: Human Monocyte-Derived Macrophages; l: length; LPS: Liposaccharide; OVA: Ovalbumin; PPD: Tuberculin purified protein derivative; t: thickness.

Flores *et al.* developed a macrophage therapy using PEG-functionalized SWCNT (4 nM) loaded with an inhibitor of the anti-phagocytic CD47-SIRP α signalling axis, SHP1i. *In vitro*, SWNT-SHP1i tend to accumulate in murine macrophages and caused an increase of apoptotic cells induced by macrophages (13,4 %), comparing to free SHP1i (6,31 %) and SWNT-Cy5.5 controls (5,98 %). *In vivo*, they have shown to reduce the expression of inflammatory genes related to the response to IL-1 and IFN- γ , with no decrease in cell viability ($\approx$ 90 %). [136] Zeinali *et al.* produced SWCNT conjugated with tuberculin purified protein derivative (PPD) to evaluate a new potential treatment for tuberculosis, directly associated to the activation of T-helper type I response. *In vitro*, both free SWCNT and PPD-conjugated SWCNT induced an increase in ROS levels (which is an indicator of an inflammatory response) and a decrease in cell viability, on macrophages. BALB/c mice immunized with SWCNT-PPD presented higher levels of IFN γ production than those immunized with non-conjugated PPD. Resulting in an increased IFN- γ /IL-5 ratio which represents a more pro-inflammatory environment, without cytotoxic effects being observed when comparing with only PPD control. [137] Ovalbumin was coupled to acid functionalized SWCNT in order to study antigens uptake by B cells. It was observed that B cells incubated with OVA-SWCNT conjugates internalized more ovalbumin than B cells incubated with free ovalbumin and a 20 % increase of antibody response to OVA-SWCNT was observed, in comparison to OVA alone. [138] Pantarotto *et al.* functionalized CNT with a peptide from the foot-and-mouth disease virus (FMDV) and OVA, seeking effective ways of administering protective antigens. Results of the mono-derivatized CNT conjugate showed a high stimulation of anti-peptide antibody responses in mice, setting off the possibility to use them in vaccine release for the application in the foot-and-mouth disease. [148] Using a whole-genome expression technology, Pescatori *et al.* investigated the effect of MWCNT functionalization, by oxidation and by 1,3-dipolar cycloaddition reaction to ammonium, and diameter on Jurkat and THP1 cell lines, which represented the adaptive and innate immune response, respectively. For this purpose, both cell lines were treated with four different types of functionalized MWCNT: ox-MWCNT-1 and ox-MWCNT-NH $_3^+$ - 1 (both with 20 - 30 nm diameter); ox-MWCNT-NH $_3^+$ - 2 and ox-MWCNT-2 (both with $\approx$ 9.5 nm diameter). The ox-MWCNT-NH $_3^+$ - 2 type affected the regulation of both monocytes and T cells by increasing IL-6, IL-1 β and TNF- α production. Also, ox-MWCNT-1 and ox-MWCNT-NH $_3^+$ - 1 induced IL-10 production in human primary monocytes, and an increase in CCL4L1, IL-1B and TNFAIP6 expression was found in the presence of MWCNT, suggesting an activation of monocytes and inflammation signalling pathways. It is possible to conclude that the inflammatory response can be related to the oxidation and/or 1,3-dipolar cycloaddition that MWCNT suffer. These types of functionalization may induce the generation of ROS that, as seen above, is a strong inflammatory mediator. However, the low diameter (9.5 nm) and the lack of ammonium groups, verified in the ox-MWCNT-2 type implies the absence of immunostimulatory properties comparing to the other species. [141] Crescio *et al.* evaluated the capacity of oxidized MWCNT to incite an immune response *in vitro*, under stimulated microgravity using a Random Positioning Machine. Authors tested primary monocytes and T cells and verified a significant increase of CD69 expression, as well as an enhancement of cytokine production, such as TNF- α , IL-10, IL-13, IFN γ and IL-2, demonstrating the role of oxidized MWCNT in controlling

microgravity-induced immune cells dysregulation (Figure 6.A). [140] Furthermore, Yandar *et al.* conjugated functionalized MWCNT with an AMA-1 N-terminus apical membrane antigen 1 (21267) peptide derived from *Plasmodium vivax* and internalized it in *Plasmodium berghei* infected mice, to be used in the therapy for malarial infection. Results showed late infection development on the groups immunized with peptides 21267 and MWCNT+21267, and a greater survival time with the complex compared to controls, although the parasitaemia (average number of parasites in blood) levels were increased (Figure 6. B). [139]

Mukherjee *et al.* studied the influence of GO with different dimensions (smaller, 50-300 nm and large, 10-40 μ m) over cytotoxicity and immune response using primary human monocyte-derived macrophages. GO showed a time-dependent effect over the release of cathepsin B and K⁺ efflux, being the smaller GO faster in this process. Also, a stimulation of caspase-dependent IL-1 β expression was observed in lipopolysaccharide (LPS)-primed macrophages in the presence of GO. LPS are a component of Gram negative bacteria which stimulates TLR4 pathways, resulting in the production of inflammatory factors. [143] Chen *et al.* investigated the potentiality of GO to induce immune response in macrophages through TLR pathways, which are associated to inflammatory responses. They found a concentration-dependent increase of LC3⁺ autophagosomes, which suggests an enhancement of autophagy. Furthermore, macrophages treated with GO manifested to be regulated by the TLR4 and TLR9 pathways, producing cytokines, such as IL-2, IL-10, IFN γ and TNF- α , important factors in the activation of the adaptive immune response. [144] A relevant factor that influences the effect of GO as an immunoadjuvant is surface functionalization. Yan *et al.* studied the potential of preparing GO functionalized with chitosan (CS) as an immunological stimulant and found high viability (~100 %) after culturing RAW264.7 cells for 24 h with this complex, comparing to GO without functionalization (~80 %). CS-GO stimulated (> 50 %) cytokine production, namely IL-6 and TNF- α , comparing to GO alone, , pointing out the importance of surface coating strategies toward enhancing immune cell response. [142]

Xu *et al.* conjugated the immunomodulating peptide tuftsin with the fullerene C₆₀, creating NH₂-tuftsin-C₆₀ and C₆₀-tuftsin-COOH. The first (NH₂-tuftsin-C₆₀) was conjugated in a solution phase, while in C₆₀-tuftsin-COOH, a pre-functionalized fullerene was covalently attached to tuftsin in a solid-phase. Comparing to natural tuftsin alone, the conjugates slightly increased macrophages phagocytosis and MHC-II expression, without showing toxicity and manifesting to completely resist to enzymatic hydrolysis. [145] Some years later, the same group investigated the possibility of using fullerenol as a vaccine adjuvant by conjugating it with DNA encoding the HIV-1 Env protein, which is the only surface-expressed viral protein in HIV-1. They found an increase in CD3⁺ T cell proliferation with the complex Fullerenol-Env *in vitro*. In mice, after immunization (intradermal, intramuscular, intraperitoneal, subcutaneous and intranasal inoculation, three times at two-week intervals), it enhanced cellular immunity resulting in an increase of TNF- α and IFN γ levels, with all mice surviving after 45 days of treatment. [146]

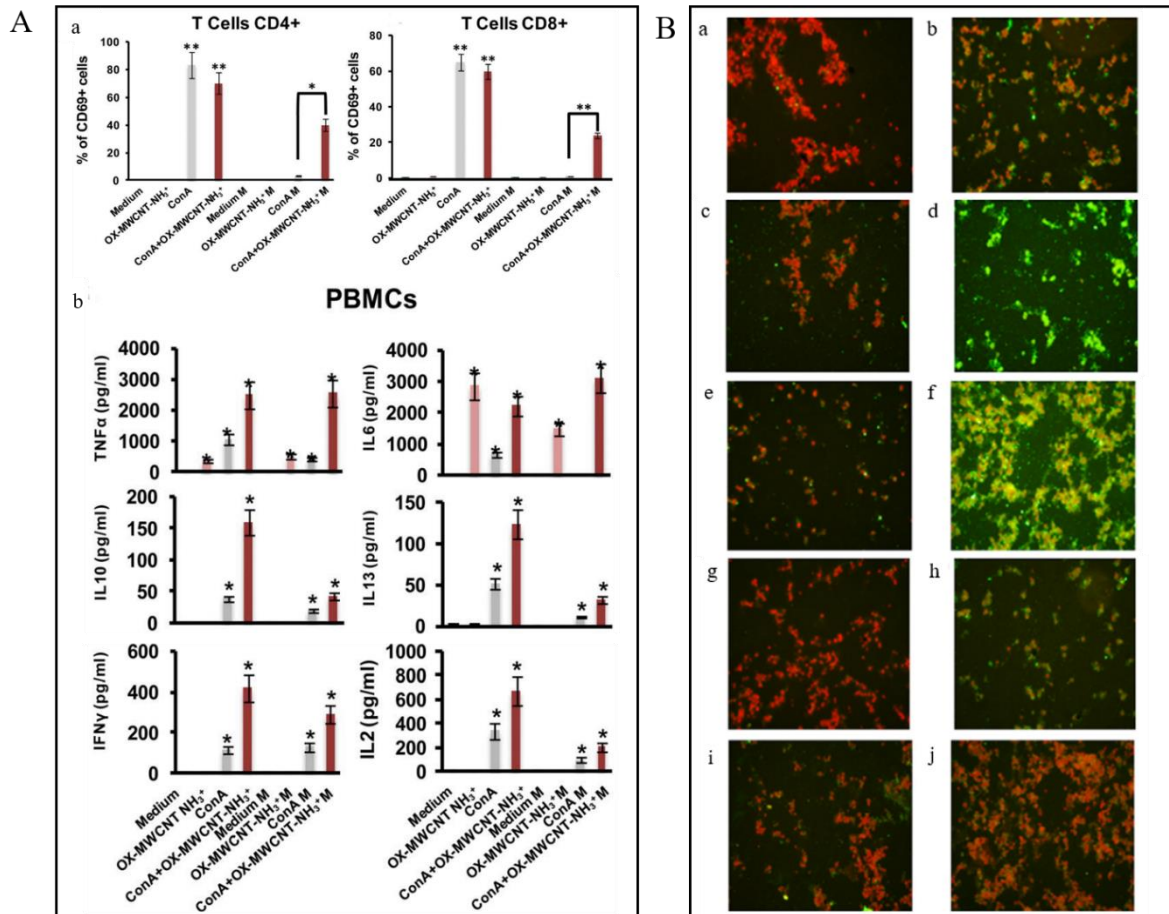


Figure 2.6. CNM induced immunostimulation. A. Evaluation of the MWCNT capability to contradict stimulated space flight immunosuppression. a) Microgravity (M) was compared with static controls (OX-MWCNT-NH₂+ alone or in the presence of cell activators, such as ConA). Activation marker assay of T cells: The expression of CD25 (left) and CD69 (right) was assessed by flow cytometry on CD3+ T cells existent on the PBMCs. ConA (10 µg mL⁻¹) was used as positive control. b) Cytokine secretion assay (TNF-α, IL-10, IFN-γ, IL-6, IL-13, and IL-2). Cytokine production was assessed by multiplex ELISA on PBMCs and on isolated human T lymphocytes. [140] B. *Plasmodium vivax* detection in the serum of the tested mice by immunofluorescence. a) and b) Mice immunized with a non-relevant peptide (33189). c) and d) Mice immunized with *Plasmodium vivax* AMA-1 peptide 21267. e) and f) Mice immunized with the CNT-21267 peptide complex. g) and h) Mice immunized with just CNT. i) and j) Placebo group: Mice immunized with a saline solution. [139] ConA, Concanavalin A; LPS, Lipopolysaccharide MWCNT, Multi-walled Carbon Nanotube, PBMCs Peripheral Blood Mononuclear Cells.

A different approach was taken by Eidi *et al.* using fluorescent nanodiamonds further functionalized by hyperbranched polyglycerol and coupled to Alhydrogel® (AluDia®) to study the possibility of building aluminium-based vaccines without causing neurologic autoimmune/inflammatory reactions. The injection of AluDia was followed *in vivo* by the presence of fluorescent nanodiamonds and was detectable in the injected muscle and other organs, like draining lymph nodes, spleen, and brain, overcoming the long-term neurotoxicity verified in other organic fluorophores. Results showed a promotion of autophagy, with 71% of granulomas disappearing and a decrease in the size of existing granulomas. [147]

CNM impact on immunostimulation has been reported for a diversity of applications, from vaccination against AIDS, treatment of infectious diseases like, tuberculosis and malaria, to immune system dysregulations. CNM exhibit a small size which facilitates their uptake by cells,

thereby stimulating an immune response. For instance, CNT present a high surface area which allows the functionalization with oxygen-containing functional groups, providing stability and allowing their cellular uptake. [112-114, 140, 141] CNT were also observed to induce ROS generation, which can lead to a pro-inflammatory activation of immune cells, contributing to the production of several pro-inflammatory cytokines. These immunostimulatory actions can be very advantageous in the treatment of infectious diseases, like tuberculosis. Size and concentration-dependent GO actions are noticed in *in vitro* studies described above, to fight against infectious agents. GO have been shown to have a time-dependent effect on the cathepsin B release, as well as, a size-dependent effect, since it was observed a faster process for sizes ~ 350 nm comparing to larger GO particles (~ 2.4 μm), because nanometric sizes are ideal for cellular uptake. This time- and size-dependent reaction has also shown to trigger inflammasome activation (NLRP3), which is directly related to caspase 1 release and, consequently, the release of pro-inflammatory cytokines. GO presented no toxicity for concentrations equal or inferior to 100 $\mu\text{g mL}^{-1}$. Functionalized with chitosan, GO revealed to stimulate TLRs pathways and consequently, the inflammatory response, although this is not observed in all the studies analysed. [142-144] Fullerenes and nanodiamonds also showed positive results, being highlighted the pro-inflammatory response of fullerenol at concentrations between 20 and 50 $\mu\text{g mL}^{-1}$, namely by increasing macrophages phagocytosis and CD3 T cells proliferation. [145] Nanodiamonds have shown to increase autophagy replicating the behaviour of common aluminium-based antigen vaccines, overcoming their toxicity limitation. In general, the positive results (pro-inflammatory activation) of CNM in this section, are seen for nanoparticles with sizes inferior to 300 nm, comparing with materials with larger sizes. Also, functionalized CNM have been shown to induce more effective immunostimulation, since suitable surface modifications facilitate uptake and increases stability. Since biological interactions change according to CNM physicochemical properties, such as size and surface composition, it is paramount to take these factors into account in order to adjust their properties to obtain desired immunostimulatory responses to the target diseases.

2.4.2. Immunosuppressive therapies

The inhibition of the immune response or immunosuppression can be beneficial in the treatment of inflammatory and autoimmune diseases and allergies, since these pathologies entail an exacerbated immune response, that often causes damage to the affected organs or tissues. Nonetheless, current immunosuppressive therapies, particularly those administered systemically, can lead to greater susceptibility to infections by pathogens, or tumour development. CNM have been reported to have anti-inflammatory properties since they can decrease the levels of oxygen reactive species through the downregulation of the signalling pathways involved. [149, 150] Also, this can occur due to specific functionalization with immunosuppressors (drugs or other molecules). [148, 151] For instance, MWCNT and fullerenes present a direct influence on immunomodulation. [152] Fullerenes (C_{60}) have the capacity to scavenge free oxygen radicals in their cage structure, reducing ROS generation. This effect can lower the release of inflammatory and/or allergic mediators from mast cells and basophils from peripheral blood. [153] Also, CNT

have been reported to induce specific suppression of the immune system, namely regarding antibody release and production, and NK cells activity. [152-154] Figure 7, points out the main pathways of immunosuppression induced by CNM for the above referred target diseases. Table 2.3 summarizes the state-of-the-art literature regarding the outcomes of using CNM for immunosuppression purposes.

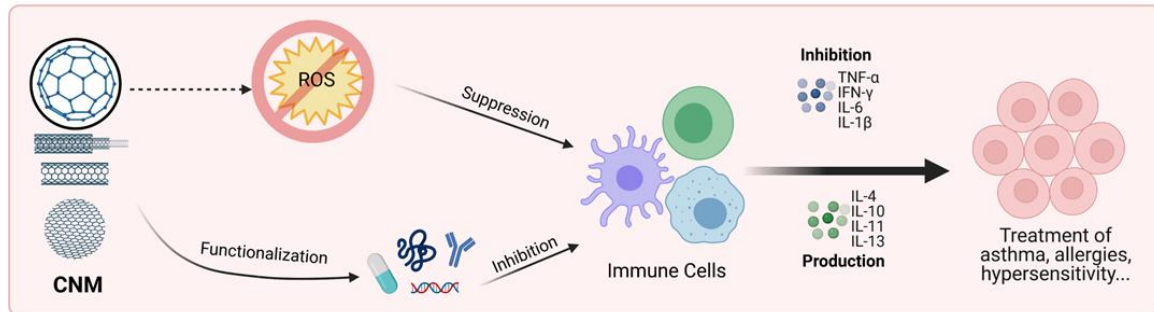


Figure 2.7. Immunosuppressive response induced by CNM through immune cells and pro-inflammatory cytokine production inhibition, and anti-inflammatory cytokines release. Fullerenes can inhibit the formation of ROS suppressing the immune cells activation (inhibition of pro-inflammatory cytokines release). [153] Alternatively, other CNM, when functionalized with specific drug or polymers, can also suppress immune cells activation and produce anti-inflammatory cytokines release. [148, 151] Created with [BioRender.com](https://www.biorender.com). CNM, Carbon Nanomaterials; ROS, Reactive Oxygen Species. Cytokines: IL-4, IL-10, IL-11, IL-13, IFN γ and TNF- α .

Table 2.3. Application of carbon nanomaterials in immunosuppression therapies.

Nanomaterial	Dimensions	Functionalization	Target Disease	Molecules Delivered	<i>In vitro</i> model	<i>In vivo</i> model	Results	Ref. No.
SWCNT	15-60 nm	Pyrrolidine ring	Foot-and-mouth disease	FMDV and OVA	Baby hamster kidney BHK 21 cells	BALB/c mice immunized w/ FMDV	<i>In vitro</i> : t=4 min, $\uparrow$ of bis-conjugate 3 reaction with the mAb ($> 100 \text{ ng/mm}^2$ of analyte) <i>In vivo</i> : $\uparrow$ anti-peptide antibody responses with peptide-CNT (bis-conjugate 3) + OVA; $\uparrow$ virus-neutralizing antibody responses (FMDV) with mono-conjugate 2 + OVA	[148]
MWCNT	d = 370 nm	-	Asthma	Toll-like receptor	PBMCs from healthy donors and mite-allergic subjects	-	$> 60 \%$ $\downarrow$ of IL-5 secretion induced by HDMs in PBMCs from mite-allergic subjects in the presence of MWCNT; MWCNT $\downarrow$ of allergen-specific T cell-mediated immune response ($> 50 \%$ $\downarrow$ the IFN- γ secretion with the $\uparrow$ of MWCNT concentration)	[149]
	d = 10-20 nm l = 5-15 μm	-	Pulmonary pathology	Concanavalin A	-	C57BL/6 mice (inhaled MWCNT)	$> 50 \%$ suppression of T-cell-dependent antigen response (14 days exposure to MWCNT); $\approx 20 \%$ $\downarrow$ NK function with 1 mg/ m^3 MWCNT inhalation	[155]
GO	14 μm	Coated w/ Titanium (Ti)	Bone scaffolds and implants	-	Bone marrow stromal cells and RAW 264.7 cell line	-	$>20\%$ $\downarrow$ expression of inflammation-related genes (TNF- α , IL-6 and IL1 β) under inflammatory conditions with Ti-GO comparing with Ti; $\approx 20\%$ $\uparrow$ osteogenic related genes in hMSCs (ALP, COL1, RUNX2) under inflammatory conditions	[150]
rGO	l = 1090 mm^2	-	Central nervous system damage	-	-	Wistar rats with a surgical hemisection on C6	I and I+SC expression of vimentin and PDGFR β ($\uparrow 20000 \mu\text{m}^2$ positive stained area comparing to control) after 30 days; $\uparrow$ of Iba1 ($>3000 \mu\text{m}^2$) and	[156]

							CD86 (>4000 μm ²) in the lesion site of I group	
GQDs	40 nm	-	Hepatitis	-	Jurkat T cells, J774 and macrophages and HepG2 hepatocytes	Male WT and ST2 ^{-/-} BALB/c mice	<i>In vitro</i> : GQDs suppress Con A hepatotoxicity (mRNA level ↓ >20 %; GQD (50 mg/kg) ↓ ≈50 % the liver numbers of CD3+ (both CD4+ and CD8+) T cells <i>In vivo</i> : ↓ in IFN- γ levels, CD4+ and CD8+ T cells (> 50 %)	[157]
Fullerenes	-	TGA	Allergic Asthma	OVA, PPOH and EET	Human lung MCs; Naive B cells from BALB/c mice spleens	C57BL/6 and BALB/c mice with induced asthma	<i>In vitro</i> : ↓ inflammatory cells; upregulation of CYP1B gene expression in MCs with TGA <i>In vivo</i> : ↓ > 70 % in asthma-promoting cytokines (IL-4 and IL-5) in TGA-treated mice; > 80 % ↑ in BALF eosinophil numbers with TGA-PPOH comparing to control	[151]
	-	Polyhydroxyl and N-ethyl-polyaminoacid	Asthma, inflammatory arthritis, heart disease and multiple sclerosis	Ag	Human skin/lung MCs and PBB	C57BL/6 mice	<i>In vitro</i> : ≈50 % ↓ ROS production in MC and PBB (with fullerenes) <i>In vivo</i> : ↓ in the MC/PBB-derived histamine response in mice injected with C ₆₀ 24 h before systemic Ag activation	[158]
	-	-	Arthritis	-	Human synovial fibroblasts, lymphocytes and macrophages	Adjuvant-induced arthritic Sprague Dawley rats	<i>In vitro</i> : treatment with C ₆₀ (10.0 μM) ↓ (≈50 %) TNF-α-induced excess production of TNF-α and IL-1β by the cells <i>In vivo</i> : C ₆₀ -treated group shows ↓ 50% of arthritis scores and - 30% of pathological tissue scores at 8 th week comparing to control	[159]
	-	Hydroxylation	-	-	UV-damaged HaCaT cells	-	60 % and 69 % ↑cell viability in cells pre-treated with HH-F or SHH-F, respectively; SHH-F ↓ the ROS levels in 52 % comparing to non-treated cells	[160]
	d = 165 nm	-	DTH	-	Splenocytes from normal and DTH mice	C57BL/6 mice with DTH	<i>In vitro</i> : > 50 % ↓ in IL-17 expression by nano-C ₆₀ in mice with DTH <i>In vivo</i> : ≈ 50 % ↑ in TNF- α level in DTH mice with nano-C ₆₀ ; not relevant results with other cytokines	[161]
ND	5.1 ≈ 2.2 nm	Octadecylamine	Rheumatoid arthritis	Dexamethasone	-	DBA/1J mice with arthritis	≈ 50 % ↓ in arthritic score progression with low ND-ODA and ND-ODA-Dex; 10 % ↑ in bone volume with Dex-, low ND-ODA- and ND-ODA-Dex after 14 days; ↑ in macrophage infiltration and in TNF- α expression	[162]

ConA: Concanavalin A; d: diameter; DTH: Delayed-type hypersensitivity; EET: Cis-epoxyeicosatrienoic acid; FMDV: Peptide from the foot-and-mouth disease virus; HH-F: C₆₀ (OH)₃₂₋₃₄.7H₂O; hMSCs: Human bone marrow stromal cells; I: Injury; I + SC: injury with implantation of rGO scaffold; MCs: Mast Cells; l: length; PBB: Peripheral Blood Basophil; ODA: Octadecylamine; PPOH: 6-(2-Propargyloxyphenyl) hexanoic acid; SHH-F: C₆₀ (OH)₄₄.8H₂O; TGA: C₇₀-tetraglycolate.

The immunomodulatory action of MWCNT was studied by Laverny *et al.* using PBMCs from healthy and allergic donors. Results showed that, upon stimulation with TLR4 and TLR2/TLR3 agonists and culture in the presence of MWCNT, PBMCs from healthy donors secreted increased

levels of pro-inflammatory cytokines, such as TNF- α , IL-6 and IL-12/23p40. A T cell mitogen (phytohemagglutinin A) stimulation led to the secretion of IL-2 and IFN- γ , while in allergic patients, allergen-induced IL-5 (pro-inflammatory cytokine) secretion was inhibited. (Figure 8. A) Also, the authors noticed that MWCNT can lead to dendritic cell differentiation. [149] Mitchell *et al.* tested the effect of MWCNT inhalation (from 0.3 to 5 mg/m³) in systemic/pulmonary immune response. A significant repression of T-cell activity and, consequently, antigen-dependent response in the presence of MWCNT was observed after 14 days of exposure. Also, inhalation of 1 mg m⁻³ of MWCNT led to a decrease in NK cell activity, supporting that this nanomaterial can be used as an immunosuppressive vector. (Figure 8. B) [155]

A very recent investigation from Su *et al.* revealed the potential of GO coated titanium (Ti-GO) to present positive immunomodulatory effects in osteogenesis. In fact, Ti-GO (0.25 mg mL⁻¹, 24h of incubation) induced macrophages activation, pro-inflammatory cytokines secretion and osteogenic genes expression under physiological conditions. However, in previously induced acute inflammatory environment, the same complex (Ti-GO) significantly suppressed pro-inflammatory cytokine production (TNF- α , IL-6, IL-1 β), suggesting a role for the surrounding microenvironment in modulating CNM-immune cells interaction and response. [150]

For the first time in 2016, López-Dolado *et al.* implanted 3D scaffolds comprising partially reduced GO in a surgery-induced spinal cord injury rat model (lesion model: hemisection at C6) and studied tissue feedback. The presence of rGO-modified scaffolds led to a significant stabilization of the injury through the reduction of early inflammatory responses. Also, new blood vessel formation and regeneration of neuronal axons at the periphery of the rGO scaffolds were noticed. On the other hand, in the control scaffolds without rGO, the quantity of blood vessels was decreased and axons were not found. Interestingly, large numbers of macrophages were found in the environment surrounding rGO scaffolds, pointing out that rGO-based materials can induce a pro-regenerative environment in the spinal cord injury. [156]

Norton *et al.* investigated the potential of a fullerene derivative, C70-tetraglycolate (C70-TGA), as a mast cell stabilizer. An upregulation of CYP1B gene expression was found *in vitro*. This gene is associated with the risk of *asthma*, in mast cells. *In vivo* results showed a decrease in inflammatory cell numbers and in eosinophil infiltration in the airways with C70-TGA tested on an ovalbumin (OVA) challenge model of asthma. The disease was induced by intraperitoneal sensitization with OVA in a first phase and through three intranasal OVA challenges in a second phase, finalizing with the collection of bronchoalveolar lavage fluid and lung tissues. C70-TGA was added three times through an intranasal way, starting on the day before disease induction. Mice treated with C70-TGA presented a decrease in asthma-related cytokines, like IL-4 and IL-5, reducing inflammation, when compared with non-sensitized control animals. [151] Ryan *et al.* reviewed the possibility of using fullerenes to inhibit allergic response, namely by suppressing the Antigen-induced type I hypersensitivity. *In vitro* tests with human mast cells and peripheral blood basophils showed a significant decrease in ROS production. Furthermore, *in vivo* assays showed a reduction in histamine response in mice treated with C₆₀, in addition to the stabilization of its

body temperature, an important indicator of possible asthma reduction in mice. [158] Yudoh *et al.* examined the possibility of using a water-soluble fullerene (obtained by mechanical milling in liquid media with no addition of polar organic solvents), C₆₀, as an inhibitor of arthritis *in vitro* and in an arthritis rat model. Fibroblasts, lymphocytes and macrophages were incubated with TNF- α and treated with C₆₀. A decrease in production of inflammatory cytokines TNF- α and IL-1 β , in response to TNF- α stimulation was reported. *In vivo*, groups treated with C₆₀ showed a reduction in the arthritis score, namely on synovitis and joints destruction, comparing to control group, without C₆₀. [159] Saitoh *et al.* investigated the radical scavenging properties and cytotoxic effects of three types of fullerenes derivatives (LH-F, HH-F and SHH-F), using a human keratinocyte cell line (HaCaT cells) previously damaged by UV radiation. They found a significant increase of cell viability in the presence of HH-F and SHH-F. The last one significantly reduced ROS levels, comparing to untreated damaged cells (by irradiation). [160] This would decrease the possibility of triggering an inflammatory response in the irradiation-induced injuries environment of human skin. In the same year, Yamashita *et al.* showed the immunomodulatory effect of fullerene, C₆₀, in a mouse model of delayed-type hypersensitivity (DTH) induced through methylbovine serum albumin (mBSA). Firstly, *in vitro* studies with murine splenocytes revealed a decrease in IL-17, a pro-inflammatory cytokine related to the activation of the innate immune response, leading to a more pronounced immunosuppressive environment, which is favourable for hypersensitivity-related diseases. Importantly, *in vivo* studies demonstrated a significant decrease of IFN γ and IL-6 levels and increased Treg/CD4Tcell ratio in DTH mice treated with C₆₀, comparing with untreated mice. Thus, despite the increased TNF- α in C₆₀ treated animals, the cytokine regulation and attenuation of disease led the authors to conclude that C₆₀ was immunomodulatory. [161]

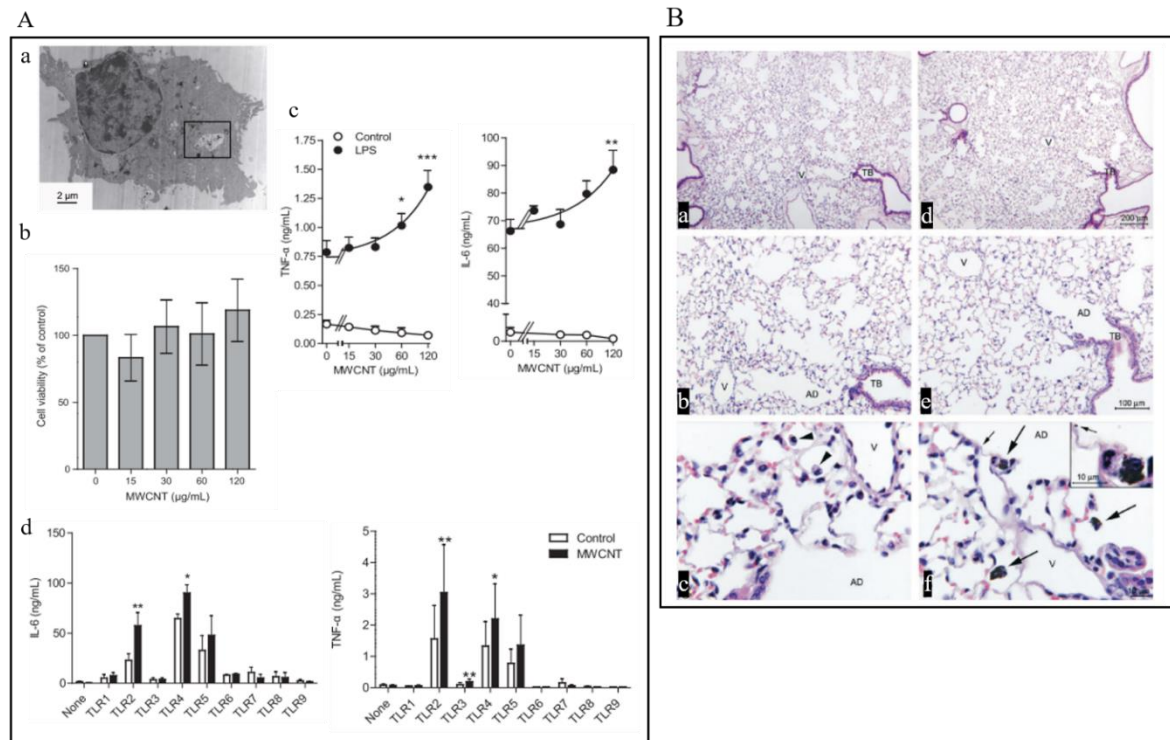


Figure 2.8. CNM induced immunosuppression. A. Uptake of MWCNTs by monocytes, seen in transmission electron microscopy images (a) Effect of increasing concentrations of MWCNTs on PBMC viability (assessed by trypan blue exclusion assay after 48 h of culture) (b) Secretion of pro-inflammatory cytokines, TNF- α and IL-6, by PBMCs from healthy donors after a 48 h stimulation with or without LPS $1 \mu\text{g mL}^{-1}$ in the presence or absence of the referred concentrations of MWCNTs (c) secretion of pro-inflammatory cytokines TNF- α and IL-6 by Toll-like receptor agonist-stimulated PBMCs from healthy donors after a 48 h stimulation in the presence or absence of MWCNTs (d) [149] B. Representative hematoxylin and eosin-stained lung sections at equivalent magnifications from a control animal (a to c) and after inhalation of 5 mg/m^3 MWCNTs for 14 consecutive days (d to f). [155] AD, alveolar duct, MWCNT, Multi-walled Carbon Nanotubes, PBMC, Peripheral Blood Mononuclear Cells, TB, terminal bronchial; TLR, Toll-like receptor; V, vessel.

Volarevic *et al.* investigated the effect of large GQD (40 nm) in a model of concanavalin A-related liver damage. GQD injection (50 mg kg^{-1} of body weight, given 30 min before before Con A injection) elicited a hepatoprotective effect against Con A hepatotoxicity, comparing to control without GQD. The authors relate this with decreased immune cell infiltration in the liver, particularly T cells and decreased serum IFN γ levels. Also, mouse splenocytes stimulated *in vitro* with $5 \mu\text{g mL}^{-1}$ Con A, incubated for 24 h with GQD showed reduced activation cytokines extracellular signal-regulated kinase and IFN γ production. [157]

A new platform for the treatment of rheumatoid arthritis was presented by Pentecost *et al.* taking advantage of the immunomodulatory capability of nanodiamonds. Mice were injected with collagen type II to induce arthritis and treated with small quantities ($15 \mu\text{g mL}^{-1}$) of octadecylamine-functionalized nanodiamond (ND-ODA) and dexamethasone-adsorbed ND-ODA (ND-ODA-Dex). Treated mice presented a relevant reduction of the arthritic score in both ND-ODA and ND-ODA-Dex (similar results) comparing to non-treated mice, as well as a small increase in bone volume after 14 days. Additionally, low doses ($15 \mu\text{g mL}^{-1}$) of ND-ODA and ND-ODA-Dex led to a reduction in macrophage infiltration and in TNF- α expression, which suggests that nanodiamonds

can be used as an advantageous immunomodulatory platform without the need of dexamethasone, an anti-inflammatory agent. [162]

In recent years, interest in new strategies using carbon-derived nanomaterials to harness the power of the immune response, has been mounting. Different materials and modifications continue to be explored for achieving immunosuppression. Fullerene derivatives are the most used type of CNM in this area, in concentrations above 500 μM and performing incubation for periods of 24 h or below, since they can reduce the levels of reactive oxygen species produced by macrophages, due to their ability to scavenge free radicals in their structure. Functionalization of fullerenes by the addition of hydrophilic components to a hydrophobic nanomaterial, for instance, with TGA or by hydroxylation, provides more stability and allows their uptake by cells. Also, MWCNT (around 100 $\mu\text{g mL}^{-1}$, after 48h of incubation) can indirectly inhibit the immune response since they can activate specific pathways that lead to the suppression of proinflammatory cytokines production (e.g., IFN γ , TNF- α and IL-5). This leads to the consequent modulation of the immune response, and results in improvements in key parameters regarding immune disorders, such as asthma and arthritis. Other CNM (GO, rGO, GQD and Nanodiamonds), also present immunosuppression capabilities, since they are able to reduce various inflammatory mediators, such as TNF- α , IL-6 and IL-1 β . However, comparing with fullerenes and MWCNT, their immunosuppressive capabilities require further investigation, since their anti-inflammatory behaviour is not very common and requires very specific conditions. In sum, CNM can act as immunomodulatory biomaterials themselves, with the possibility of adjusting their effect by tuning their physicochemical properties or combining them with bioactive substances, to induce a specific desired effect, being versatile tools that bring new perspectives to the field of immunotherapy.

2.5. Overview on Translational Challenges and Clinical Concerns

Immunotherapy is an essential and unique approach to the treatment of cancer and infectious diseases, where immune activation is required, but also in inflammatory and allergic disorders, where immune suppression is needed. It presents high specificity, long-term efficacy, reduces the need for high quantities of drugs and provides less invasive treatments, comparing to traditional therapies. The use of carbon nanomaterials, alone or as drug carriers, to modulate the immune response has great potential for clinical application, as discussed in the previous sections. However, important questions remain about the long-term effects of the presence of the carbon nanomaterials on the human body, such as regarding their biodegradation and elimination routes or potential long-term toxicity. [163] This is particularly relevant for patients with chronic diseases and requiring repeated treatments. The following bullet points summarize the current challenges pressing the need for novel developments, so that the results obtained in the present studies regarding CNM for immunotherapy can be translated to clinical application, something that is not seen yet.

- Translation of the results obtained in animal models to human applications;

- Understand the environment of an organ-specific tumour/disease for an effective therapy;
- Overcome CNM toxicity and guarantee their biodegradation;
- Maximization of the synergistic effects and minimization of the side effects CNM-mediated immunotherapy.

The use of adequate pre-clinical models is crucial to understand the mechanisms of action, the targeted delivery of drugs and the safety of the treatments. Though the commonly used rodent models do not always translate the real context of the human immune response, they are only an approximation. [164, 165] Considerable differences exist between the immune system of laboratory animals and the human species, including in immune cell differentiation and function, its complexity and recognition and other characteristics that need to be considered in order to choose the most adequate animal model. Choosing the right model is a crucial factor in a preclinical evaluation of new therapies to understand the biological mechanisms and reduce risks before clinical trials. [166, 167] Particularly, interactions between CNM and immune cells depend on several factors, like structure, dimensions, functionalization and others. The same nanomaterial can activate an immune response in one application and a simple modification on its structure can lead to immune suppression. Although parameters like toxicity can be evaluated *in vitro*, with good *in vitro-in vivo* correlation, sometimes the functional impact *in vitro*, does not translate to *in vivo*, since factors like the microenvironment, which influence immune activation are more complex, when comparing to the *in vitro* controlled settings. [168, 169] The communication between various types of immune cells and other cells of the body is necessary and crucial, being difficult to replicate those interactions *in vitro*. [166] A tumour, for instance, emerges due to somatic mutations in the cell genome, that lead to malignant cell transformation and uncontrolled cell proliferation. Importantly, interactions between these malignant cells and the surrounding milieu, including immune cells, can stimulate tumour growth and development. [170, 171] The tumour microenvironment is a critical factor that influences tumour progression, invasion, and metastatic capacity, as well as the immune response to the tumour. Therefore, the challenge is to understand the specific-tissue immunity and the constraints to an appropriate therapy. [172]

A very important concern that must be taken into account is the toxic effect of nanomaterials on humans that is specific to the material, its size and structure. [173] In many cases, *in vivo* toxicity is the main challenge for material application on the biomedical field and a meticulous research in this topic is needed to overcome this problem. For instance, the length of CNT can be related to a strong toxicity since longer ones can provoke an intense inflammatory response, which if uncontrolled will be deleterious. The inhalation of MWCNT can trigger inflammation in the lungs. A recent report indicates that acute lung inflammation is mediated by neutrophils and later involves natural pro-resolving mediators and the macrophage population transition from M1 to M2 phenotypes, in a long resolution phase. [174] Additionally, a rigid type of MWCNT (MWCNT-7) can provoke carcinogenic effects. [175, 176] Thorough investigations are needed to understand the

limitations in terms of structure, size, and concentrations, as it is possible to engineer CNM in order to enhance their biocompatibility and degradation in the human body. [177-179]

There are very few studies about the long-term toxicity related to the accumulation of carbon-derived nanomaterials in the organism. Most of the investigations reveal toxicity related to CNT size, as well as dose-dependent toxicity in the case of both G and CNT. The dose-dependent toxicity, in the majority of studies, depends on the material used, the cell or animal models and on the final application. [180-183] In recent years, several materials have been designed for biomedical applications, targeting specific tissues/cells and thus far the long-term risk associated to the accumulation of these compounds in the body is not fully determined. Nevertheless, several studies demonstrated that the carbon-nanomaterials referred above are easily biodegraded by various chemical and enzymatic reactions. For instance, pre-treated CNT with sodium hypochlorite, NaClO, and hydrogen peroxide, H₂O₂, are degraded by oxidation, resulting in large carbon particles agglomerates. [45, 184] GO can be degraded by neutrophils through degranulation leading to non-toxic products. Also, photo-Fenton reaction (oxidation) can reduce GO, forming mainly GQD, which are sooner or later decomposed in CO₂ and water. [185, 186] GO, GQD and fullerenes have been shown to be degraded by a catalysis mediated by human myeloperoxidase, which is produced by neutrophils. [44, 187]

Comparing to traditional therapies, like chemotherapy for cancer treatment, immunotherapy has shown several advantages which can be more effective with the conjugation of both approaches. [188, 189] The multidrug resistance is one of the most important barriers in the treatment of several tumours. The use of carbon nanomaterials in cancer immunotherapy can reduce the need for drugs in the treatment, since it modulates the immune system to fight against the threat, decreasing associated side effects, the symptoms, and the evolution of the disease. Also, the CNM's ability to deliver drugs or small therapeutic molecules to the tumour cells, can increase the immunotherapeutic potential and reduce the side effects. [190] In hypersensitivity cases, CNM's based immunotherapy induces long-term allergen-specific tolerance, something that does not happen with pharmacotherapy which consists in short-time symptom-relieving therapy. The use of immunotherapy reduces the need of constant administration of pharmacological adjuvants. [191, 192] With a one-stand use of immunotherapy, the positive results are immediate and last longer, making the treatment cheaper and more effective, since the commonly used pharmacotherapy, normally stands a life-long and it only avoids the progression of the disease. Immunotherapy can also prevent disease progression and even provide the complete eradication of the problem, something that is not possible with the use of drugs only. [193, 194]

CNM have high potential to target the immune response and modulate it towards immunity or tolerance, for different therapeutic outcomes. Although side effects are always present, scientists continue to study new mechanisms to minimize them, maximizing the synergistic effects between drugs and their carriers, and reduce concerns related to toxicity, drug resistance and adverse consequences. [195]

2.6. Conclusions of the state-of-the-art

CNM are known to incite a response by the immune system. Depending on their properties and conjugation with different drugs, they are able to trigger immunosuppression and/or immunostimulation, which can be very positive to the development of new nanoscale systems for immunotherapy.

In this review, it is discussed the many CNM properties influencing the immune response, such as particle size, surface coating, functionalization, and others. Due to their small sizes, CNT and G derivatives can be easily internalized by cells making them great vehicles for delivery of therapeutics, including drugs or other biomolecules. In general, CNT have good absorption in the near-infrared region, which makes them important tools in targeted cancer photothermal therapy and immunotherapy. Surface functionalization of GO and GQD allows them to circulate in the body and can also enable specific targeting using molecules/antigens, in order to activate a selective immune response. The induction of reactive oxygen species production as a consequence of an activation of the immune cells is very important to stimulate an inflammatory reaction. On the other side, fullerene's structure allows the reduction of those oxidative species, inhibiting the release of inflammatory factors, which can be very useful in inflammatory or allergic diseases. These CNM, as presented, are explored as tools for cancer and autoimmune diseases treatment, namely, as drug delivery systems, and vaccine adjuvants or carriers, although there is still have side effects associated, like toxicity. Several studies need to be made to evaluate all the conditions that make the same nanomaterial, in specific conditions, an immunosuppressant and, in other circumstances, an immunostimulatory. Also, information related to long-term toxicity is still missing since the use of carbon nanomaterials in the field of immunotherapy is quite novel.

Combinations of CNM and drugs/antibodies are still in earlier stages (*in vitro* assays of toxicity, evaluation of immune cells response *in vitro* and *in vivo*). The results so far are very promising and immunotherapy using CNM is already seen as a solution, since the nanomaterials can play a role in directing immune cells, but further investigation is essential to achieve their full potential and revolutionize the future of medicine.

Chapter 3 : Materials and Methods

3.1. Production and characterization of graphene-based materials

3.1.1 GBM production

Nanographene Oxide (GOn) was produced by chemical oxidation of graphite using the modified Hummers method [63]. In a first phase, the oxidation of graphite powder (size $\leq 20\ \mu\text{m}$, Sigma Aldrich, St. Louis, MO, USA) were performed in a Lenz reactor with permanent stirring (around 400 rpm) with the addition of 160 mL of sulfuric acid, H_2SO_4 (VWR, Darmstadt, Germany, 40 mL of phosphoric acid, H_3PO_4 (Chem-Lab, Zedelgem, Belgium) to 4 g of graphite. 24g of potassium permanganate, KMnO_4 (JMGS, Odivelas, Portugal), 600 mL of water and 26.5 g of hydrogen peroxide, H_2O_2 (VWR, Darmstadt, Germany) were added to finish the reaction, resulting in dispersion of graphene oxide and some acid remaining. All the reaction temperature (below $40\ ^\circ\text{C}$) was controlled using Cooling Bath (Julabo F12). After overnight resting, in a second phase, the necessary washes with water (to achieve pH 7) were performed in order to remove all the acid that resulted from the reaction. In the last phase, under continuous propulsion performed by a Peristaltic Pump, Matson, was made an 8h ultrasonication to reduce the particle size of GOn, using an Industrial Ultrasonic Probe, Hielscher, resulting in Nanographene Oxide dispersion. GOn was photo-reduced to p-rGOn. Figure 3.1. describes the production of both GOn and p-rGOn by the methods explained above.

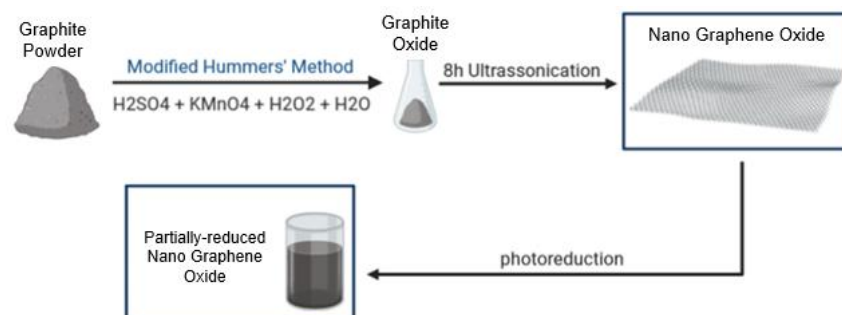


Figure 3.1. Nanographene Oxide (GOn) and partially-reduced nanographene Oxide (p-rGOn) production methods. Created with [BioRender.com](https://www.biorender.com)

3.1.2. GBM Characterization

The physico-chemical properties of developed materials were characterized by ZetaSizer, FTIR, TGA and UV-Vis Spectroscopy and the results obtained are presented next, in Chapter 4. The dispersion stability of GOn and p-rGOn, for 0 and 8h of UV reduction, were analysed after 40 days of the production.

3.1.2.1. Transmission Electron Microscopy

Morphology and lateral dimensions of GOn and p-rGOn dispersions were analysed by transmission electron microscopy (TEM, JEOL JEM 1400 TEM, Tokyo, Japan). Aqueous dispersions were prepared at a concentration of $50 \mu\text{g mL}^{-1}$. For each sample, $10 \mu\text{L}$ were deposited on a carbon coated TEM grid and allowed to sediment for 1 min. The excess material was removed by capillarity using filter paper. Nanomaterial lateral dimensions were measured on several TEM images using ImageJ software.

3.1.2.2. Dynamic Light Scattering

Particle size distributions for GOn and p-rGOn were determined by dynamic light scattering (DLS, LS230 particle size analyser, Beckman Coulter, Brea, CA, USA), without any previous treatment or dilution. Data were collected performing two scans of 60 s, including polarization intensity differential scattering, and using Fraunhofer's model. This model assumes spherical shape for particles in suspension. This evaluation of size distributions does not provide precise estimations of particle size and this result must be completed with the evaluation of the materials deagglomeration in water, with ZetaSizer equipment. [62]

3.1.2.3. Zeta potential measurements

GOn and p-rGOn dispersions were prepared at a concentration of $25 \mu\text{g mL}^{-1}$. Both size and zeta potential of their aqueous dispersions were determined using a Zetasizer Nano-ZS (Malvern Instruments, Worcestershire, UK) in a disposable Zetasizer cuvette (Malvern Instruments, Worcestershire, UK). The measurements were performed in triplicate, after 15 min sonication, at room temperature.

3.1.2.4. Thermogravimetric analysis

Thermogravimetric analysis (TGA) (Netzsh STA 449 F3 Jupiter, Selb, Germany) was performed to evaluate the thermal stability, through weight loss of dry samples (around 6 mg) of GOn and p-rGOn under a constant temperature increase. At a constant heating rate, $10 \text{ }^{\circ}\text{C min}^{-1}$, the temperature was increased from 30 to $1000 \text{ }^{\circ}\text{C}$, under nitrogen flow.

3.1.2.5. Fourier transform infrared Spectroscopy

GOn and p-rGOn dry samples were prepared by 24h heating, at 60 °C, of the dispersions obtained, to perform the infrared spectroscopy analysis, using a VERTEX 70 FTIR spectrometer (Bruker, Karlsruhe, Germany) in transmittance mode at room temperature. Samples were measured in attenuated total reflection (ATR) with an A225/Q PLATINUM ATR Diamond crystal with a single reflection accessory. Spectra were recorded from 4000 to 400 cm^{-1} with 60 scans and a resolution of 4 cm^{-1} .

3.1.2.6. UV-visible spectroscopy

Absorption spectra of GOn and p-rGOn were acquired using a Lambda 35 UV/vis spectrometer (Perkin-Elmer, Waltham, MA, USA). Samples were prepared in a concentration of 250 $\mu\text{g mL}^{-1}$, and they were transferred to a 160 μL quartz cuvette (Hellma Analytics, Analytica Munich, Germany) with 10 mm light path length and spectra were recorded in the range of 200-850 nm. Measurements were performed at room temperature by averaging three scans with baseline correction based on water as a blank control.

3.2. Biological Studies

3.2.1. Biocompatibility

In vitro biocompatibility of GOn and p-rGOn was evaluated on primary human macrophages. All obtained human samples and procedures were performed in agreement with the principles of the Declaration of Helsinki and following approval by the CHUSJ Ethics Committee for Health (reference 90/19). Monocytes were isolated from buffy coats from anonymous healthy blood donors, provided by Serviço de Imunohemoterapia, Centro Hospitalar Universitário de São João (CHUSJ), Porto.

3.2.2. Monocyte Isolation

The procedure was performed as previously described in [196]. Briefly, buffy coats were centrifuged for 20 min, at 1200 rcf, at room temperature, with acceleration 5 and without brake. The peripheral blood mononuclear cell layer was collected and incubated with RosetteSep human monocyte enrichment isolation kit (StemCell Technologies SARL, Grenoble, France), for 20 min, on a roller shaker. The mixture was then diluted with equal volume of 2% foetal bovine serum (FBS, Biowest) in sterile PBS and it was layered over Histopaque®-1077 (Sigma-Aldrich) and centrifuged for 20 min, at 1200 rcf, at room temperature, with acceleration 2 and without brake. The enriched monocyte layer was collected carefully and washed three times with PBS, at 1300 rpm for 7 min. Lastly, the cell pellet was resuspended in complete culture media (CM-RPMI 1640 + Glutamax (Invitrogen, Paisley, UK)) supplemented with 1 % penicillin G-streptomycin (Invitrogen) and 10 % of FBS. Cells were then counted using a Neubauer chamber and plated on tissue culture plates and cultured in a humidified incubator at 37 °C/5 %.

3.2.3. Macrophage differentiation and stimulation

Monocytes differentiated in the presence of 50 ng mL⁻¹ of macrophage colony-stimulating factor (M-CSF, Immunotools, Friesoythe, Germany). After 7 days, cell culture medium was replaced, M-CSF was removed, and it was added 50 ng mL⁻¹ of Interferon- γ (IFN- γ , ImmunoTools). At day 10 cells were left unstimulated or stimulated with 10 ng mL⁻¹ of LPS (*E. coli*, Sigma) and activated with different concentrations (50, 100 and 250 μ g mL⁻¹) of GOn or p-rGOn and incubated for an additional 72h-period. After 13 days of culture, cells culture supernatants and macrophages were collected.

3.2.4. UV irradiation

After macrophages differentiation, subsequently to the addition of the GBM, the cells were exposed to low-power UVC radiation (270 ± 5 nm, 3 mW cm⁻²) for 30 min, using a custom-built irradiation system composed of 12 LEDs, which fit the wells of a cell culture plate.

3.2.5. Cytotoxicity - Lactate dehydrogenase (LDH) Assay

50 μ L of the supernatant collected from the macrophages culture after 24h of activation corresponding to each condition was added to a 96-well plate, as well as 50 μ L of Assay Buffer (CytoTox 96® Non-Radioactive Cytotoxicity Assay, Promega) and incubated for 30 min at room temperature, protected from the light. Following, 50 μ L of Stop Solution (CytoTox 96® Non-Radioactive Cytotoxicity Assay, Promega) were added to the wells and the absorbance signal was measured at 490 nm in a plate reader (Synergy Mx, software Gen5). The cytotoxicity calculations were made according to the manufacturer's instructions (CytoTox 96® Non-Radioactive Cytotoxicity Assay, Promega).

3.2.6. Live and Dead Macrophages Viability Assay

At day 13 of culture, the samples were stained with Hoescht at 2.5 μ g mL⁻¹ (Invitrogen) and Calcein at 2 μ g mL⁻¹ (Invitrogen), both for 30 minutes. 5 minutes before the analysis, the cells were stained with phosphate iodide (PI) 2 μ g mL⁻¹ (BD Biosciences). The acquisition was made using IN Cell Analyzer 2000 (GE Healthcare) using a Nikon 20x/0.45 NA Plan Fluor objective (1x1 binning) with a pixel array of 2048 x 2048. The acquisition mode was 2D with a Z section of around 3 μ m. Exposure times were maintained for all samples within experiments, with the aim of permitting the comparison between results. In order to detect Hoescht, Calcein and Propidium Iodide (PI), the excitation and emission filters used were DAPI, FITC, and Cy5, respectively. Images' analysis was made using the IN Cell Investigator Developer Toolbox 1.9.2 (GE Healthcare). The macrophages were individualized in the form of "seed" targets, in order to avoid errors associated to the association of adjacent cells as a unique cell. "Erosion" and "clump breaking" were performed to diminish the cells exterior boundary and to identify the closer cells and calculate their limit, respectively. After this, the nuclear outer boundaries were enlarged to allow the pixel

quantification and it was performed a separation in the intensity, for each staining, by segmenting the pixels according to their fluorescence limit, being equal to each experiment. Finally, using fluorescence intensity levels calculated and pretend under the form of histograms, in Spotfire® data software, the limits of the mean pixel intensities for each staining were chosen.

The acquisition and analysis were performed at the BioSciences Screening platform at i3S, University of Porto, Portugal with the assistance of André Maia.

3.2.7. Cell Morphology

To evaluate the cells morphology, the samples were stained for f-actin using phalloidin (Alexa Fluor 488 nm, Invitrogen) and nuclei using DAPI. The cells were washed three times with PBS for 5 minutes and fixed with 4 % PFA (paraformaldehyde) for 15 min at room temperature. Cells were washed three times and were incubated for 15 min with PBS + 0.2 % Triton X-100 and washed three times with PBS for 5 min, at room temperature. This step was made to guarantee the cell permeation. To reduce the non-specific background, the cells were blocked with PBS + 1 % Bovine Serum Albumin (BSA) at room temperature, for 1 hour and washed again three times with PBS. Lastly, cells were incubated for 1 hour at room temperature with a 16:1000 solution of phalloidin in PBS and washed three times, for 5 minutes each wash, with PBS. All steps were performed under orbital shaking and covered from the light. Samples were mounted with Fluoromount with DAPI and stored at 4°C until the observation in the Inverted Fluorescence Microscope (Carl Zeiss, Axiovert 200M). Images were taken with AxioVision Software and processed with ImageJ.

3.2.8. Transmission Electron Microscopy

Macrophages were cultured on a Lab-Tek chamber slide and activated with GOn and p-rGOn, at 100 µg mL⁻¹, at day 10 of differentiation. Samples were fixed by immersion in 2.5 % glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) solution for 1 day. After washing and two hours in post-fixating 2 % osmium tetroxide in 0.1 M sodium cacodylate buffer (pH 7.4) solution, cells were washed in water, incubated with 1 % Uranyl acetate overnight, washed in water and dehydrated through graded series of ethanol, and embedded in Epon (EMS). Ultrathin sections were cut at 50 nm and prepared on an RMC Ultramicrotome (PowerTome, USA) using a diamond knife and recovered to 300 mesh copper grids, followed by double contrast method with uranyl acetate substitute (#11000; Electron Microscopy Sciences) and lead citrate (#11300; Electron Microscopy Sciences) for 5 min each. Visualization was performed at 80 kV in a (JEOL JEM 1400 microscope, Japan) and digital images were acquired using a CCD digital camera Orious 1100 W (Tokyo, Japan). The transmission electronic microscopy was performed at the HEMS core facility at i3S, University of Porto, Portugal with the assistance of Ana Rita Malheiro e Rui Fernandes.

3.2.9. Enzyme-Linked Immunosorbent Assay (ELISA)

Culture medium collected at day 13 was centrifuged (400 rcf, 5 min) and kept at -20 °C for posterior analysis. Human IL-10 and IL-1 β , for the macrophages, and human IFN- γ and IL-4, for the dendritic cells, were quantified by ELISA (ELISA MAX Deluxe Set Human, Biolegend) according to manufacturer's instructions. Cytokine (pg/mL) were determined using a standard calibration curve.

3.2.10. Quantitative Real-Time Polymerase Chain Reaction (qPCR)

Total RNA was extracted using TRIzol® Reagent (Invitrogen), following the manufacturer's instructions. RNA concentration was determined using the NanoDrop Spectrophotometer ND-1000 (ThermoFisher Scientific). The integrity was evaluated by gel electrophoresis. For gene expression analysis, RNA was treated with TURBO DNA-free Kit (Invitrogen) and cDNA was synthesized using Random Hexamers (Invitrogen), dNTPs (Bioline) and SuperScript III Reverse Transcriptase (Invitrogen). qPCR was carried out in CFX96 Touch Real-Time PCR Detection System (Bio-Rad, USA) using cDNA, primers (Appendix A, Table A) and iQ SYBR Green Supermix (Bio-Rad). All runs were performed in duplicate. Relative expression levels from 6 independent experiments were calculated according to MIQE guidelines, through the quantification cycle (Cq) method. [197]

3.2.11. Flow Cytometry

Fully differentiated macrophages, at day 13, were incubated with PBS-EDTA at room temperature during 10 min and gently harvested, washed, and resuspended in FACS buffer (PBS, 2 % FBS, 0.01 % azide) with the following fluorescent conjugated antibodies, in a total volume of 50 μ L: anti-CD86-FITC, anti-CD14-APC, HLA-DR-Pac. Blue (all from Immunotools) and anti-CD163-PE (from R&D Systems). Then, they were incubated protected from the light for 30 min at 4°C. Isotype matched antibodies (negative controls) were used to define background staining. After 3 washes, fluorescence was measured using a FACS Canto Flow Cytometer (BD Biosciences). The analysis was performed using FlowJo software.

3.2.12. Reactive Oxygen Species Quantification

To measure the levels of ROS in conditioned media from LPS+IFN- γ -stimulated macrophages, in the presence of GOn and p-rGOn materials, the Acridan Lumigen PS-3 assay was developed according to the protocols adapted from [198]. The ALPS-3 substrate was made by combining reagent A (H₂O₂ in Tris buffer, Amersham ECL Plus kit, GE Healthcare) and reagent B (acridan solution in dioxane and ethanol, Amersham ECL Plus kit, GE Healthcare) in a 40:1 ratio. 20 μ L of conditioned media from macrophages 80 μ L for each sample were placed in a 96 well plate. Following, it was added 50 μ L of ALPS-3 substrate in each well and the plate was incubated in the dark for 5 min at room temperature. The light intensity spectra (photons counts) were recorded from 350 to 500 nm using a Synergy Mx plate reader and Gen5 software. The mean of the records from 455, 460 and 465 were used for comparison between samples.

3.2.13. Mixed Lymphocyte Reactions

Monocyte-derived DC were differentiated in the presence of IL-4 (50 ng mL⁻¹) and GM-CSF (50 ng mL⁻¹) (both from Immunotools, Friesoythe, Germany) for 6 days, before being exposed to GMB materials in presence/absence of 100 ng mL⁻¹ of TNF- α , adapting previously described protocols. [199] At day 7 lymphocytes were isolated from fresh buffy coats from different blood donors to DC. These were diluted in an equal volume of PBS 1x and then, the mixture was layered over Lymphoprep (Axis-Shield PoC, Oslo, Norway), followed by a centrifugation (800 xg for 30 min, at room temperature, without brake). Peripheral Blood Mononuclear Cells (PBMCs) were collected after density gradient centrifugation, counted with a Neubauer Chamber, and plated on three TCPS petri dishes at 50 x 10⁶ cells mL⁻¹. After 2 hours, lymphocytes in the supernatant were separated from the monocytic population, that adhered to the plates. The lymphocytes were then counted with a Neubauer chamber and labelled with 1 μ M carboxyfluorescein diacetate succinimidyl ester (CFSE, Invitrogen) for 15 min, at 37 °C. Following, two washes were performed, for 5 min at 1500 rpm, with PBS supplemented with 20 % FBS. The cells were resuspended in complete medium, CM-RPMI 1640 + Glutamax (Invitrogen, Paisley, UK) supplemented with 1 % penicillin G-streptomycin (Invitrogen) and 10 % of FBS and added in a 3:1 proportion (0.9 x 10⁶ cells mL⁻¹) to the pre-cultured DC. The following controls were also set up: a control of T cells without CFSE staining, T cells stained with CFSE alone (both negative controls) and T cells stained with CFSE and stimulated with phytohaemmagglutinin (PHA) at 1, 2.5 and 5 μ g mL⁻¹ (Sigma-Aldrich), as positive proliferation controls. After 5 days of co-culture, the T cells on the supernatant were collected and labelled with anti-CD3-PE conjugated. Unlabelled T cells were used to determine background staining and cells with only CD3-PE staining and with only CFSE were used to define samples compensation. The acquisition was made on a FACS Calibur (BD Biosciences) using Cell Quest software, and the analysis was made with FlowJo software. T cell proliferation was determined by CFSE halving of CD3+ cells.

3.2.14. Statistical Analysis

Statistical analysis was performed using GraphPad Prism 8 software. Results were analysed for Gaussian distribution using the D'Agostino and Pearson normality test and samples were non-parametric. The Friedman and the Kruskal-Wallis test were used to compare between samples followed by uncorrected Dunn's comparison test. A value of $p < 0.05$ was considered statistically significant: * < 0.05 ; ** < 0.01 ; *** < 0.001 . Data are presented showing the mean of all donors and the respective maximum and minimum values. Only the significative comparisons are reported in the figures.

Chapter 4 : Results

4.1. Physico-chemical properties of the GBM

After graphite oxidation by the modified Hummers method and processing through high-power sonication, GOn has been obtained. Subsequent irradiation with UV allowed to produce p-rGOn. The morphology and size of both GBM was evaluated by TEM. Figure 4.1 shows that both nanomaterials are composed of well exfoliated and individualized nanosheets. High-power ultrasonication processing allowed achieving sizes (measured from TEM images) of 206.969 ± 125.793 nm, and 276.399 ± 187.314 nm for GOn and p-rGOn, respectively.

The size distribution of GOn and p-rGOn was also evaluated using a ZetaSizer (Table 4.1). An average size of $284,2 \pm 38,4$ nm and $218,5 \pm 25,7$ nm for GOn and p-rGOn were obtained, respectively. Polydispersity indexes of $0,49 \pm 0,06$ for GOn and of $0,431 \pm 0,061$ for p-rGOn were determined, being the range of PDI from 0, for a perfectly uniform sample regarding particle size, to 1, for a very much polydisperse sample with various particle size populations. [200] Zeta potential values of $-44,5 \pm 4,7$ mV and $-37,9 \pm 2,6$ mV were obtained for GOn and p-rGOn, respectively, which indicates good water stability for both materials, regardless the partial reduction performed, it didn't affect p-rGOn water stability in way that would render it not suitable for desired phototherapy applications. [201]

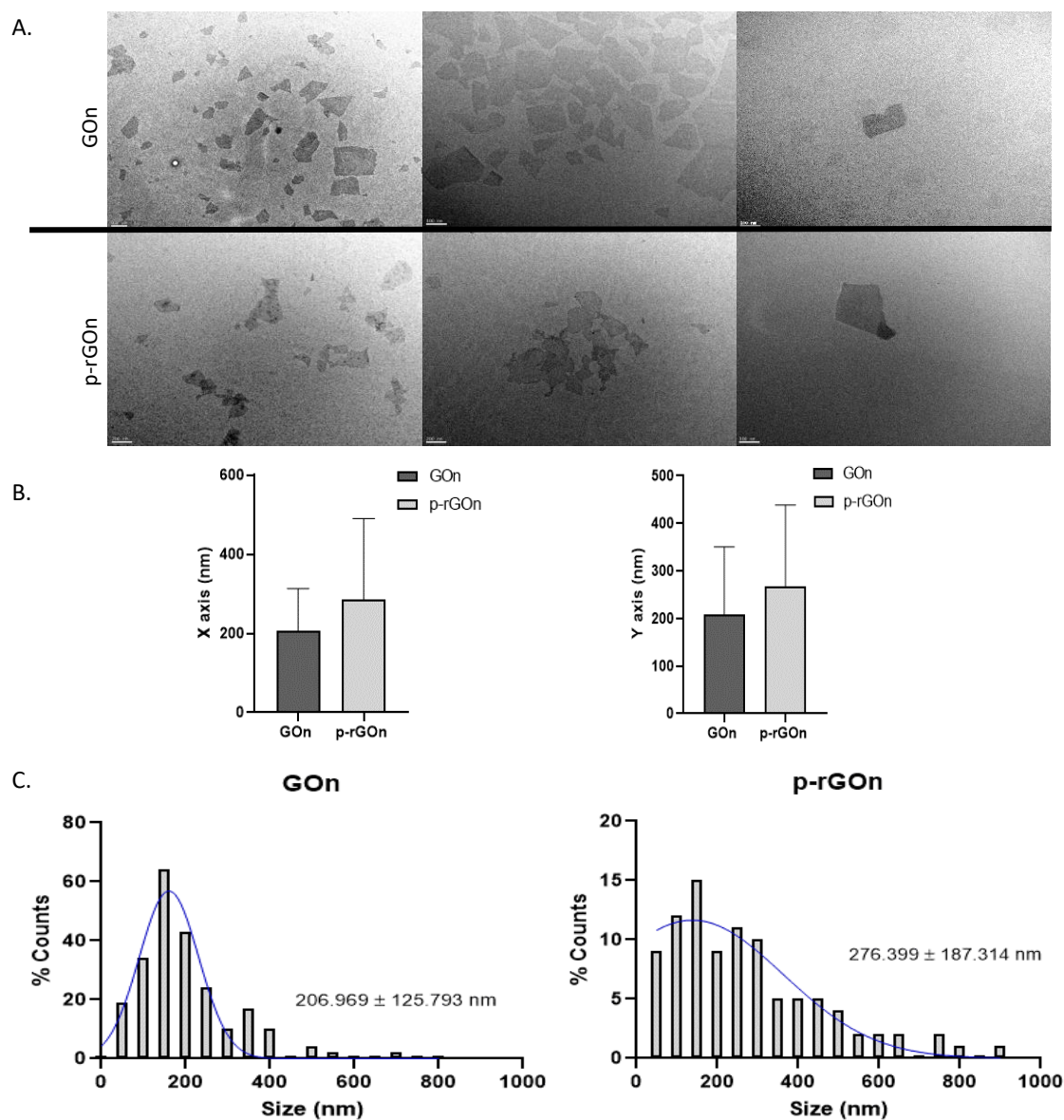


Figure 4.1. A) GOn and p-rGOn particle morphology and size determined by TEM images analysis. Scale bar represents 200 nm, except in the bottom images where it represents 100 nm; B) Particle size for the X and Y axis; C) Particle size distribution histograms. GBM aqueous dispersions at a concentration of $50 \mu\text{g mL}^{-1}$ were deposited at the surface of TEM grids to obtain the images.

Table 4.1. Particle size, polydispersity index, and zeta potential for GOn and p-rGOn aqueous dispersions diluted at a concentration of $25 \mu\text{g mL}^{-1}$, determined by DLS with a Zetasizer equipment ($n = 3$).

	GOn	p-rGOn
Average size (nm)	$284,2 \pm 38,4$	$218,5 \pm 25,7$
Polydispersity index	$0,49 \pm 0,06$	$0,431 \pm 0,061$
Zeta potential (mV)	$-44,5 \pm 4,7$	$-37,9 \pm 2,6$

The water stability of both GBM was also analysed visually comparing both materials at the day of production and after 4 months of storage. Both GOn and p-rGOn were stable dark brown dispersions after production and during the storage time of 4 months, as observed in Figure 4.2, with no phase separation or sedimentation having occurred. This is relevant regarding the storage of GBM containing formulations for medical applications, such as phototherapy.

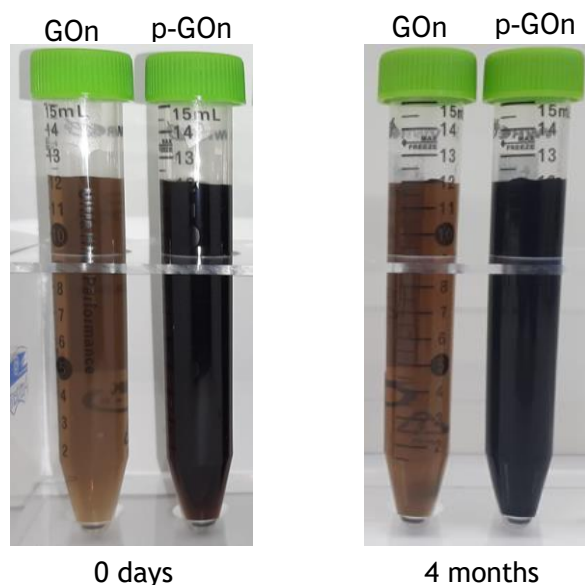


Figure 4.2. GBM water dispersions stability visual evaluation. GOn (8.9 mg mL^{-1}) and p-rGOn (0.8 mg mL^{-1}) dispersions, at day 0 and 4 months after production.

Thermogravimetric Analysis (Figure 4.3) was performed in order to evaluate GOn and p-rGOn thermal stability and degree of oxidation. Both materials presented a similar thermodegradation profile until around 600°C being achieved. The first step of weight loss ($\approx 25 \text{ wt.}\%$) occurred between 100°C to 225°C , with the loss of carboxyl and epoxy functional groups. Weight lost before 100°C is attributed to water evaporation. Next, from around 225°C to 600°C , around $50 \text{ wt.}\%$ loss has been reached with the elimination of carbonyls, hydroxyls, and combustion of the carbon skeleton. The total weight loss at 1000°C was of 95.5% for GOn and of 66.2% for p-rGOn. This step corresponds to the continuous combustion of the carbon skeleton. Oxygenated groups are thermolabile so the lower amount of these functional groups in p-rGOn results in higher thermal stability, when compared to GOn that contains a higher content of oxygen-functional groups. [91]

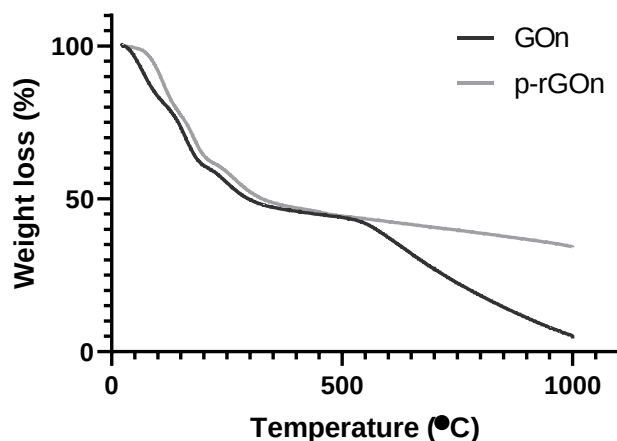


Figure 4.3. Thermogravimetric analysis of GOn and p-rGOn, performed under nitrogen atmosphere.

Fourier Transform Infra-Red (FTIR) spectra of the GBM (Figure 4.4) were obtained to confirm the successful introduction of the characteristic oxygen-containing functional groups in GOn and then its effective photo-reduction to p-rGOn. The peaks at 1166 cm^{-1} and 1041 cm^{-1} correspond to C-O stretching vibrations attributed to ethers, while C-O bending vibrations present at 970 cm^{-1} are related to epoxy groups. The peak at 1621 cm^{-1} corresponds to the stretching of cyclic alkenes (C=C). Around 1731 cm^{-1} a peak is assigned to carbonyl and carboxyl groups C=O stretching vibrations. Finally, a the wide band between 3000 and 3600 cm^{-1} , results from O-H stretching vibrations, typical from adsorbed water molecules, hydroxyls and carboxyls. [202] The decrease of the intensity of bands assigned to oxygen-containing functional groups confirms the successful UV-reduction of GOn to achieve p-rGOn.

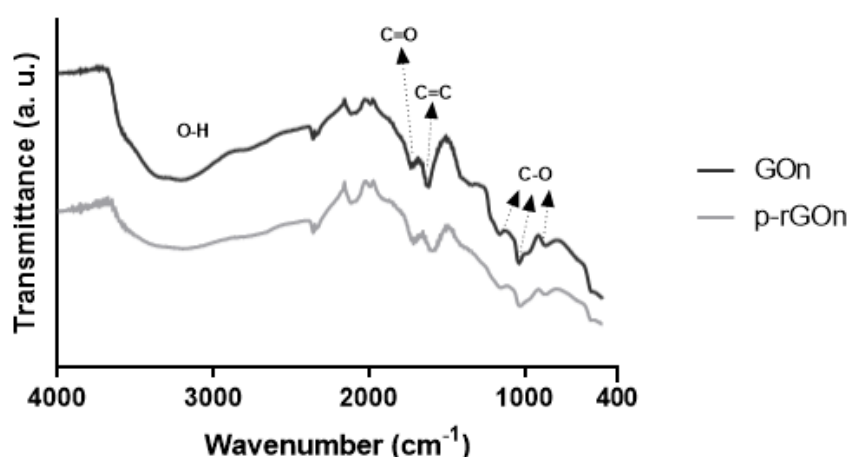


Figure 4.4. Fourier Transform Infra-Red spectra of GOn and p-rGOn.

To assess the potential of the developed materials to be used for photothermal therapy, namely in terms of UV absorption capacity, the optical properties of both GOn and p-rGOn were characterized by UV/vis spectroscopy (Figure 4.5). The results showed an absorbance peak at $\lambda_{max} = 230\text{ nm}$ for GOn, which is assigned to $\pi\text{-}\pi^*$ electronic transitions in sp^2 clusters, and a shoulder

peak at 300 nm, which is attributed to $n-\pi^*$ transitions of free electron pairs in oxygen atoms in C=O bonds from carbonyl and carboxyl groups. [203] The same peak at $\lambda_{max} = 230$ nm was observed for p-rGOn, being identified the absence of a shoulder peak which can be related to the reduction in oxygen atoms, namely from the carbonyl and carboxyl groups. Also, p-rGOn presented approximately a 2-fold increase in comparison to GOn in UV absorbance, at the wavelength (270 nm) used for phototherapy in the present study.

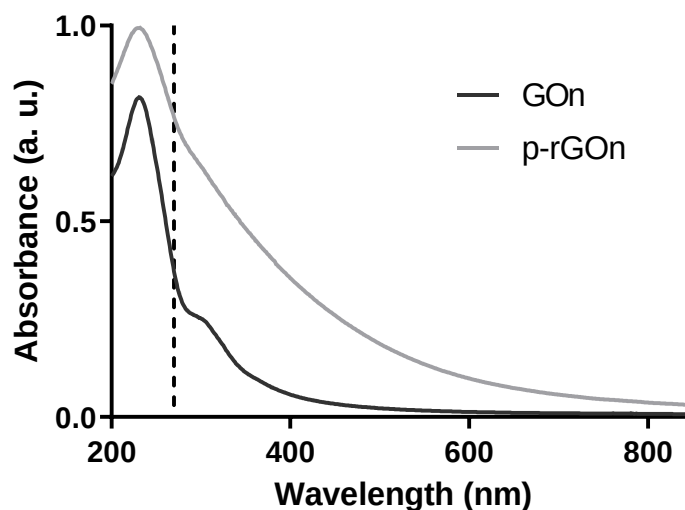


Figure 4.5. UV-Visible Spectroscopy analysis of GOn and p-rGOn. The dashed line at 270 nm marks the wavelength used for phototherapy in the present study.

4.2. Impact of GBM concentration on Macrophage's viability

Next it was performed the evaluation of the potential of using the developed innovative GBM to stimulate immune cells. First, it was determined the range of non-toxic concentrations for human primary monocyte-derived macrophages. Cells were exposed to increasing concentrations of each nanomaterial (50, 100 and 250 $\mu\text{g mL}^{-1}$) for 72 h. Macrophage's morphology is illustrated in figure 4.5, being manifest no normal conformation or disintegration, with cell uptake being observed for both GBM and significant biomaterial accumulation for the higher concentrations, more extensively for p-rGOn.

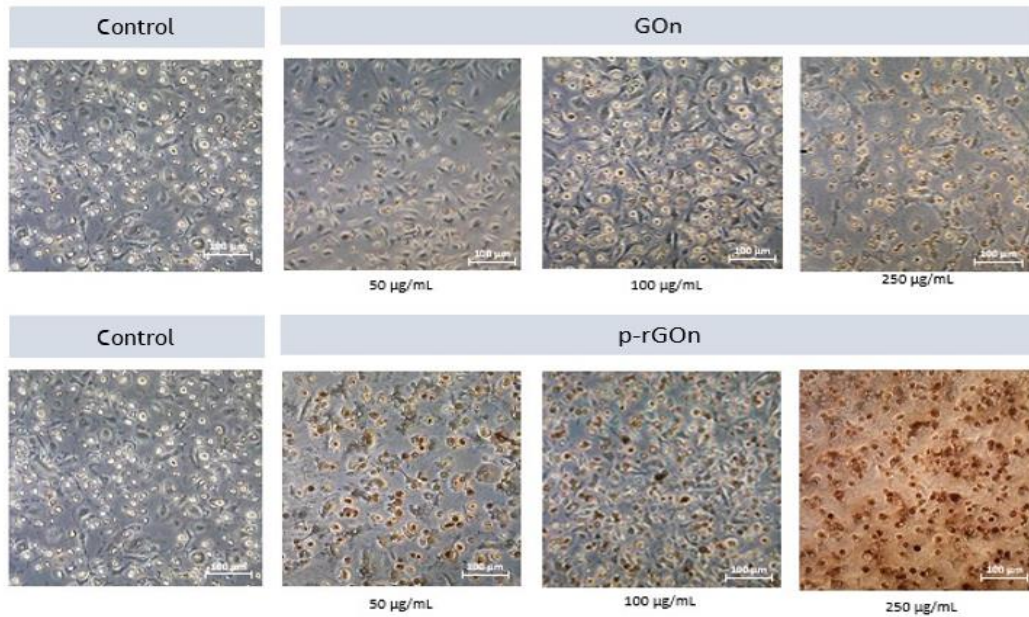


Figure 4.6. Optical microscopy images of macrophages cultured in the presence of different concentrations of GOn (right, top) and p-rGOn (right, bottom). Control cells were incubated in the absence of GBM (left). Scale bar represents 100 μm for all images.

To evaluate cytotoxicity towards macrophages it was analysed the LDH release, with 24h of GBM incubation with increasing concentrations of both GBM (50, 100 and 250 $\mu\text{g mL}^{-1}$), with and without 30 min of UVB/C led source ($270 \pm 5 \text{ nm}$, 3 mW cm^{-2}) irradiation. Results are presented in Figure 4.7.A and show increased LDH release for both materials, and particularly for rGOn, at 250 $\mu\text{g mL}^{-1}$ with almost 20 % of cytotoxicity. This result was similar without and with UV irradiation. Next, Live/Dead assays were performed after 72h of GBM incubation, at the same concentrations as above, also with 30 min of UV irradiation (with the exception of the highest concentration, 250 $\mu\text{g mL}^{-1}$). Results show that both irradiation and biomaterial presence reduced the number of live cells, those that could hydrolyse calcein, thus showing green fluorescence (Figure 4.7.B). When the numbers of cells were analysed across different donors, a significant decrease in the total number of cells could be observed, when they were incubated with GBM and/or irradiated, when compared to the control. When the percentage of live and dead cells was analysed, elevated cytotoxicity was found, for the highest concentration of both GBM (more than 25 % for GOn and around 50 % for rGOn), while no more than ~5 % toxicity was seen the remaining conditions (Figure 4.7.C). By analysing both LDH and Live/Dead results, it was possible to identify that both GBM, at 250 $\mu\text{g mL}^{-1}$ are toxic for macrophages. Based on these results the 100 $\mu\text{g mL}^{-1}$ concentration was selected for the following experiments.

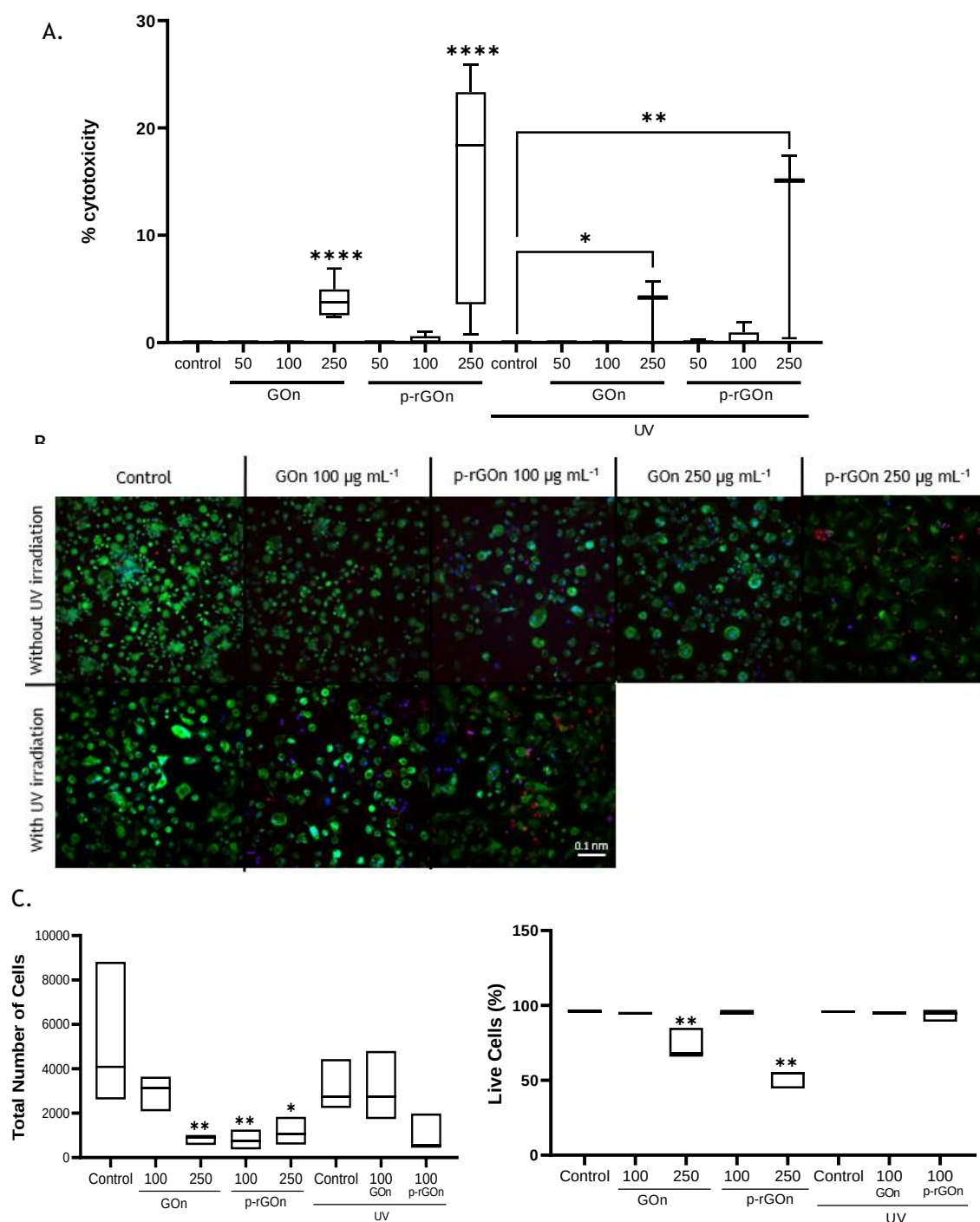


Figure 4.7. Cytotoxicity of GBM for human primary macrophages. A) GOn and rGOn (50, 100 and 250 $\mu\text{g mL}^{-1}$) cytotoxicity results using LDH assay on the supernatants from 13 day of culture (24h of incubation with GBM): B) Representative images of Live/Dead assay after 72h of incubation with GOn and rGOn (50, 100 and 250 $\mu\text{g mL}^{-1}$). Scale bar: 0.1 nm; C) Quantification of Li/Dead assay results across different donors, in number of cells (left table) and in percentage (right graph). Data represent arithmetic mean $\pm$ SD, analysed by Kruskal-Wallis test followed by uncorrected Dunn's test ($n=6$). * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$. The asterisks above the box plots represents a comparison with the control.

4.3. Macrophage's morphology

Having chosen the $100 \mu\text{g mL}^{-1}$ as the working concentration, next, macrophage cell integrity and morphology were evaluated. The cytoskeleton of macrophages exposed to GBM for 72h, were stained with phalloidin (green staining) and the nuclei with PI (red staining) and observed by fluorescence microscopy. Figure 4.8 shows that GBM, particularly rGO may still cause some cell deterioration.

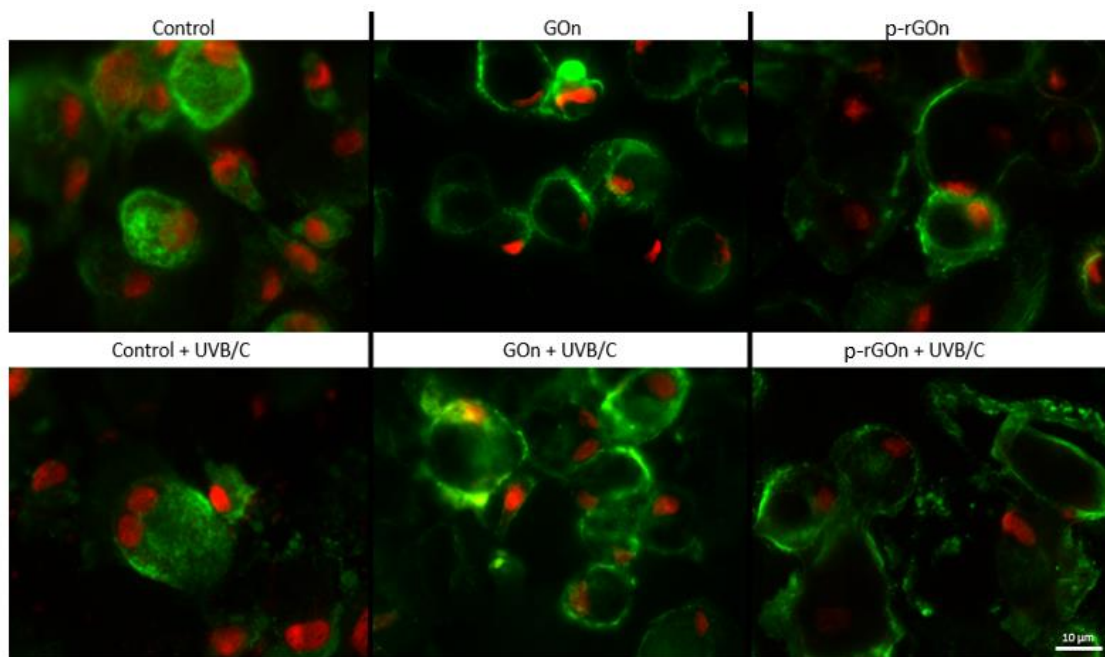


Figure 4.8. Fluorescence microscopy images of macrophages cultured in the presence of GOn and p-rGOn at $100 \mu\text{g mL}^{-1}$, with and without UV irradiation. Control cells were incubated in the absence of GBM. Green staining: Phalloidin; Red staining: DAPI. Scale bar: $10 \mu\text{m}$.

To evaluate cell integrity in more detail, macrophages were exposed to both nanomaterials at $100 \mu\text{g mL}^{-1}$ and analysed using Transmission Electron Microscopy. Results are presented in figure 4.9, and show that cells exposed to both nanomaterials, GOn and p-rGOn, displayed elevated membrane fragmentation in comparison with control cells, not incubated with GBM, being visible the existence of several vesicles in the exterior part of the membrane and in the interior part, the loss of organelles near to the membrane.

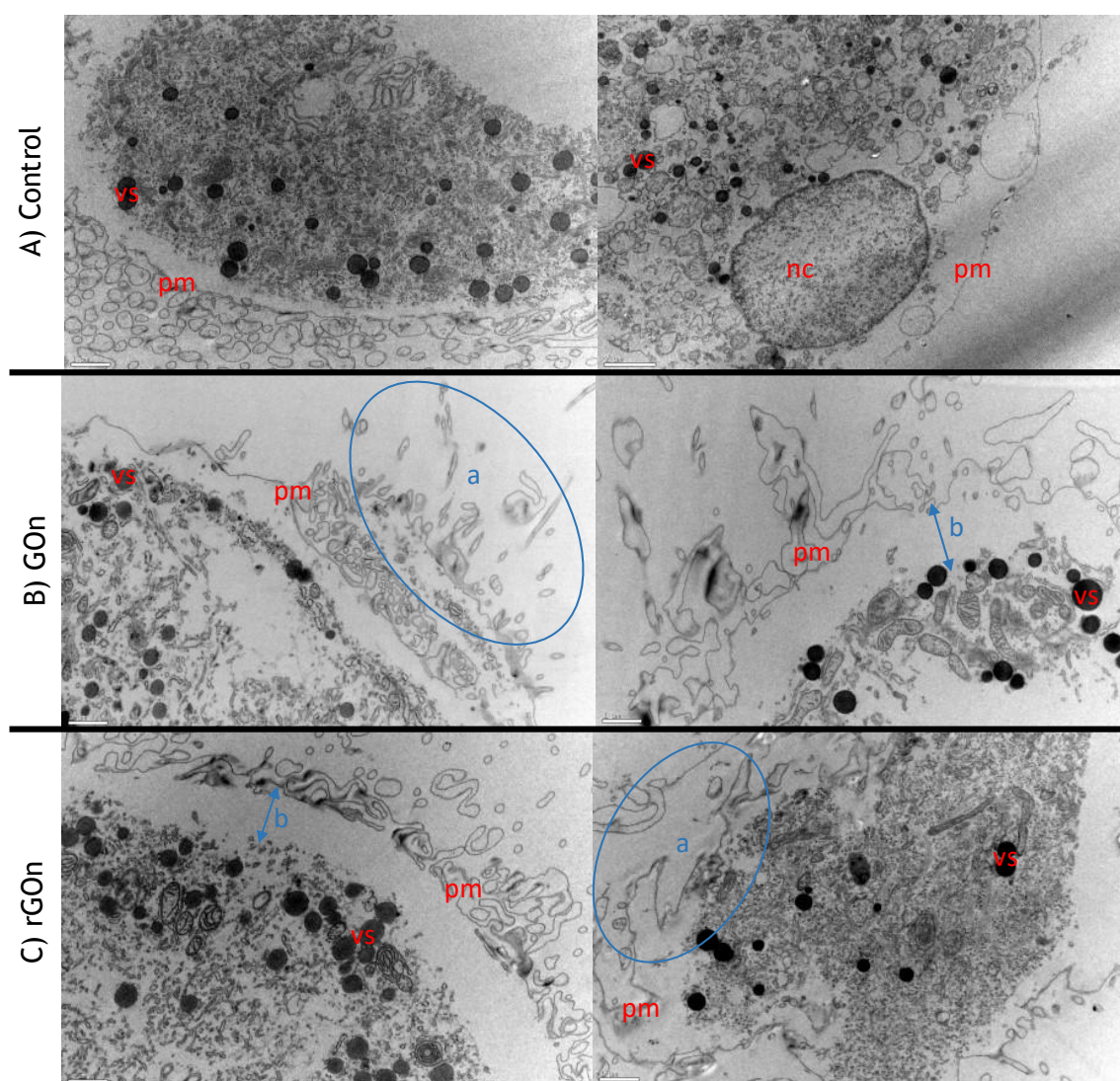


Figure 4.9. TEM images of macrophages incubated for 72 h with $100 \mu\text{g mL}^{-1}$ GBMs. A) Control of cells without GBM, being visible the plasma membrane; B) Macrophages incubated with GOn with visible membrane disruption (a) and loss of organelles near to the membrane (b); C) Macrophages incubated with p-rGOn with visible loss of organelles near to the membrane (b) and membrane disruption (a). nc-nuclei; pm-plasma membrane; vs-vesicle. Scale bar represents $1 \mu\text{m}$ for all images, except the image from control in the left panel that represents $2 \mu\text{m}$.

By analysing the TEM images, it was possible to identify the presence of both nanomaterials (Figure 4.10) in the form of small darker non-defined substances (highlighted in the blue and red circles) with $\sim 300 \text{ nm}$. Comparing to control conditions, cells exposed to either GOn or p-rGOn show mitochondrial disruption (Figure 4.10, a and b). Further study is required to confirm and understand the possible impact of the reported findings.

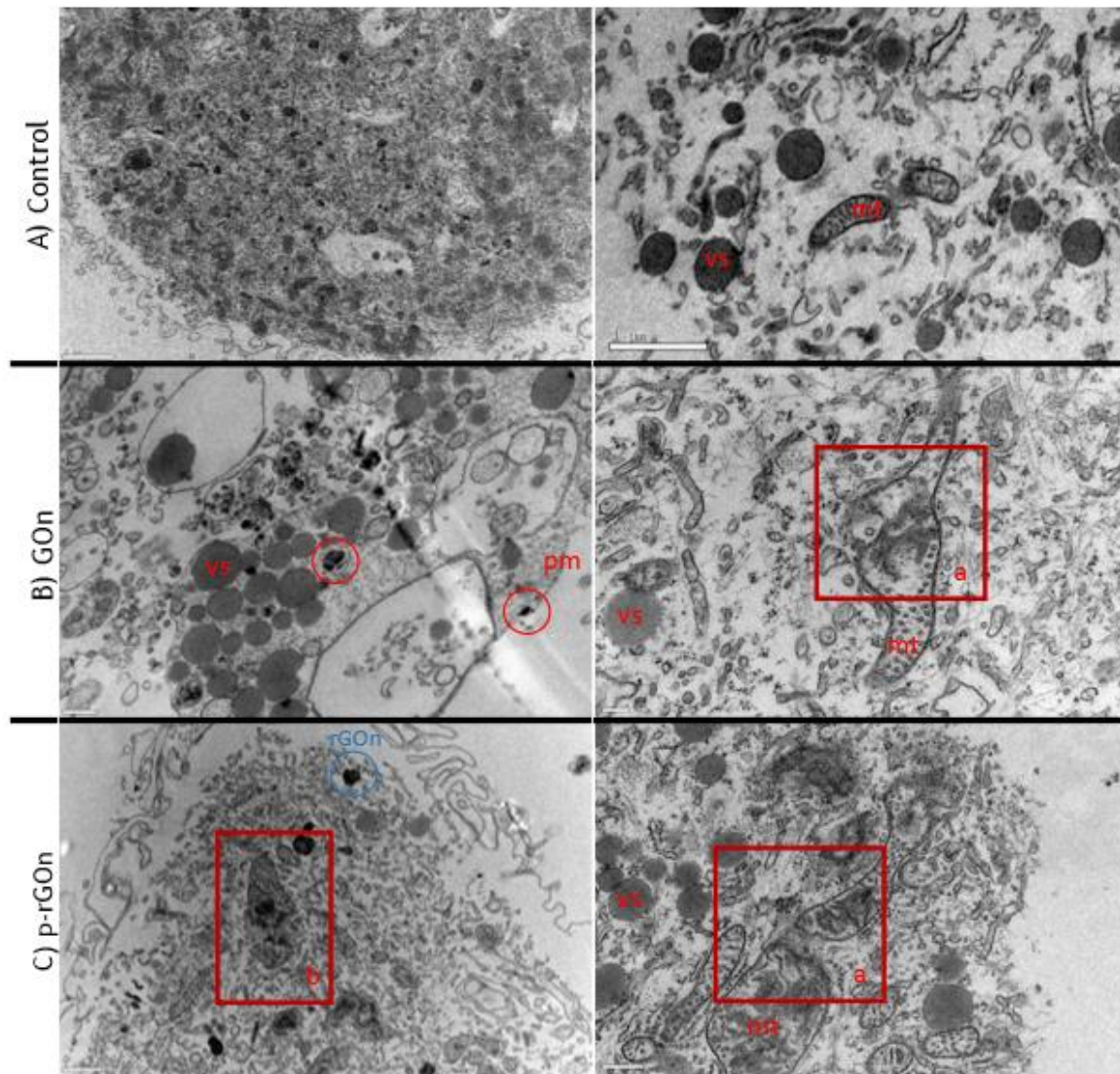


Figure 4.10. TEM images of macrophages incubated for 72 h with GOn and p-rGOn, at $100 \mu\text{g mL}^{-1}$ GBMs. A) Normal mitochondria observed in the control of macrophages without GBM (right panel) and normal macrophage (left panel); B) Fragmented mitochondrion (a) observed in macrophages incubated with GOn (right panel) and GOn particles in the cytoplasm (red circles, left panel); C) Fragmented mitochondrion (a) observed in macrophages incubated with p-rGOn (right panel), p-rGOn particles in the cytoplasm (blue circles, left panel) interacting with a disintegrated mitochondrion (b, left panel). Scale bar represents $1 \mu\text{m}$ for Control (right) and p-rGOn (left), $2 \mu\text{m}$ for GOn (right), $0.2 \mu\text{m}$ for GOn (right), $0.5 \mu\text{m}$ for GOn (left) and p-rGOn (right). mt: mitochondrion; pm: plasma membrane; vs: vesicle.

4.4. The impact of GBM on Macrophage Polarization

Our next step was to evaluate the ability of GBM to modulate macrophage polarisation, by analysing cell surface expression of specific phenotypic markers for M1 and M2 macrophages, in combination with the lineage marker CD14.

After 72h of exposure to GBM the SSC/FSC ratio of the cells showed significant changes, and less cells are found for conditions exposed to GBM (Figure 4.11, top row). These results agree with

those obtained for the live-dead assays and preclude a definitive interpretation of cell surface marker expression, Figure 4.10 bottom panel.

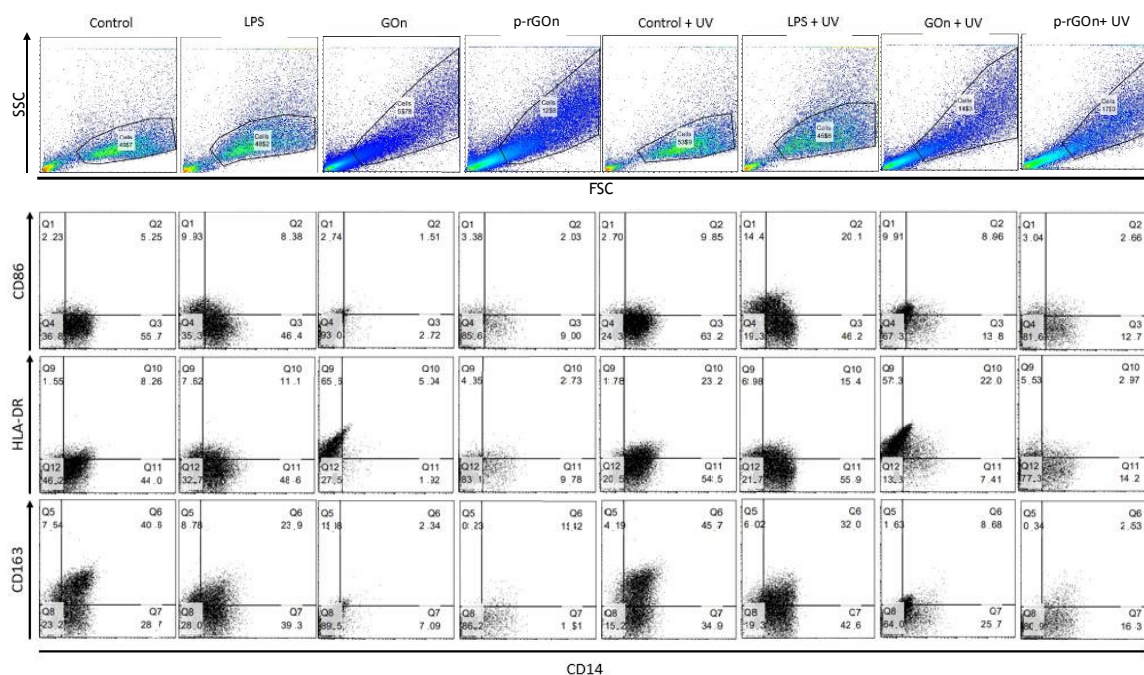


Figure 4.11. Human primary monocyte-derived macrophages incubated in the presence of GOn and p-rGOn at $100 \mu\text{g mL}^{-1}$ were harvested and cell surface stained with the indicated antibodies before FACS analysis. LPS-IFN- γ was used as positive control. Top row of plots shows cell size and complexity/granularity (FSC versus SSC), and bottom panel show the different markers in the population selected as cells in the top row graphs.

Given the difficulties in performing flow cytometry, to confirm the results obtained gene expression analysis was performed.

After 10 days of culture, macrophages were exposed to the GBM at $100 \mu\text{g mL}^{-1}$ and irradiated with UV irradiation for 30 min. After 3 days, the RNA was extracted from the cells and converted in cDNA. The expression of genes related to Transforming growth factor beta (TGF- β), an anti-inflammatory mediator, and to Interleukin-6 (IL-6), a pro-inflammatory cytokine, were evaluated. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene, to normalize the results, since its production on macrophages is quite constant, without significant variations. Results are presented in Figure 4.12.A and show that IL-6 expression increased significantly upon LPS+IFN- γ stimulation, as expected for a M1, pro-inflammatory, stimulation. In presence of GBM the statistically significant difference was lost. Significant increases were also observed when all the stimuli were combined, pro-inflammatory stimulation, GBM exposure and irradiation, when compared to the control condition, potentially indicating a synergistic effect between them. The lack of significant changes between the positive M1 control, LPS+IFN- γ , and the activated and GBM exposed cells, both with and without UV irradiation, indicate that the materials did not have a significant anti-inflammatory impact. TGF- β , expression was only

significantly decreased in LPS+IFN- γ stimulated cells exposed to GOn, when compared with LPS+IFN- γ stimulation alone.

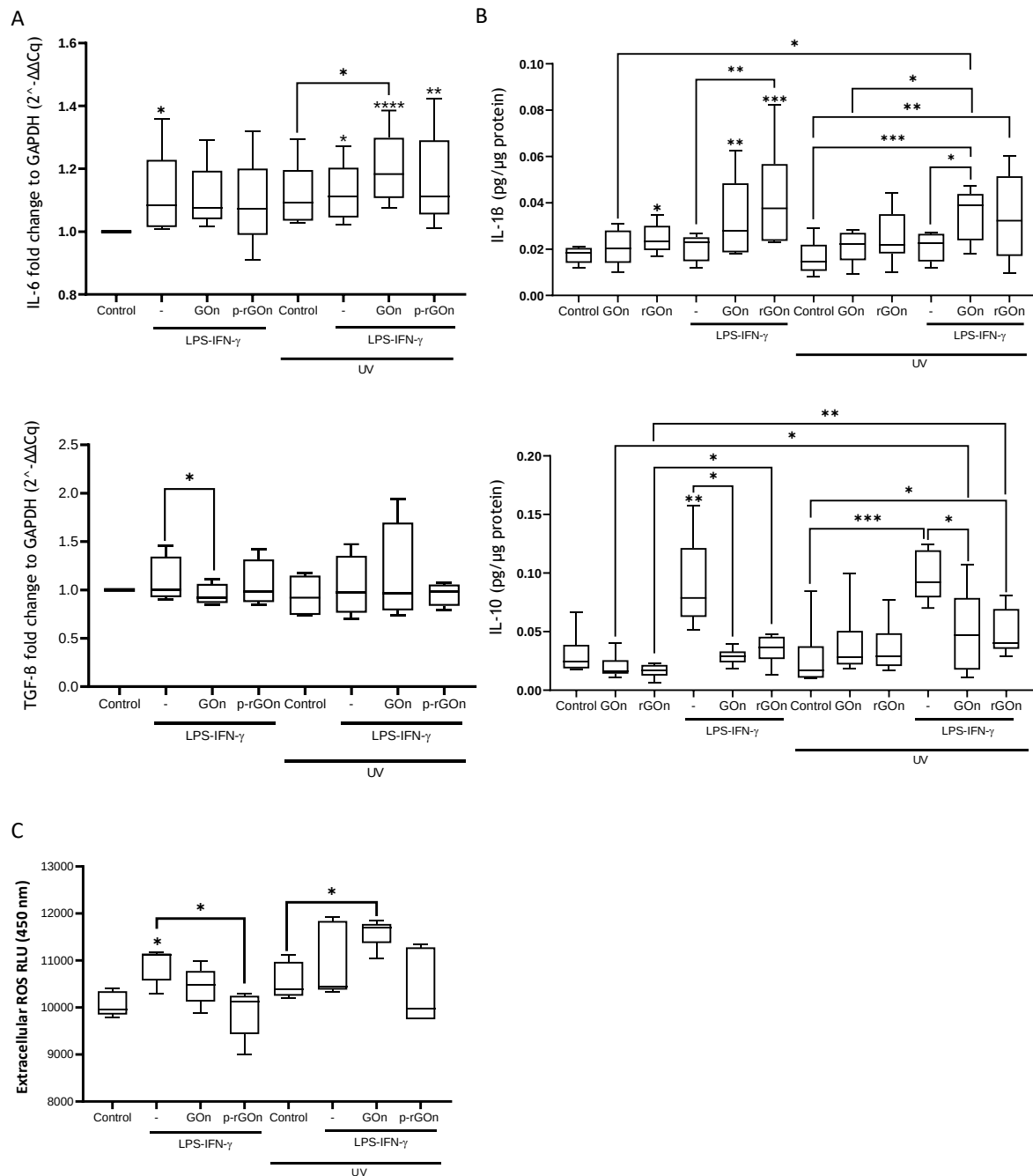


Figure 4.12. Macrophages incubated with GOn ($100 \mu\text{g mL}^{-1}$) and rGOn ($100 \mu\text{g mL}^{-1}$), with and without 30 min of UV irradiation. A) Gene Expression evaluation of IL-6 (top graph) TGF- β (bottom graph). Gene expression was evaluated by RT-PCR; B) Cytokine production: IL-10 (left panel) and IL-1 β (right panel). Cytokine release was determined by ELISA, using cell culture supernatants from 13 day differentiated human primary monocyte-derived macrophages; C) Reactive Oxygen Species (ROS) production: ROS quantity was determined by a Luminescence Assay, using cell culture supernatants from 13 day differentiated human primary monocyte-derived macrophages. LPS+IFN- γ was used as positive control. Data represent arithmetic mean $\pm$ SD, analysed by Friedman test followed by uncorrected Dunn's test ($n = 6$). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. The asterisks above the box plots represents a comparison with the control.

The secretion profile of macrophages is crucial for their contribution to the microenvironment. In order to understand how GBM affected macrophages secretion of IL-1 β and IL-10, as pro- and anti-inflammatory cytokines, were analysed. The results obtained are presented in Figure 4.12.B, and show that IL-1 β , was significantly increased by exposure to rGO alone or upon LPS+IFN- γ activation, in comparison to unstimulated control. It was also significantly increased in presence of rGOn and LPS+IFN- γ , when compared to LPS+IFN- γ alone. The exposure to GOn significantly increased IL-1 β , but only when combined with LPS+IFN- γ stimulation. Upon irradiation GOn exposure in LPS+IFN- γ activated cells significantly increased IL-1 β , when compared with either GOn or LPS+IFN- γ stimulation. IL-10 secretion significantly increased in LPS+IFN- γ stimulated conditions, when compared to the respective controls. Exposure to GBM per se did not change IL-10 secretion significantly. When cells were incubated with GBM and activated with LPS+IFN- γ IL-10 was reduced in comparison with LPS+IFN- γ alone but increased in comparison to GBM exposure alone. UV irradiated cells showed a similar pattern. The elevated secretion of IL-10 in response to LPS+IFN- γ stimulation has been previously reported and likely corresponds to a feedback loop. [196]

Finally, the impact of GBM exposure in Reactive Oxygen Species (ROS) production was evaluated since the ROS act as signalling molecules as well as inflammation mediators. As expected LPS+IFN- γ stimulation increased ROS. Interestingly presence of rGO significantly decreased detectable ROS, indicating that rGOn might be scavenging the oxygen free radicals. In UV irradiated conditions only the combination of LPS+IFN- γ stimulation in presence of GO led to a significant increase in ROS, when compared to the respective control (Figure 4.12.C).

Taken together, these results suggest that both nanomaterials tend to induce a pro-inflammatory response on macrophages. Also, there appears to be a synergistic activation of an inflammatory mechanism by both GBM, with and without UV irradiation, when in the presence of a pro-inflammatory environment.

4.5. The impact of GBM on DC-induced T cell proliferation

To evaluate the functional consequences of GBM stimulation of antigen presenting Cells (APC), their impact on DC-induced T cell proliferation and cytokine secretion were evaluated.

Dendritic cells were exposed to GBM in naïve or pro-inflammatory conditions, in presence of the pro-inflammatory cytokine TNF- α . Then, CFSE-labelled lymphocytes from a different donor were added to the cultures to obtain a Mixed Lymphocyte Reaction (MLR). CFSE is metabolized by live lymphocytes making them fluorescent, and the dye is divided equally among daughter cells, allowing for cell division to be monitored (Figure 4.13.A). At the end of the experiment, after collection of lymphocytes, large adherent DC and the occasional lymphocyte could still be observed in the wells (Supplementary Results, Figure A)

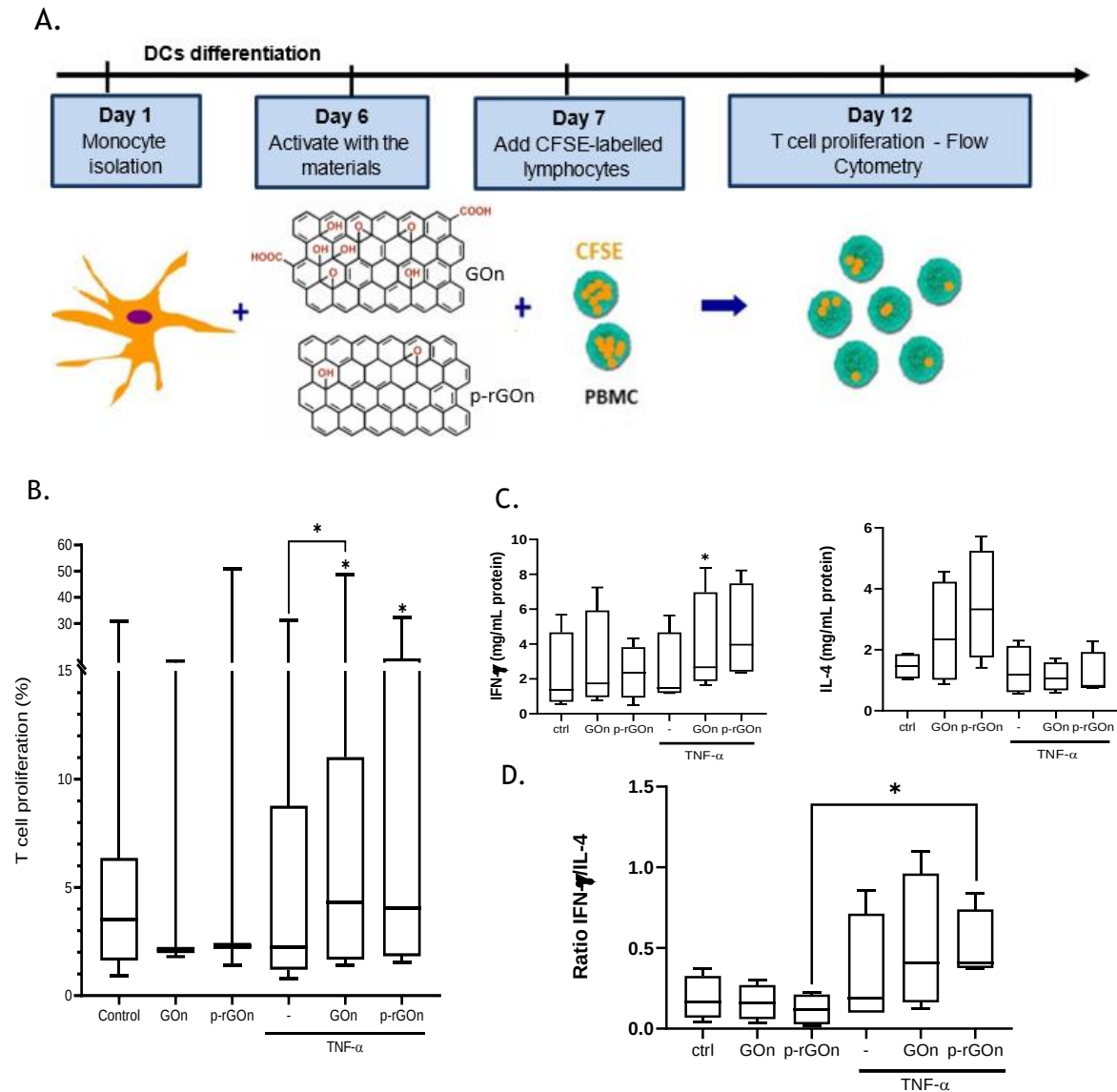


Figure 4.13. A) Schematic representation of a mixed lymphocyte reaction, explaining the T cell polarization induced by GBM-activated DC; B) T cell proliferation in response to DC activation after incubation with the GBM (100 μ g mL⁻¹). To evaluate T cell proliferation, mixed lymphocyte reactions were set up and T cell proliferation evaluated by CFSE halving of CD3+ cells. C) Cytokine production: IFN- γ (left) and IL-4 (right). Cytokine release was determined by ELISA, using cell culture supernatants from 13 day differentiated human primary monocyte-derived macrophages; D) Ratio IFN- γ /IL-4 obtained from cytokine production obtained in C). Data represent arithmetic mean $\pm$ SD, analysed by Friedman test followed by uncorrected Dunn's test (n=4). *p < 0.05; **p < 0.01; ***p<0.001; ****p<0.0001. The asterisks above the box plots represents a comparison with the control.

The MLR results obtained are illustrated in Figure 4.13.B and show that GBM alone do not increase DC ability to promote T cell proliferation. However, when combined with TNF- α exposure, led to significant increases in T cell proliferation, when compared to unstimulated DC. Also, TNF- α -activated DC exposed to GOx led to significantly increased T cell proliferation, comparing to TNF- α -stimulated DC. The supernatants collected from T cells after 5 days of co-culture with TNF- α -activated Dendritic Cells in presence/absence of GBM, were collected and used to evaluate the GBM effect on the polarization of the T cell subsets, by measuring secretion of IFN- γ as a Th1 (pro-

inflammatory) and IL-4 as a Th2 (anti-inflammatory) cytokine (Figure 4.13.C). IFN- γ levels were higher in MLR with TNF- α stimulated DC, and increased further by exposure to GBM, reaching statistical significance for TNF- α combined with GO. Interestingly, although the comparison is not statistically significant it is possible to observe an increase in IL-4 secretion for cell stimulated by GBM-exposed DC, particularly for rGO, which may indicate the tendency of this nanomaterial alone to induce Th2 polarization. The IFN- γ /IL-4 ratio showed a significant increased for MLR with TNF- α -activated DC exposed to rGO, in comparison to those exposed to rGO alone (Figure 4.12.D).

Chapter 5 : Discussion

The main goal of this dissertation work was to develop graphene-based nanomaterials, namely, nanographene oxide (GOn) and partially-reduced nanographene oxide (p-rGOn), with the purpose to study their immunomodulatory effect on macrophages and dendritic cells, as a complement of UV phototherapy for chronic skin diseases and/or cancer.

To accomplish that goal, the first objective was the production and characterization of GOn and p-rGOn. Graphene oxide is commonly obtained from graphite by exfoliation methods, being the most commonly used the modified Hummer's method. [57] This exfoliation procedure leads to the introduction of oxygen-containing functional groups that increase water stability and usually improve biological properties, however, at the expenses of graphene's benzene rings π - π conjugation disruption, which decreases optical absorption, relevant for GBM to act as photothermal therapy agents. However, those properties can be partially restored by reducing GO. Usually obtained rGO becomes unstable in water and sediments, preventing its use for biomedical applications, unless further chemical functionalization is performed, as for example PEGylation. [204, 205] In this work, besides graphite oxide being processed through a high-power sonicator in recirculation to reduce size further than in most studies found in literature, another innovative approach was taken, by only partially reducing GO, developing a material which is both water stable and that possesses increased light absorption (p-rGOn).

Particle size determinations performed by DLS revealed that GOn and p-rGOn were constituted by particles with sizes of $284,2 \pm 38,4$ nm and $218,5 \pm 25,7$ nm for GOn and p-rGOn, respectively. Regarding these results, it must be taken in account the fact that the DLS equipment calculates the size assuming that all the particles are spherical, which it is not true as we can see in the TEM images. However, when the particle size was directly measured from the particles using TEM images, different values (although in the same range were identified) of 206.969 ± 125.793 nm and 276.399 ± 187.314 nm for GOn and for p-rGOn, respectively. This discrepancy can be seen through frequency distribution histograms and explained by the high polydispersity index obtained, since the samples analysed present subpopulations with diverse sizes. Finally, the zeta potential values demonstrated that the materials are water stable, which is also observable visually until 4 months

of storage for both materials. [200, 201] it is also relevant to mention that materials were stored at very high concentrations for GBM, of 8.9 mg mL^{-1} for GOn and of 0.8 mg mL^{-1} for p-rGOn, and still presented an outstanding water stability, that places them as suitable active components for formulations to be used in medical applications, such as phototherapy in the context of the present study. Silva, *et al.* demonstrated that GOn has the ability to penetrate through human skin and has the potential to be used for skin photothermal therapy. [206, 207]

The successful oxidation of GOn and its photo-reduction to produce p-rGOn were assessed by performing TGA, FTIR, and UV-Visible Spectroscopy. The decrease of the intensity of the bands assigned to oxygen-containing functional groups confirms the successful UV-reduction of GOn to achieve p-rGOn. A similar result was reported in the literature by Alam *et al.*, which have found lower intensity peaks, attributed to oxygen functional groups, in rGO comparing to GO. [208] Furthermore, p-rGOn also shown to have higher thermal stability, as observed in the TGA analysis. The previous authors also report similar results, attributing it to the lower defect density of rGOn comparing to GOn. [208] Comparisons were made with rGOn as the material reported in the context of this dissertation, p-rGOn, has no precedent, being rGOn the most similar one found in the literature.

UV-Visible spectroscopy analysis revealed that both GBM present their maximum absorption wavelength at 230 nm, however, p-rGOn lacks a shoulder peak at 300 nm, which is present in p-rGOn spectrum, attributed to $n-\pi^*$ transitions of free electron pairs in oxygen atoms in $C=O$ bonds from carbonyl and carboxyl groups, which further confirms that an effective partial reduction took place. [203] Similar results were observed by Lai *et al.* for different types of GOn, being the peaks at 230 and 300 nm associated to $n-\pi^*$ and $\pi-\pi^*$ plasmon peaks. [209] Ding *et al.* also reported the disappearance of the shoulder peak at $\sim 300 \text{ nm}$ with increase of the time of UV exposure in GO photo-reduction, which is in agreement with results obtained for p-rGOn. [210] Also, p-rGOn presented approximately a 2-fold increase in comparison to GOn in UV absorbance, at the wavelength (270 nm) used for phototherapy in the present study. [211] Therefore, in sum, all desired physicochemical features were achieved in developed GBM, namely small nanometric size, water stability, and optical absorption in the region of interest.

Regarding GBM application in future immunotherapeutic approaches, first their biocompatibility and impact on immune cells were evaluated. Using LDH quantification at 24h and live/dead assay at 72h of exposure it was determined that both GOn and rGOn at $250 \text{ } \mu\text{g mL}^{-1}$ presented cytotoxicity for macrophages, which was not consistently verified for lower concentrations. The live/dead assay confirmed a decrease in viability to around 20 % and 50%, in the presence of $250 \text{ } \mu\text{g mL}^{-1}$ of GOn and rGOn, respectively. Li *et al.* and Wang *et al.* also demonstrated no toxicity for $100 \text{ } \mu\text{g mL}^{-1}$ concentrations in macrophages. [212, 213] Of note, by analysing in number of cells it was possible to observe a significant decrease ($\sim 50 \%$) of the total number of cells comparing to the control without materials. This leads us to the conclusion that 50 % of cells detach and are lost in the washes performed before the analysis, probably due to cell death. The lower toxicity observed in LDH, versus live/dead assay is likely related to the different

exposure times analysed, as LDH was measured 24h after biomaterial exposure and the live/dead assay was performed at the maximum 72h of exposure.

To understand the effect of the GBM on the macrophages cellular structure, fluorescent microscopy images and TEM were performed. Although the nanomaterials were not clearly visible, it was possible to see membrane disintegration and the loss of organelles, particularly close to the plasma membrane, which was not verified in the controls without nanomaterials exposure. Macrophages are specialised in phagocytosis, and based on the literature it was expected that the GBM were captured by them. [55, 214] This could implicate that the cells while in the process of up taking the GBM, were damaged, leading to the disruption of the membrane, as well as some organelles. This membrane damaging was already verified with similar materials in bacterial cell membrane by Lim *et al.* [215] and by Sengupta *et al.* [216]. The TEM images also, showed the existence of damaged mitochondrion, being possible to observe the material inside of a fractured one. Pinto *et al.* reported that graphene and GO, provoked mitochondrial dysfunction, leading to the increase of ROS. [37] Also, based on the literature analysis, this could mean that the sharpened edges of the nanomaterials are damaging the mitochondria, which is reported to affect the metabolic activity and the intracellular signalling, altering macrophages immune response. [217] Alsaedi *et al.* found that graphene nanoparticles induced mitochondria damage, by the constant disruption of their membrane potential, through the induction of pro-apoptotic proteins or through the formation of pores. [218]

Therefore, it was attempted to determine the ability of GBM to modulate macrophage polarisation by cell surface expression of specific phenotypic markers for human M1 (CD86, HLA-DR) and M2 (CD163) macrophages, in combination with the lineage marker CD14. The decrease in the number of cells in the presence of both GBM, and their changes in size and granularity did not allow for a conclusive interpretation of the results obtained. To overcome these difficulties, gene expression analysis was performed, and a tendency was uncovered for both GBM, activated with LPS+IFN- γ to induce the expression of IL-6 (pro-inflammatory profile), when compared to control. Ma *et al.* studied the effect of Graphene Oxide sheets dimensions on macrophages polarization, using macrophage cell lines and *in vivo* models. They found that this material tends to induce M1 polarization, although higher size particles (750 to 1300 nm) caused higher M1 macrophage induction than smaller particles (50 to 350 nm). On the other side, Feito *et al.* found that that PEG-Graphene Oxide nanosheets decrease the number of M1 macrophages, in basal conditions, in comparison to macrophages control. [219, 220] Zhou *et al.* also demonstrated that macrophage exposure to graphene triggered significant transcription of pro-inflammatory factors in macrophages, namely genes related to the secretion of IL-6 and TNF- α . [221] Their results also showed non-significant variations in TGF- β levels.

In agreement with the evaluation of the macrophage's polarisation and gene expression, the cytokine secretion analysis, demonstrated a general induction of a pro-inflammatory profile, since the GBM both with and without LPS+IFN- γ activation and UV irradiation, led to an increase of IL-1 β production. Also, Mukherjee *et al.* found that macrophages primed with LPS+IFN- γ , and exposed to Graphene Oxide with 2 different dimensions (small: 50-300 nm; large: 10-40 μ m) expressed high

levels of IL-1 β , comparing to LPS+IFN- γ -activated cells. [143] Regarding the production of IL-10, it was observed a significative increase in the secretion of IL-10, in the cells exposed to the LPS+IFN- γ -activated GBM and UV irradiation and in comparison to GBM alone. These results may indicate that the UV irradiation combined with the LPS+IFN- γ activation may be inducing some anti-inflammatory synergistic activation on macrophages, although it must be considered a probable the feedback loop induced in the presence of LPS+IFN- γ , that was also reported by Bessa-Gonçalves *et al.*, that results in an increase of IL-10 production. [196] [196] [194] [194] Interestingly, this anti-inflammatory activation (production of IL-10) on macrophages induced by UV irradiation alone was also verified by Toichi *et al.* [222] and in keratinocytes by Shreedhar *et al.* [223], although it is not yet reported in the literature their effect combined with GBM.

Next, given the important of ROS to the inflammatory environment, and the potential for GBM to act as ROS scavengers, the effect of GBM with and without LPS+IFN- γ activation and UV irradiation on extracellular ROS levels was evaluated. The results showed increased ROS in the supernatant of M1 activated macrophages, as expected, upon irradiation GOn contributed to this increased ROS levels. The increase in released ROS with exposure to GOn is reported in the literature by several authors. [120, 224, 225] Interestingly, LPS+IFN- γ -activated macrophages, exposed to rGOn showed decreased ROS levels in the supernatants, in comparison to LPS+IFN- γ stimulation alone. Although rGOn was presented in the previous results as a strong pro-inflammatory activator, this outcome may reveal the capability of rGOn to scavenge the oxygen free radicals and the possibility of using it as a potentiator of an anti-inflammatory activation. This scavenging capacity of reduced GO, as well as its redox mediating potential, was previously reported by comparing the effect of GO and rGO effect on the activity/stability of horseradish peroxidase by Zhang *et al.* [226]

To evaluate the functional consequences of GBM exposure for immune responses, mixed lymphocyte reaction was set up between DC from one donor and labelled lymphocytes from a different donor. DC exposure to GBM led to increased T cell proliferation, in comparison to controls. According to Foged *et al.* Graphene Oxide with nanometric sizes is effectively taken up by DC. [227] Being its principal function the activation of antigen-specific T cells, and taking into account the reports from Russier *et al.* and Zhi *et al.*, it is possible to presume that GOn may activate an immune response from DC, consequently, inducing T cell proliferation and Th1 polarization. [55, 228] Which is also in agreement with the increased INF- γ levels observed in the current work. The results obtained suggest an increased Th1/Th2 ratio, upon co-culture with TNF- α -activated rGOn-exposed DC, suggesting that this GBM has a propensity to potentiate the inflammatory reaction. Shurin *et al.* tested Graphene Oxide in OVA-induced asthma and found that it diminished Th2 immune response. [229] Interestingly, although it was not statistically significative, a slight increase of Th2 cytokine IL-4 was observed in the supernatants of co-cultures between lymphocytes and DC exposed only to rGOn, likely reflecting their predisposition to induce Th2 polarization. There are no reports regarding reduced Graphene Oxide induction of Th1 polarisation, namely through variations in the IL-4/IFN- γ ratio, in the literature.

Taken together, the results obtained so far support the induction of a pro-inflammatory activation by GBM. However, it is important to highlight that these should be performed with more donors, in order to strengthen the results.

Chapter 6 : Conclusions

Immunotherapy combined with phototherapy (UV irradiation) using GOn/p-rGOn for the treatment of chronic skin diseases and/or cancer was the focus of the dissertation work. Regarding the state-of-the-art, it is possible to conclude that CNM, due to their unique structure and properties, and possible modifications revealed to be valuable tools in cancer treatment, immunostimulation and immunosuppression of some inflammatory or autoimmune diseases. However, like all the innovations, all the risks and possible concerns must be cleared to accomplish an effective and favourable result, without side effects.

The first results comprised the production and characterization of GOn and p-rGOn, which were successfully obtained with the desired size range that assures biocompatibility and tissue penetration (between 200 and 300 nm), being stable in water at least 4 months after the production day. GOn and p-rGOn displayed the typical oxygen-containing functional groups, being their quantity reduced in p-rGOn in comparison to GOn, confirming both a successful oxidization and a partial photo-reduction as desired. It was also concluded, that both GBM can absorb in the region of interest for phototherapy (270 nm), furthermore, photo-absorption has been increased by 2-fold for p-rGOn, without affecting its water stability, essential for biomedical applications. Also, the $100 \mu\text{g mL}^{-1}$ concentration of either GOn or rGOn is non-toxic for macrophages at 24h of exposure, although toxicity increases with exposure time, in presence or absence of irradiation.

The overall results obtained here indicate a propension of both GBM to induce a pro-inflammatory response, which might be more interesting for the treatment of cancer and infectious diseases, than for the treatment of chronic skin diseases, as was our initial objective. Nonetheless, in some specific conditions it was also demonstrated the possibility of rGOn to be used as a scavenger of ROS and decrease the IFN- γ /IL-4 ratio, thus favouring an anti-inflammatory response.

In order to achieve more detailed and specified response, the future work should include assays with more donors and intermediate GBM concentrations, to achieve a biocompatible and immunomodulatory effect of GBM and irradiation. Also, a complete cytokine profile and gene

expression characterization needs to be performed. Additionally, a more profound study of irradiation must be done, with more times points, different intensities, and other conditions of irradiations, as well as the combination with specific drugs, for the desired effect. Finally, future evaluation of GBM combination with the irradiation *in vivo* could be important to understand their capacity to promote or inhibit inflammation.

Regarding future outcomes of the present work, a review article has been written and will be submitted soon. Additionally, a scientific paper including the results obtained in this dissertation work will be prepared for submission to a scientific journal.

Appendix A

Supplementary Results

Table A. List of primers used for quantitative RT-PCR analysis.

Gene		Origin	Primer Sequence
TGF- β	Transforming growth factor beta	human	Fw: CGGACTACTACGCCAAAGAA Rv: CCCGAATGTCTGACGTATTG
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase	human	Fw: CCTCAAGATCATCAGCAAT Rv: CCATCCACAGTCTTCTGGGT
IL-6	Interleukin-6	human	Fw: AGCCAGAGTCATTGAGAGCA Rv: AGTTGGATGGTCTTGGTCCT

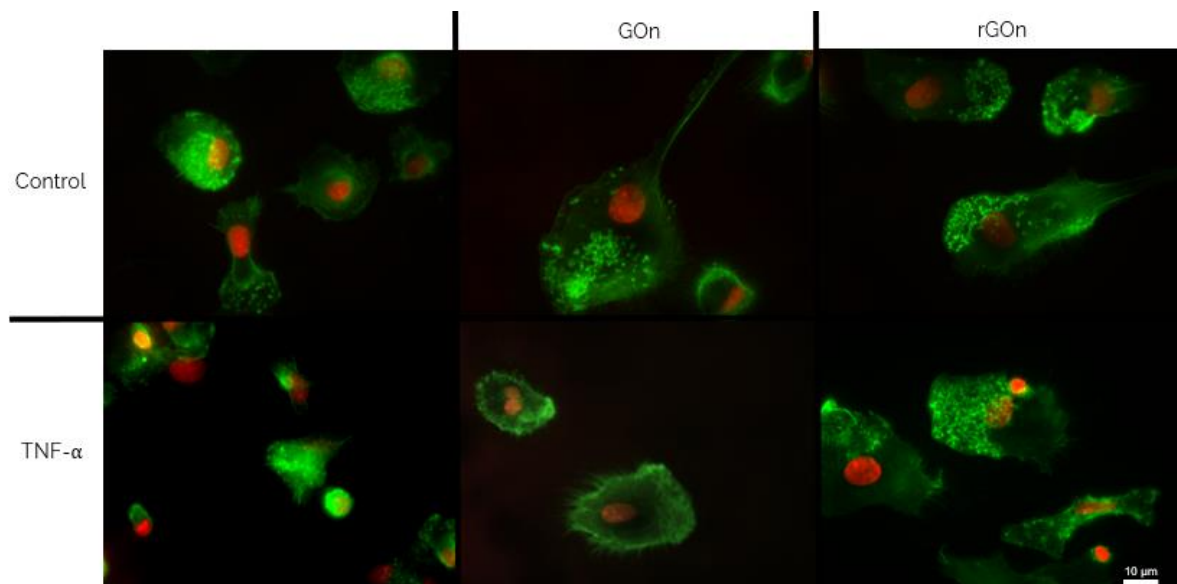


Figure A. Fluorescence microscopy images of macrophages cultured in the presence of GOn and p-rGOn at $100 \mu\text{g mL}^{-1}$ and TNF- α at 100 ng mL^{-1} . Control cells were incubated in the absence of GBM. Green staining: Phalloidin; Red Staining: DAPI. Scale bar represents $10 \mu\text{m}$.

Appendix B

List of Presentations

- S. Azevedo, L. Timochenco, F. Silva, S. I. Amaral, M. Bessa-Gonçalves, K. Chattahy, J. R. Fernandes, F. D. Magalhães, R. Costa-Almeida, S. G. Santos, A. M. Pinto, “Immunomodulatory effects of nanographene oxide and reduced nanographene oxide in combination with phototherapy”, IJUP 2021.
- S. I. Amaral, F. Silva, L. Timochenco, S. Azevedo, J. R. Fernandes, B. Sarmento, F. D. Magalhães, I. C. Gonçalves, R. Costa-Almeida, A. M. Pinto, “Reduced nanographene oxide hydrogels for combined chemotherapy and photothermal therapy of skin cancer”, IJUP, 2021.

Appendix C

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References

- [1] R. J. Hay, N. E. Johns, H. C. Williams, I. W. Bolliger, R. P. Dellavalle, D. J. Margolis, R. Marks, L. Naldi, M. A. Weinstock, and S. K. J. J. o. I. D. Wulf, "The global burden of skin disease in 2010: an analysis of the prevalence and impact of skin conditions," *Journal of Investigative Dermatology*, vol. 134, no. 6, pp. 1527-1534, 2014.
- [2] H. W. Lim, S. A. Collins, J. S. Resneck Jr, J. L. Bolognia, J. A. Hodge, T. A. Rohrer, M. J. Van Beek, D. J. Margolis, A. J. Sober, and M. A. Weinstock, "The burden of skin disease in the United States," *Journal of the American Academy of Dermatology*, vol. 76, no. 5, pp. 958-972. e2, 2017.
- [3] M. J. Bhosle, A. Kulkarni, S. R. Feldman, R. J. H. Balkrishnan, and q. o. l. outcomes, "Quality of life in patients with psoriasis," *Health and Quality of Life Outcomes*, vol. 4, no. 1, p. 35, 2006.
- [4] Ö. Bilgiç, A. Bilgiç, H. Akiş, F. Eskioğlu, E. J. C. Kiliç, and E. D. C. dermatology, "Depression, anxiety and health - related quality of life in children and adolescents with vitiligo," *Clinical Experimental Dermatology: Clinical dermatology*, vol. 36, no. 4, pp. 360-365, 2011.
- [5] C. J. A. o. N. Lifschitz and Metabolism, "The impact of atopic dermatitis on quality of life," *Annals of Nutrition Metabolism*, vol. 66, no. Suppl. 1, pp. 34-40, 2015.
- [6] Y. Matsumura and H. N. Ananthaswamy, "Toxic effects of ultraviolet radiation on the skin," *Toxicology applied pharmacology*, vol. 195, no. 3, pp. 298-308, 2004.
- [7] U. R. Hengge, T. Ruzicka, R. A. Schwartz, and M. J. Cork, "Adverse effects of topical glucocorticosteroids," *Journal of the American Academy of Dermatology*, vol. 54, no. 1, pp. 1-15, 2006.
- [8] A. Baylet, M. Laclaverie, L. Marchand, S. Bordes, B. Closs-Gonthier, and L. Delpy, "Immunotherapies in cutaneous pathologies: an overview," *Drug Discovery Today*, 2020.
- [9] R. K. Pandey and V. K. Prajapati, "Molecular and immunological toxic effects of nanoparticles," *International journal of biological macromolecules*, vol. 107, pp. 1278-1293, 2018.

- [10] P. C. Henriques, I. Borges, A. M. Pinto, F. D. Magalhães, and I. C. Gonçalves, "Fabrication and antimicrobial performance of surfaces integrating graphene-based materials," *Carbon*, vol. 132, pp. 709-732, 2018.
- [11] D. C. Marcano, D. V. Kosynkin, J. M. Berlin, A. Sinitskii, Z. Sun, A. Slesarev, L. B. Alemany, W. Lu, and J. M. Tour, "Improved synthesis of graphene oxide," *ACS nano*, vol. 4, no. 8, pp. 4806-4814, 2010.
- [12] A. Battigelli, C. Ménard-Moyon, and A. Bianco, "Carbon nanomaterials as new tools for immunotherapeutic applications," *Journal of Materials Chemistry B*, vol. 2, no. 37, pp. 6144-6156, 2014.
- [13] V. C. Sanchez, A. Jachak, R. H. Hurt, and A. B. Kane, "Biological interactions of graphene-family nanomaterials: an interdisciplinary review," *Chemical research in toxicology*, vol. 25, no. 1, pp. 15-34, 2012.
- [14] J. T. Robinson, F. K. Perkins, E. S. Snow, Z. Wei, and P. E. Sheehan, "Reduced graphene oxide molecular sensors," *Nano letters*, vol. 8, no. 10, pp. 3137-3140, 2008.
- [15] M. Seredych, B. Haines, V. Sokolova, P. Cheung, F. Meng, L. Stone, L. Mikhalovska, S. Mikhalovsky, V. N. Mochalin, and Y. Gogotsi, "Graphene-based materials for the fast removal of cytokines from blood plasma," *ACS Applied Bio Materials*, vol. 1, no. 2, pp. 436-443, 2018.
- [16] J. Han, Y. S. Kim, M.-Y. Lim, H. Y. Kim, S. Kong, M. Kang, Y. W. Choo, J. H. Jun, S. Ryu, and H.-y. Jeong, "Dual roles of graphene oxide to attenuate inflammation and elicit timely polarization of macrophage phenotypes for cardiac repair," *ACS nano*, vol. 12, no. 2, pp. 1959-1977, 2018.
- [17] A. V. Tkach, N. Yanamala, S. Stanley, M. R. Shurin, G. V. Shurin, E. R. Kisin, A. R. Murray, S. Pareso, T. Khaliullin, and G. P. Kotchey, "Graphene oxide, but not fullerenes, targets immunoproteasomes and suppresses antigen presentation by dendritic cells," *Small*, vol. 9, no. 9 - 10, pp. 1686-1690, 2013.
- [18] Q. Li, F. Li, X. Qi, F. Wei, H. Chen, and T. Wang, "Pluronic® F127 stabilized reduced graphene oxide hydrogel for the treatment of psoriasis: In vitro and in vivo studies," *Colloids Surfaces B: Biointerfaces*, vol. 195, p. 111246, 2020.
- [19] X. Deng, H. Liang, W. Yang, and Z. Shao, "Polarization and function of tumor-associated macrophages mediate graphene oxide-induced photothermal cancer therapy," *Journal of Photochemistry and Photobiology B: Biology*, p. 111913, 2020.
- [20] L. Xu, J. Xiang, Y. Liu, J. Xu, Y. Luo, L. Feng, Z. Liu, and R. Peng, "Functionalized graphene oxide serves as a novel vaccine nano-adjuvant for robust stimulation of cellular immunity," *Nanoscale*, vol. 8, no. 6, pp. 3785-3795, 2016.
- [21] X. Wang, F. Cao, M. Yan, Y. Liu, X. Zhu, H. Sun, and G. Ma, "Alum-functionalized graphene oxide nanocomplexes for effective anticancer vaccination," *Acta biomaterialia*, vol. 83, pp. 390-399, 2019.
- [22] M. Schuster, A. Nechansky, and R. Kircheis, "Cancer immunotherapy," *Biotechnology Journal: Healthcare Nutrition Technology*, vol. 1, no. 2, pp. 138-147, 2006.

- [23] M. Jutel, I. Agache, S. Bonini, A. W. Burks, M. Calderon, W. Canonica, L. Cox, P. Demoly, A. J. Frew, and R. O'Hehir, "International consensus on allergy immunotherapy," *Journal of Allergy Clinical Immunology*, vol. 136, no. 3, pp. 556-568, 2015.
- [24] M. Feldmann and L. Steinman, "Design of effective immunotherapy for human autoimmunity," *Nature*, vol. 435, no. 7042, pp. 612-619, 2005.
- [25] P. d. M. Silva, J. Bier, L. N. Paiatto, C. Galdino Albuquerque, C. Lopes Souza, L. G. R. Fernandes, W. M. d. S. C. Tamashiro, and P. U. Simioni, "Tolerogenic dendritic cells on transplantation: immunotherapy based on second signal blockage," *Journal of immunology research*, vol. 2015, 2015.
- [26] K. Naran, T. Nundalall, S. Chetty, and S. Barth, "Principles of immunotherapy: implications for treatment strategies in cancer and infectious diseases," *Frontiers in microbiology*, vol. 9, p. 3158, 2018.
- [27] J. Kubackova, J. Zbytovska, and O. Holas, "Nanomaterials for direct and indirect immunomodulation: A review of applications," *European Journal of Pharmaceutical Sciences*, vol. 142, p. 105139, 2020.
- [28] I. J. Lebish and R. M. Moraski, "Mechanisms of immunomodulation by drugs," *Toxicologic Pathology*, vol. 15, no. 3, pp. 338-345, 1987.
- [29] M. Rahmati, E. A. Silva, J. E. Reseland, C. A. Heyward, and H. J. Haugen, "Biological responses to physicochemical properties of biomaterial surface," *Chemical Society Reviews*, vol. 49, no. 15, pp. 5178-5224, 2020.
- [30] L. Martínez-Lostao, A. Anel, and J. Pardo, "How do cytotoxic lymphocytes kill cancer cells?," *Clinical Cancer Research*, 2015.
- [31] L. Gu, P. A. Khadaroo, H. Su, L. Kong, L. Chen, X. Wang, X. Li, H. Zhu, X. Zhong, and J. Pan, "The safety and tolerability of combined immune checkpoint inhibitors (anti-PD-1/PD-L1 plus anti-CTLA-4): a systematic review and meta-analysis," *BMC cancer*, vol. 19, no. 1, pp. 1-10, 2019.
- [32] S. Hussain, J. A. Vanoirbeek, and P. H. Hoet, "Interactions of nanomaterials with the immune system," *Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology*, vol. 4, no. 2, pp. 169-183, 2012.
- [33] K. Tsuge, T. Inazumi, A. Shimamoto, and Y. Sugimoto, "Molecular mechanisms underlying prostaglandin E2-exacerbated inflammation and immune diseases," *International immunology*, vol. 31, no. 9, pp. 597-606, 2019.
- [34] M. Orecchioni, D. Bedognetti, F. Sgarrella, F. M. Marincola, A. Bianco, and L. G. Delogu, "Impact of carbon nanotubes and graphene on immune cells," *Journal of translational medicine*, vol. 12, no. 1, p. 138, 2014.
- [35] B. S. Zolnik, Á. González-Fernández, N. Sadrieh, and M. A. Dobrovolskaia, "Minireview: nanoparticles and the immune system," *Endocrinology*, vol. 151, no. 2, pp. 458-465, 2010.
- [36] J. M. Silva, M. Videira, R. Gaspar, V. Pr  at, and H. F. Florindo, "Immune system targeting by biodegradable nanoparticles for cancer vaccines," *Journal of controlled release*, vol. 168, no. 2, pp. 179-199, 2013.

- [37] A. M. Pinto, I. C. Goncalves, and F. D. Magalhaes, "Graphene-based materials biocompatibility: a review," *Colloids Surfaces B: Biointerfaces*, vol. 111, pp. 188-202, 2013.
- [38] Q. Jiao, L. Li, Q. Mu, and Q. Zhang, "Immunomodulation of nanoparticles in nanomedicine applications," *BioMed research international*, vol. 2014, 2014.
- [39] J. A. Hubbell, S. N. Thomas, and M. A. Swartz, "Materials engineering for immunomodulation," *Nature*, vol. 462, no. 7272, pp. 449-460, 2009.
- [40] M. Elsabahy and K. L. Wooley, "Cytokines as biomarkers of nanoparticle immunotoxicity," *Chemical Society Reviews*, vol. 42, no. 12, pp. 5552-5576, 2013.
- [41] S. Barillet, E. Fattal, S. Mura, N. Tsapis, M. Pallardy, H. Hillaireau, and S. Kerdine-Römer, "Immunotoxicity of poly (lactic-co-glycolic acid) nanoparticles: influence of surface properties on dendritic cell activation," *Nanotoxicology*, vol. 13, no. 5, pp. 606-622, 2019.
- [42] E. Bracho - Sanchez, C. Q. Xia, M. J. Clare - Salzler, and B. G. Keselowsky, "Micro and nano material carriers for immunomodulation," *American Journal of Transplantation*, vol. 16, no. 12, pp. 3362-3370, 2016.
- [43] B. Fadeel, C. Bussy, S. Merino, E. Vázquez, E. Flahaut, F. Mouchet, L. Evariste, L. Gauthier, A. J. Koivisto, and U. Vogel, "Safety assessment of graphene-based materials: focus on human health and the environment," *ACS nano*, vol. 12, no. 11, pp. 10582-10620, 2018.
- [44] E. Litasova, V. Iljin, A. Sokolov, V. Vasilyev, M. Dumpis, and L. Piotrovskiy, "The biodegradation of fullerene C 60 by myeloperoxidase," in *Doklady Biochemistry and Biophysics*, 2016, vol. 471, no. 1, pp. 417-420: Springer.
- [45] Y. Peng and H. Liu, "Effects of oxidation by hydrogen peroxide on the structures of multiwalled carbon nanotubes," *Industrial Engineering Chemistry Research*, vol. 45, no. 19, pp. 6483-6488, 2006.
- [46] B. P. Jiang, B. Zhou, Z. Lin, H. Liang, and X. C. Shen, "Recent advances in carbon nanomaterials for cancer phototherapy," *Chemistry-A European Journal*, vol. 25, no. 16, pp. 3993-4004, 2019.
- [47] A. Bianco, K. Kostarelos, and M. Prato, "Opportunities and challenges of carbon-based nanomaterials for cancer therapy," *Expert opinion on drug delivery*, vol. 5, no. 3, pp. 331-342, 2008.
- [48] H. Qian, B. Liu, and X. Jiang, "Application of nanomaterials in cancer immunotherapy," *Materials today chemistry*, vol. 7, pp. 53-64, 2018.
- [49] N. J. Coville, S. D. Mhlanga, E. N. Nxumalo, and A. Shaikjee, "A review of shaped carbon nanomaterials," *South African Journal of Science*, vol. 107, no. 3-4, pp. 01-15, 2011.
- [50] W. R. Wagner, S. E. Sakiyama-Elbert, G. Zhang, and M. J. Yaszemski, A. Press, Ed. *Biomaterials Science: An Introduction to Materials in Medicine*, 4 ed. Elsevier Science, 2020.
- [51] X. Huang, X. Qi, F. Boey, and H. Zhang, "Graphene-based composites," *Chemical Society Reviews*, vol. 41, no. 2, pp. 666-686, 2012.

- [52] M. Orecchioni, R. Cabizza, A. Bianco, and L. G. Delogu, "Graphene as cancer theranostic tool: progress and future challenges," *Theranostics*, vol. 5, no. 7, p. 710, 2015.
- [53] Y. Zhu, S. Murali, W. Cai, X. Li, J. W. Suk, J. R. Potts, and R. S. Ruoff, "Graphene and graphene oxide: synthesis, properties, and applications," *Advanced materials*, vol. 22, no. 35, pp. 3906-3924, 2010.
- [54] D. Li and R. B. Kaner, "Graphene-based materials," *Science*, vol. 320, no. 5880, pp. 1170-1171, 2008.
- [55] J. Russier, E. Treossi, A. Scarsi, F. Perrozzi, H. Dumortier, L. Ottaviano, M. Meneghetti, V. Palermo, and A. Bianco, "Evidencing the mask effect of graphene oxide: a comparative study on primary human and murine phagocytic cells," *Nanoscale*, vol. 5, no. 22, pp. 11234-11247, 2013.
- [56] S. K. Rastogi, G. Raghavan, G. Yang, and T. Cohen-Karni, "Effect of graphene on nonneuronal and neuronal cell viability and stress," *Nano Letters*, vol. 17, no. 5, pp. 3297-3301, 2017.
- [57] A. M. Pinto, C. Gonçalves, D. M. Sousa, A. R. Ferreira, J. A. Moreira, I. C. Gonçalves, and F. D. Magalhaes, "Smaller particle size and higher oxidation improves biocompatibility of graphene-based materials," *Carbon*, vol. 99, pp. 318-329, 2016.
- [58] A. M. Pinto, I. C. Goncalves, and F. D. Magalhaes, "Graphene-based materials biocompatibility: a review," *Colloids Surfaces B: Biointerfaces*, vol. 111, pp. 188-202, 2013.
- [59] C. Wu, X. Guan, J. Xu, Y. Zhang, Q. Liu, Y. Tian, S. Li, X. Qin, H. Yang, and Y. Liu, "Highly efficient cascading synergy of cancer photo-immunotherapy enabled by engineered graphene quantum dots/photosensitizer/CpG oligonucleotides hybrid nanotheranostics," *Biomaterials*, vol. 205, pp. 106-119, 2019.
- [60] K. L. Schroeder, R. V. Goreham, and T. Nann, "Graphene quantum dots for theranostics and bioimaging," *Pharmaceutical research*, vol. 33, no. 10, pp. 2337-2357, 2016.
- [61] D. Iannazzo, A. Pistone, M. Salamò, S. Galvagno, R. Romeo, S. V. Giofrè, C. Branca, G. Visalli, and A. Di Pietro, "Graphene quantum dots for cancer targeted drug delivery," *International journal of pharmaceuticals*, vol. 518, no. 1-2, pp. 185-192, 2017.
- [62] W. S. Yeap, S. Chen, and K. P. Loh, "Detonation nanodiamond: an organic platform for the suzuki coupling of organic molecules," *Langmuir*, vol. 25, no. 1, pp. 185-191, 2009.
- [63] E. Perevedentseva, Y.-C. Lin, M. Jani, and C.-L. Cheng, "Biomedical applications of nanodiamonds in imaging and therapy," *Nanomedicine*, vol. 8, no. 12, pp. 2041-2060, 2013.
- [64] Y. Xing, W. Xiong, L. Zhu, E. Osawa, S. Hussin, and L. Dai, "DNA damage in embryonic stem cells caused by nanodiamonds," *ACS nano*, vol. 5, no. 3, pp. 2376-2384, 2011.
- [65] Y. Yuan, Y. Chen, J.-H. Liu, H. Wang, and Y. Liu, "Biodistribution and fate of nanodiamonds in vivo," *Diamond and related materials*, vol. 18, no. 1, pp. 95-100, 2009.
- [66] Y.-R. Chang, H.-Y. Lee, K. Chen, C.-C. Chang, D.-S. Tsai, C.-C. Fu, T.-S. Lim, Y.-K. Tzeng, C.-Y. Fang, and C.-C. Han, "Mass production and dynamic imaging of fluorescent nanodiamonds," *Nature nanotechnology*, vol. 3, no. 5, pp. 284-288, 2008.

- [67] C.-C. Fu, H.-Y. Lee, K. Chen, T.-S. Lim, H.-Y. Wu, P.-K. Lin, P.-K. Wei, P.-H. Tsao, H.-C. Chang, and W. Fann, "Characterization and application of single fluorescent nanodiamonds as cellular biomarkers," *Proceedings of the National Academy of Sciences*, vol. 104, no. 3, pp. 727-732, 2007.
- [68] D. M. Smith, J. K. Simon, and J. R. Baker Jr, "Applications of nanotechnology for immunology," *Nature Reviews Immunology*, vol. 13, no. 8, pp. 592-605, 2013.
- [69] N. Shershakova, E. Baraboshkina, S. Andreev, D. Purgina, I. Struchkova, O. Kamysnikov, A. Nikonova, and M. Khaitov, "Anti-inflammatory effect of fullerene C 60 in a mice model of atopic dermatitis," *Journal of nanobiotechnology*, vol. 14, no. 1, p. 8, 2016.
- [70] A. W. Jensen, S. R. Wilson, and D. I. Schuster, "Biological applications of fullerenes," *Bioorganic medicinal chemistry*, vol. 4, no. 6, pp. 767-779, 1996.
- [71] M. Sola, J. Mestres, and M. Duran, "Molecular size and pyramidalization: two keys for understanding the reactivity of fullerenes," *The Journal of Physical Chemistry*, vol. 99, no. 27, pp. 10752-10758, 1995.
- [72] P. Anilkumar, F. Lu, L. Cao, P. G Luo, J.-H. Liu, S. Sahu, K. N Tackett II, Y. Wang, and Y.-P. Sun, "Fullerenes for applications in biology and medicine," *Current Medicinal Chemistry*, vol. 18, no. 14, pp. 2045-2059, 2011.
- [73] Z. Chen, L. Ma, Y. Liu, and C. Chen, "Applications of functionalized fullerenes in tumor theranostics," *Theranostics*, vol. 2, no. 3, p. 238, 2012.
- [74] R. Bakry, R. M. Vallant, M. Najam-ul-Haq, M. Rainer, Z. Szabo, C. W. Huck, and G. K. Bonn, "Medicinal applications of fullerenes," *International journal of nanomedicine*, vol. 2, no. 4, p. 639, 2007.
- [75] J. Ferlay, M. Colombet, I. Soerjomataram, C. Mathers, D. Parkin, M. Piñeros, A. Znaor, and F. Bray, "Estimating the global cancer incidence and mortality in 2018: GLOBOCAN sources and methods," *International Journal of Cancer*, vol. 144, no. 8, pp. 1941-1953, 2019.
- [76] R. R. Love, H. Leventhal, D. V. Easterling, and D. R. Nerenz, "Side effects and emotional distress during cancer chemotherapy," *Cancer*, vol. 63, no. 3, pp. 604-612, 1989.
- [77] C. Taylor and A. Kirby, "Cardiac side-effects from breast cancer radiotherapy," *Clinical Oncology*, vol. 27, no. 11, pp. 621-629, 2015.
- [78] S. Farkona, E. P. Diamandis, and I. M. Blasutig, "Cancer immunotherapy: the beginning of the end of cancer?," *BMC medicine*, vol. 14, no. 1, pp. 1-18, 2016.
- [79] R. S. Riley, C. H. June, R. Langer, and M. J. Mitchell, "Delivery technologies for cancer immunotherapy," *Nature reviews Drug discovery*, vol. 18, no. 3, pp. 175-196, 2019.
- [80] K. C. Conlon, M. D. Miljkovic, and T. A. Waldmann, "Cytokines in the treatment of cancer," *Journal of Interferon & Cytokine Research*, vol. 39, no. 1, pp. 6-21, 2019.
- [81] B. Simon and U. Uslu, "CAR - T cell therapy in melanoma: A future success story?," *Experimental dermatology*, vol. 27, no. 12, pp. 1315-1321, 2018.

- [82] Q. Chen, Q. Hu, E. Dukhovlinova, G. Chen, S. Ahn, C. Wang, E. A. Ogunnaike, F. S. Ligler, G. Dotti, and Z. Gu, "Photothermal therapy promotes tumor infiltration and antitumor activity of CAR T cells," *Advanced materials*, vol. 31, no. 23, p. 1900192, 2019.
- [83] S. Tan, D. Li, and X. Zhu, "Cancer immunotherapy: Pros, cons and beyond," *Biomedicine Pharmacotherapy*, vol. 124, p. 109821, 2020.
- [84] E. Ames and W. J. Murphy, "Advantages and clinical applications of natural killer cells in cancer immunotherapy," *Cancer Immunology, Immunotherapy*, vol. 63, no. 1, pp. 21-28, 2014.
- [85] H. A. Hassan, L. Smyth, J. T.-W. Wang, P. M. Costa, K. Ratnasothy, S. S. Diebold, G. Lombardi, and K. T. Al-Jamal, "Dual stimulation of antigen presenting cells using carbon nanotube-based vaccine delivery system for cancer immunotherapy," *Biomaterials*, vol. 104, pp. 310-322, 2016.
- [86] T. Wang, D. Wang, H. Yu, B. Feng, F. Zhou, H. Zhang, L. Zhou, S. Jiao, and Y. Li, "A cancer vaccine-mediated postoperative immunotherapy for recurrent and metastatic tumors," *Nature communications*, vol. 9, no. 1, pp. 1-12, 2018.
- [87] L. Cassetta and T. Kitamura, "Macrophage targeting: opening new possibilities for cancer immunotherapy," *Immunology*, vol. 155, no. 3, pp. 285-293, 2018.
- [88] F. L. Komdeur, A. Singh, S. van de Wall, J. J. Meulenberg, A. Boerma, B. N. Hoogeboom, S. T. Paijens, C. Oyarce, M. de Bruyn, and E. Schuurin, "First-in-Human Phase I Clinical Trial of an SFV-Based RNA Replicon Cancer Vaccine against HPV-Induced Cancers," *Molecular Therapy*, vol. 29, no. 2, pp. 611-625, 2021.
- [89] K. Kim, H. S. Kim, J. Y. Kim, H. Jung, J.-M. Sun, J. S. Ahn, M.-J. Ahn, K. Park, S.-H. Lee, and J. K. Choi, "Predicting clinical benefit of immunotherapy by antigenic or functional mutations affecting tumour immunogenicity," *Nature communications*, vol. 11, no. 1, pp. 1-11, 2020.
- [90] R. Zhang, M. M. Billingsley, and M. J. Mitchell, "Biomaterials for vaccine-based cancer immunotherapy," *Journal of Controlled Release*, vol. 292, pp. 256-276, 2018.
- [91] R. Costa-Almeida, D. Bogas, J. R. Fernandes, L. Timochenko, F. A. Silva, J. Meneses, I. C. Gonçalves, F. D. Magalhães, and A. M. Pinto, "Near-Infrared Radiation-Based Mild Photohyperthermia Therapy of Non-Melanoma Skin Cancer with PEGylated Reduced Nanographene Oxide," *Polymers*, vol. 12, no. 8, p. 1840, 2020.
- [92] N. Shao, S. Lu, E. Wickstrom, and B. Panchapakesan, "Integrated molecular targeting of IGF1R and HER2 surface receptors and destruction of breast cancer cells using single wall carbon nanotubes," *Nanotechnology*, vol. 18, no. 31, p. 315101, 2007.
- [93] C. Guilbaud-Chéreau, B. Dinesh, R. Schurhammer, D. Collin, A. Bianco, and C. c. Ménard-Moyon, "Protected Amino Acid-Based Hydrogels Incorporating Carbon Nanomaterials for Near-Infrared Irradiation-Triggered Drug Release," *ACS applied materials interfaces*, vol. 11, no. 14, pp. 13147-13157, 2019.

- [94] X. Dong, C. Wei, J. Liang, T. Liu, D. Kong, and F. Lv, "Thermosensitive hydrogel loaded with chitosan-carbon nanotubes for near infrared light triggered drug delivery," *Journal of Industrial and Engineering Chemistry*, vol. 154, pp. 253-262, 2017.
- [95] J. T. Robinson, K. Welscher, S. M. Tabakman, S. P. Sherlock, H. Wang, R. Luong, and H. Dai, "High performance in vivo near-IR ($> 1 \mu\text{m}$) imaging and photothermal cancer therapy with carbon nanotubes," *Nano research*, vol. 3, no. 11, pp. 779-793, 2010.
- [96] E. B. Kang, I. In, K.-D. Lee, and S. Y. Park, "Mitochondria-targeted fluorescent carbon nano-platform for NIR-triggered hyperthermia and mitochondrial inhibition," *Journal of Industrial Engineering Chemistry*, vol. 55, pp. 224-233, 2017.
- [97] I. A. Zárate, H. Aguilar-Bolados, M. Yazdani-Pedram, G. d. C. Pizarro, and A. Neira-Carrillo, "In Vitro Hyperthermia Evaluation of Electrospun Polymer Composite Fibers Loaded with Reduced Graphene Oxide," *Polymers*, vol. 12, no. 11, p. 2663, 2020.
- [98] A. D. Manasrah, T. Laoui, S. J. Zaidi, and M. A. Atieh, "Effect of PEG functionalized carbon nanotubes on the enhancement of thermal and physical properties of nanofluids," *Experimental Thermal Fluid Science*, vol. 84, pp. 231-241, 2017.
- [99] W. Song, S. N. Musetti, and L. Huang, "Nanomaterials for cancer immunotherapy," *Biomaterials*, vol. 148, pp. 16-30, 2017.
- [100] L. Sanchez, Y. Yi, and Y. Yu, "Effect of partial PEGylation on particle uptake by macrophages," *Nanoscale*, vol. 9, no. 1, pp. 288-297, 2017.
- [101] K. H. Bae, H. J. Chung, and T. G. Park, "Nanomaterials for cancer therapy and imaging," *Molecules and cells*, vol. 31, no. 4, pp. 295-302, 2011.
- [102] B. Hosnedlova, M. Kepinska, C. Fernandez, Q. Peng, B. Ruttkay - Nedecky, H. Milnerowicz, and R. Kizek, "Carbon nanomaterials for targeted cancer therapy drugs: A critical review," *The Chemical Record*, vol. 19, no. 2-3, pp. 502-522, 2019.
- [103] J. Pardo, Z. Peng, and R. M. Leblanc, "Cancer targeting and drug delivery using carbon-based quantum dots and nanotubes," *Molecules*, vol. 23, no. 2, p. 378, 2018.
- [104] I. Srivastava, S. K. Misra, F. Ostadhossein, E. Daza, J. Singh, and D. Pan, "Surface chemistry of carbon nanoparticles functionally select their uptake in various stages of cancer cells," *Nano Research*, vol. 10, no. 10, pp. 3269-3284, 2017.
- [105] M. E. Aikins, C. Xu, and J. J. Moon, "Engineered Nanoparticles for Cancer Vaccination and Immunotherapy," *Accounts of chemical research*, vol. 53, no. 10, pp. 2094-2105, 2020.
- [106] Y. Liu, Y. Xu, Y. Tian, C. Chen, C. Wang, and X. Jiang, "Functional nanomaterials can optimize the efficacy of vaccines," *Small*, vol. 10, no. 22, pp. 4505-4520, 2014.
- [107] Y. Xiao, X. Gao, O. Taratula, S. Treado, A. Urbas, R. D. Holbrook, R. E. Cavicchi, C. T. Avedisian, S. Mitra, and R. Savla, "Anti-HER2 IgY antibody-functionalized single-walled carbon nanotubes for detection and selective destruction of breast cancer cells," *BMC cancer*, vol. 9, no. 1, p. 351, 2009.
- [108] R. Marches, P. Chakravarty, I. H. Musselman, P. Bajaj, R. N. Azad, P. Pantano, R. K. Draper, and E. S. Vitetta, "Specific thermal ablation of tumor cells using single - walled

- carbon nanotubes targeted by covalently - coupled monoclonal antibodies," *International journal of cancer*, vol. 125, no. 12, pp. 2970-2977, 2009.
- [109] P. Chakravarty, R. Marches, N. S. Zimmerman, A. D.-E. Swafford, P. Bajaj, I. H. Musselman, P. Pantano, R. K. Draper, and E. S. Vitetta, "Thermal ablation of tumor cells with antibody-functionalized single-walled carbon nanotubes," *Proceedings of the National Academy of Sciences*, vol. 105, no. 25, pp. 8697-8702, 2008.
- [110] C. H. Villa, T. Dao, I. Ahearn, N. Fehrenbacher, E. Casey, D. A. Rey, T. Korontsvit, V. Zakhaleva, C. A. Batt, and M. R. Philips, "Single-walled carbon nanotubes deliver peptide antigen into dendritic cells and enhance IgG responses to tumor-associated antigens," *ACS nano*, vol. 5, no. 7, pp. 5300-5311, 2011.
- [111] D. Zhao, D. Alizadeh, L. Zhang, W. Liu, O. Farrukh, E. Manuel, D. J. Diamond, and B. Badie, "Carbon nanotubes enhance CpG uptake and potentiate antiglioma immunity," *Clinical Cancer Research*, vol. 17, no. 4, pp. 771-782, 2011.
- [112] T. R. Fadel, E. R. Steenblock, E. Stern, N. Li, X. Wang, G. L. Haller, L. D. Pfefferle, and T. M. Fahmy, "Enhanced cellular activation with single walled carbon nanotube bundles presenting antibody stimuli," *Nano letters*, vol. 8, no. 7, pp. 2070-2076, 2008.
- [113] T. R. Fadel, M. Look, P. A. Staffier, G. L. Haller, L. D. Pfefferle, and T. M. Fahmy, "Clustering of stimuli on single-walled carbon nanotube bundles enhances cellular activation," *Langmuir*, vol. 26, no. 8, pp. 5645-5654, 2010.
- [114] T. R. Fadel, N. Li, S. Shah, M. Look, L. D. Pfefferle, G. L. Haller, S. Justesen, C. J. Wilson, and T. M. Fahmy, "Adsorption of Multimeric T Cell Antigens on Carbon Nanotubes: Effect on Protein Structure and Antigen - Specific T Cell Stimulation," *Small*, vol. 9, no. 5, pp. 666-672, 2013.
- [115] J. Meng, J. Meng, J. Duan, H. Kong, L. Li, C. Wang, S. Xie, S. Chen, N. Gu, and H. Xu, "Carbon nanotubes conjugated to tumor lysate protein enhance the efficacy of an antitumor immunotherapy," *small*, vol. 4, no. 9, pp. 1364-1370, 2008.
- [116] J. Meng, M. Yang, F. Jia, H. Kong, W. Zhang, C. Wang, J. Xing, S. Xie, and H. Xu, "Subcutaneous injection of water-soluble multi-walled carbon nanotubes in tumor-bearing mice boosts the host immune activity," *Nanotechnology*, vol. 21, no. 14, p. 145104, 2010.
- [117] T. Mocan and C. Iancu, "Effective colon cancer prophylaxis in mice using embryonic stem cells and carbon nanotubes," *International journal of nanomedicine*, vol. 6, p. 1945, 2011.
- [118] M. Čolić, T. Džopalić, S. Tomić, J. Rajković, R. Rudolf, G. Vuković, A. Marinković, and P. Uskoković, "Immunomodulatory effects of carbon nanotubes functionalized with a Toll-like receptor 7 agonist on human dendritic cells," *Carbon*, vol. 67, pp. 273-287, 2014.
- [119] H. Yue, W. Wei, Z. Gu, D. Ni, N. Luo, Z. Yang, L. Zhao, J. A. Garate, R. Zhou, and Z. Su, "Exploration of graphene oxide as an intelligent platform for cancer vaccines," *Nanoscale*, vol. 7, no. 47, pp. 19949-19957, 2015.
- [120] S. Gurunathan, M.-H. Kang, M. Jeyaraj, and J.-H. Kim, "Differential immunomodulatory effect of graphene oxide and vanillin-functionalized graphene oxide nanoparticles in

- human acute monocytic leukemia cell line (THP-1)," *International journal of molecular sciences*, vol. 20, no. 2, p. 247, 2019.
- [121] G. Wei, M. Yan, R. Dong, D. Wang, X. Zhou, J. Chen, and J. Hao, "Covalent modification of reduced graphene oxide by means of diazonium chemistry and use as a drug - delivery system," *Chemistry-A European Journal*, vol. 18, no. 46, pp. 14708-14716, 2012.
- [122] S. Tomić, K. Janjetović, D. Mihajlović, M. Milenković, T. Kravić-Stevović, Z. Marković, B. Todorović-Marković, Z. Spitalsky, M. Micusik, and D. Vučević, "Graphene quantum dots suppress proinflammatory T cell responses via autophagy-dependent induction of tolerogenic dendritic cells," *Biomaterials*, vol. 146, pp. 13-28, 2017.
- [123] Y. Qin, Z.-W. Zhou, S.-T. Pan, Z.-X. He, X. Zhang, J.-X. Qiu, W. Duan, T. Yang, and S.-F. Zhou, "Graphene quantum dots induce apoptosis, autophagy, and inflammatory response via p38 mitogen-activated protein kinase and nuclear factor- κ B mediated signaling pathways in activated THP-1 macrophages," *Toxicology*, vol. 327, pp. 62-76, 2015.
- [124] T.-F. Li, K. Li, Q. Zhang, C. Wang, Y. Yue, Z. Chen, S.-J. Yuan, X. Liu, Y. Wen, and M. Han, "Dendritic cell-mediated delivery of doxorubicin-polyglycerol-nanodiamond composites elicits enhanced anti-cancer immune response in glioblastoma," *Biomaterials*, vol. 181, pp. 35-52, 2018.
- [125] L. P. Suarez-Kelly, A. R. Campbell, I. V. Rampersaud, A. Bumb, M. S. Wang, J. P. Butchar, S. Tridandapani, L. Yu, A. A. Rampersaud, and W. E. Carson III, "Fluorescent nanodiamonds engage innate immune effector cells: a potential vehicle for targeted anti-tumor immunotherapy," *Nanomedicine: Nanotechnology, Biology and Medicine*, vol. 13, no. 3, pp. 909-920, 2017.
- [126] J. Zhu, Z. Ji, J. Wang, R. Sun, X. Zhang, Y. Gao, H. Sun, Y. Liu, Z. Wang, and A. Li, "Tumor - inhibitory effect and immunomodulatory activity of fullerol C60 (OH) x ," *Small*, vol. 4, no. 8, pp. 1168-1175, 2008.
- [127] Y. Liu, F. Jiao, Y. Qiu, W. Li, Y. Qu, C. Tian, Y. Li, R. Bai, F. Lao, and Y. Zhao, "Immunostimulatory properties and enhanced TNF- α mediated cellular immunity for tumor therapy by C60 (OH) 20 nanoparticles," *Nanotechnology*, vol. 20, no. 41, p. 415102, 2009.
- [128] Y. Wang, Z. Iqbal, and S. Mitra, "Rapidly functionalized, water-dispersed carbon nanotubes at high concentration," *Journal of the American Chemical Society*, vol. 128, no. 1, pp. 95-99, 2006.
- [129] X. Sui, C. Luo, C. Wang, F. Zhang, J. Zhang, and S. Guo, "Graphene quantum dots enhance anticancer activity of cisplatin via increasing its cellular and nuclear uptake," *Nanomedicine: Nanotechnology, Biology and Medicine*, vol. 12, no. 7, pp. 1997-2006, 2016.
- [130] X. Q. Zhang, R. Lam, X. Xu, E. K. Chow, H. J. Kim, and D. Ho, "Multimodal nanodiamond drug delivery carriers for selective targeting, imaging, and enhanced chemotherapeutic efficacy," *Advanced materials*, vol. 23, no. 41, pp. 4770-4775, 2011.
- [131] P. Saroj, M. Verma, K. Jha, and M. Pal, "An overview on immunomodulation," *J Adv Sci Res*, vol. 3, no. 1, pp. 7-12, 2012.

- [132] X. Feng, W. Xu, Z. Li, W. Song, J. Ding, and X. Chen, "Immunomodulatory nanosystems," *Advanced Science*, vol. 6, no. 17, p. 1900101, 2019.
- [133] J. Saleem, L. Wang, and C. Chen, "Immunological effects of graphene family nanomaterials," *NanoImpact*, vol. 5, pp. 109-118, 2017.
- [134] R. Gottardi and B. Douradinha, "Carbon nanotubes as a novel tool for vaccination against infectious diseases and cancer," *Journal of nanobiotechnology*, vol. 11, no. 1, pp. 1-7, 2013.
- [135] A. A. Shvedova, A. Pietroiusti, B. Fadeel, and V. E. Kagan, "Mechanisms of carbon nanotube-induced toxicity: focus on oxidative stress," *Toxicology applied pharmacology*, vol. 261, no. 2, pp. 121-133, 2012.
- [136] A. M. Flores, N. Hosseini-Nassab, K.-U. Jarr, J. Ye, X. Zhu, R. Wirka, A. L. Koh, P. Tsantilas, Y. Wang, and V. Nanda, "Pro-efferocytic nanoparticles are specifically taken up by lesional macrophages and prevent atherosclerosis," *Nature nanotechnology*, vol. 15, no. 2, pp. 154-161, 2020.
- [137] M. Zeinali, M. Jammalan, S. K. Ardestani, and N. Mosaveri, "Immunological and cytotoxicological characterization of tuberculin purified protein derivative (PPD) conjugated to single-walled carbon nanotubes," *Immunology letters*, vol. 126, no. 1-2, pp. 48-53, 2009.
- [138] T. S. Dutt and R. K. Saxena, "Enhanced antibody response to ovalbumin coupled to poly-dispersed acid functionalized single walled carbon nanotubes," *Immunology Letters*, vol. 217, pp. 77-83, 2020.
- [139] N. Yandar, G. Pastorin, M. Prato, A. Bianco, M. E. Patarroyo, and J. M. Lozano, "Immunological profile of a Plasmodium vivax AMA-1 N-terminus peptide-carbon nanotube conjugate in an infected Plasmodium berghei mouse model," *Vaccine*, vol. 26, no. 46, pp. 5864-5873, 2008.
- [140] C. Crescio, M. Orecchioni, C. Ménard-Moyon, F. Sgarrella, P. Pippia, R. Manetti, A. Bianco, and L. G. Delogu, "Immunomodulatory properties of carbon nanotubes are able to compensate immune function dysregulation caused by microgravity conditions," *Nanoscale*, vol. 6, no. 16, pp. 9599-9603, 2014.
- [141] M. Pescatori, D. Bedognetti, E. Venturelli, C. Ménard-Moyon, C. Bernardini, E. Muresu, A. Piana, G. Maida, R. Manetti, and F. Sgarrella, "Functionalized carbon nanotubes as immunomodulator systems," *Biomaterials*, vol. 34, no. 18, pp. 4395-4403, 2013.
- [142] T. Yan, H. Zhang, D. Huang, S. Feng, M. Fujita, and X.-D. Gao, "Chitosan-functionalized graphene oxide as a potential immunoadjuvant," *Nanomaterials*, vol. 7, no. 3, p. 59, 2017.
- [143] S. P. Mukherjee, K. Kostarelos, and B. Fadeel, "Cytokine Profiling of Primary Human Macrophages Exposed to Endotoxin - Free Graphene Oxide: Size - Independent NLRP3 Inflammasome Activation," *Advanced healthcare materials*, vol. 7, no. 4, p. 1700815, 2018.
- [144] G.-Y. Chen, H.-J. Yang, C.-H. Lu, Y.-C. Chao, S.-M. Hwang, C.-L. Chen, K.-W. Lo, L.-Y. Sung, W.-Y. Luo, and H.-Y. Tuan, "Simultaneous induction of autophagy and toll-like

- receptor signaling pathways by graphene oxide," *Biomaterials*, vol. 33, no. 27, pp. 6559-6569, 2012.
- [145] Y. Xu, J. Zhu, K. Xiang, Y. Li, R. Sun, J. Ma, H. Sun, and Y. Liu, "Synthesis and immunomodulatory activity of [60] fullerene-tuftsinn conjugates," *Biomaterials*, vol. 32, no. 36, pp. 9940-9949, 2011.
- [146] L. Xu, Y. Liu, Z. Chen, W. Li, Y. Liu, L. Wang, L. Ma, Y. Shao, Y. Zhao, and C. Chen, "Morphologically virus-like fullerenol nanoparticles act as the dual-functional nanoadjuvant for HIV-1 vaccine," *Advanced Materials*, vol. 25, no. 41, pp. 5928-5936, 2013.
- [147] H. Eidi, M.-O. David, G. Cr peaux, L. Henry, V. Joshi, M.-H. Berger, M. Sennour, J. Cadusseau, R. K. Gherardi, and P. A. Curmi, "Fluorescent nanodiamonds as a relevant tag for the assessment of alum adjuvant particle biodisposition," *BMC medicine*, vol. 13, no. 1, pp. 1-13, 2015.
- [148] D. Pantarotto, C. D. Partidos, J. Hoebeke, F. Brown, E. Kramer, J.-P. Briand, S. Muller, M. Prato, and A. Bianco, "Immunization with peptide-functionalized carbon nanotubes enhances virus-specific neutralizing antibody responses," *Chemistry & biology*, vol. 10, no. 10, pp. 961-966, 2003.
- [149] G. Laverny, A. Casset, A. Purohit, E. Schaeffer, C. Spiegelhalter, F. de Blay, and F. Pons, "Immunomodulatory properties of multi-walled carbon nanotubes in peripheral blood mononuclear cells from healthy subjects and allergic patients," *Toxicology Letters*, vol. 217, no. 2, pp. 91-101, 2013.
- [150] J. Su, Z. Du, L. Xiao, F. Wei, Y. Yang, M. Li, Y. Qiu, J. Liu, J. Chen, and Y. Xiao, "Graphene oxide coated titanium surfaces with osteoimmunomodulatory role to enhance osteogenesis," *Materials Science and Engineering: C*, p. 110983, 2020.
- [151] S. K. Norton, D. S. Wijesinghe, A. Dellinger, J. Sturgill, Z. Zhou, S. Barbour, C. Chalfant, D. H. Conrad, and C. L. Kepley, "Epoxyeicosatrienoic acids are involved in the C70 fullerene derivative-induced control of allergic asthma," *Journal of Allergy and Clinical Immunology*, vol. 130, no. 3, pp. 761-769. e2, 2012.
- [152] A. Ilinskaya and M. Dobrovolskaia, "Immunosuppressive and anti-inflammatory properties of engineered nanomaterials," in *HANDBOOK OF IMMUNOLOGICAL PROPERTIES OF ENGINEERED NANOMATERIALS: Volume 3: Engineered Nanomaterials and the Immune Cell Function*: World Scientific, 2016, pp. 139-163.
- [153] T. A. Ngobili and M. A. Daniele, "Nanoparticles and direct immunosuppression," *Experimental Biology Medicine*, vol. 241, no. 10, pp. 1064-1073, 2016.
- [154] X. Yuan, X. Zhang, L. Sun, Y. Wei, and X. Wei, "Cellular toxicity and immunological effects of carbon-based nanomaterials," *Particle fibre toxicology*, vol. 16, no. 1, p. 18, 2019.
- [155] L. A. Mitchell, J. Gao, R. V. Wal, A. Gigliotti, S. W. Burchiel, and J. D. McDonald, "Pulmonary and systemic immune response to inhaled multiwalled carbon nanotubes," *Toxicological sciences*, vol. 100, no. 1, pp. 203-214, 2007.

- [156] E. López-Dolado, A. González-Mayorga, M. C. Gutiérrez, and M. C. Serrano, "Immunomodulatory and angiogenic responses induced by graphene oxide scaffolds in chronic spinal hemisectioned rats," *Biomaterials*, vol. 99, pp. 72-81, 2016.
- [157] V. Volarevic, V. Paunovic, Z. Markovic, B. Simovic Markovic, M. Misirkic-Marjanovic, B. Todorovic-Markovic, S. Bojic, L. Vucicevic, S. Jovanovic, and N. Arsenijevic, "Large graphene quantum dots alleviate immune-mediated liver damage," *Acs Nano*, vol. 8, no. 12, pp. 12098-12109, 2014.
- [158] J. J. Ryan, H. R. Bateman, A. Stover, G. Gomez, S. K. Norton, W. Zhao, L. B. Schwartz, R. Lenk, and C. L. Kepley, "Fullerene nanomaterials inhibit the allergic response," *The Journal of immunology*, vol. 179, no. 1, pp. 665-672, 2007.
- [159] K. Yudoh, R. Karasawa, K. Masuko, and T. Kato, "Water-soluble fullerene (C60) inhibits the development of arthritis in the rat model of arthritis," *International journal of nanomedicine*, vol. 4, p. 217, 2009.
- [160] Y. Saitoh, A. Miyanishi, H. Mizuno, S. Kato, H. Aoshima, K. Kokubo, and N. Miwa, "Super-highly hydroxylated fullerene derivative protects human keratinocytes from UV-induced cell injuries together with the decreases in intracellular ROS generation and DNA damages," *Journal of Photochemistry Photobiology B: Biology*, vol. 102, no. 1, pp. 69-76, 2011.
- [161] K. Yamashita, M. Sakai, N. Takemoto, M. Tsukimoto, K. Uchida, H. Yajima, S. Oshio, K. Takeda, and S. Kojima, "Attenuation of delayed-type hypersensitivity by fullerene treatment," *Toxicology*, vol. 261, no. 1-2, pp. 19-24, 2009.
- [162] A. Pentecost, M. J. Kim, S. Jeon, Y. J. Ko, I. C. Kwon, Y. Gogotsi, K. Kim, and K. L. Spiller, "Immunomodulatory nanodiamond aggregate-based platform for the treatment of rheumatoid arthritis," *Regenerative Biomaterials*, vol. 6, no. 3, pp. 163-174, 2019.
- [163] P. S. Hegde and D. S. Chen, "Top 10 challenges in cancer immunotherapy," *Immunity*, vol. 52, no. 1, pp. 17-35, 2020.
- [164] S. E. Gould, M. R. Junttila, and F. J. de Sauvage, "Translational value of mouse models in oncology drug development," *Nature medicine*, vol. 21, no. 5, pp. 431-439, 2015.
- [165] L. A. Emens, P. A. Ascierto, P. K. Darcy, S. Demaria, A. M. Eggermont, W. L. Redmond, B. Seliger, and F. M. Marincola, "Cancer immunotherapy: opportunities and challenges in the rapidly evolving clinical landscape," *European Journal of Cancer*, vol. 81, pp. 116-129, 2017.
- [166] W. C. Zamboni, J. Szebeni, S. V. Kozlov, A. T. Lucas, J. A. Piscitelli, and M. A. Dobrovolskaia, "Animal models for analysis of immunological responses to nanomaterials: challenges and considerations," *Advanced drug delivery reviews*, vol. 136, pp. 82-96, 2018.
- [167] G. Dranoff, "Experimental mouse tumour models: what can be learnt about human cancer immunology?," *Nature Reviews Immunology*, vol. 12, no. 1, pp. 61-66, 2012.
- [168] P. Orsu and A. Koyyada, "Recent progresses and challenges in graphene based nano materials for advanced therapeutical applications: a comprehensive review," *Materials Today Communications*, vol. 22, p. 100823, 2020.

- [169] T. R. Fadel and T. M. Fahmy, "Immunotherapy applications of carbon nanotubes: from design to safe applications," *Trends in biotechnology*, vol. 32, no. 4, pp. 198-209, 2014.
- [170] L. B. Alexandrov, S. Nik-Zainal, D. C. Wedge, S. A. Aparicio, S. Behjati, A. V. Biankin, G. R. Bignell, N. Bolli, A. Borg, and A.-L. Børresen-Dale, "Signatures of mutational processes in human cancer," *Nature*, vol. 500, no. 7463, pp. 415-421, 2013.
- [171] F. R. Balkwill, M. Capasso, and T. Hagemann, "The tumor microenvironment at a glance," *Journal of cell science*, 2012.
- [172] A. J. Oliver, P. K. Lau, A. S. Unsworth, S. Loi, P. K. Darcy, M. H. Kershaw, and C. Y. Slaney, "Tissue-dependent tumor microenvironments and their impact on immunotherapy responses," *Frontiers in immunology*, vol. 9, p. 70, 2018.
- [173] P. C. Ray, H. Yu, and P. P. Fu, "Toxicity and environmental risks of nanomaterials: challenges and future needs," *Journal of Environmental Science and Health Part C*, vol. 27, no. 1, pp. 1-35, 2009.
- [174] C. S. Lim, D. W. Porter, M. S. Orandle, B. J. Green, M. A. Barnes, T. L. Croston, M. G. Wolfarth, L. A. Battelli, M. E. Andrew, D. H. Beezhold, P. D. Siegel, and Q. Ma, "Resolution of Pulmonary Inflammation Induced by Carbon Nanotubes and Fullerenes in Mice: Role of Macrophage Polarization," (in English), *Frontiers in Immunology*, Original Research vol. 11, no. 1186, 2020-June-12 2020.
- [175] J. Bornholdt, A. T. Saber, B. Lilje, M. Boyd, M. Jørgensen, Y. Chen, M. Vitezic, N. R. Jacobsen, S. S. Poulsen, and T. Berthing, "Identification of gene transcription start sites and enhancers responding to pulmonary carbon nanotube exposure in vivo," *ACS nano*, vol. 11, no. 4, pp. 3597-3613, 2017.
- [176] E. D. Kuempel, M.-C. Jaurand, P. Møller, Y. Morimoto, N. Kobayashi, K. E. Pinkerton, L. M. Sargent, R. C. Vermeulen, B. Fubini, and A. B. Kane, "Evaluating the mechanistic evidence and key data gaps in assessing the potential carcinogenicity of carbon nanotubes and nanofibers in humans," *Critical reviews in toxicology*, vol. 47, no. 1, pp. 1-58, 2017.
- [177] C. A. Poland, R. Duffin, I. Kinloch, A. Maynard, W. A. Wallace, A. Seaton, V. Stone, S. Brown, W. MacNee, and K. Donaldson, "Carbon nanotubes introduced into the abdominal cavity of mice show asbestos-like pathogenicity in a pilot study," *Nature nanotechnology*, vol. 3, no. 7, p. 423, 2008.
- [178] K. A. Russ, J. A. Thompson, M. Kashon, D. W. Porter, S. A. Friend, W. McKinney, and J. S. Fedan, "Comparison of multi-wall carbon nanotube and nitrogen-doped multi-wall carbon nanotube effects on lung function and airway reactivity in rats," *Toxicology applied pharmacology*, vol. 364, pp. 153-163, 2019.
- [179] Y. Sato, A. Yokoyama, K.-i. Shibata, Y. Akimoto, S.-I. Ogino, Y. Nodasaka, T. Kohgo, K. Tamura, T. Akasaka, and M. Uo, "Influence of length on cytotoxicity of multi-walled carbon nanotubes against human acute monocytic leukemia cell line THP-1 in vitro and subcutaneous tissue of rats in vivo," *Molecular BioSystems*, vol. 1, no. 2, pp. 176-182, 2005.

- [180] S.-T. Yang, X. Wang, G. Jia, Y. Gu, T. Wang, H. Nie, C. Ge, H. Wang, and Y. Liu, "Long-term accumulation and low toxicity of single-walled carbon nanotubes in intravenously exposed mice," *Toxicology letters*, vol. 181, no. 3, pp. 182-189, 2008.
- [181] S. Tang, Y. Tang, L. Zhong, K. Murat, G. Asan, J. Yu, R. Jian, C. Wang, and P. Zhou, "Short - and long - term toxicities of multi - walled carbon nanotubes in vivo and in vitro," *Journal of Applied Toxicology*, vol. 32, no. 11, pp. 900-912, 2012.
- [182] S. Xu, Z. Zhang, and M. Chu, "Long-term toxicity of reduced graphene oxide nanosheets: effects on female mouse reproductive ability and offspring development," *Biomaterials*, vol. 54, pp. 188-200, 2015.
- [183] X. Ding, J. Wang, Q. Rui, and D. Wang, "Long-term exposure to thiolated graphene oxide in the range of $\mu\text{g/L}$ induces toxicity in nematode *Caenorhabditis elegans*," *Science of the Total Environment*, vol. 616, pp. 29-37, 2018.
- [184] J. C. Yang, C. H. Yen, W. J. Wang, J. J. Horng, and Y. P. Tsai, "Assessment of adequate sodium hypochlorite concentration for pre - oxidation of multi - walled carbon nanotubes," *Journal of Chemical Technology Biotechnology*, vol. 85, no. 5, pp. 699-707, 2010.
- [185] H. Bai, W. Jiang, G. P. Kotchey, W. A. Saidi, B. J. Bythell, J. M. Jarvis, A. G. Marshall, R. A. Robinson, and A. Star, "Insight into the mechanism of graphene oxide degradation via the photo-Fenton reaction," *The Journal of Physical Chemistry C*, vol. 118, no. 19, pp. 10519-10529, 2014.
- [186] S. P. Mukherjee, A. R. Gliga, B. Lazzaretto, B. Brandner, M. Fielden, C. Vogt, L. Newman, A. F. Rodrigues, W. Shao, and P. M. Fournier, "Graphene oxide is degraded by neutrophils and the degradation products are non-genotoxic," *Nanoscale*, vol. 10, no. 3, pp. 1180-1188, 2018.
- [187] R. Kurapati, S. P. Mukherjee, C. Martín, G. Bepete, E. Vázquez, A. Pénicaud, B. Fadeel, and A. Bianco, "Degradation of Single - Layer and Few - Layer Graphene by Neutrophil Myeloperoxidase," *Angewandte Chemie International Edition*, vol. 57, no. 36, pp. 11722-11727, 2018.
- [188] R. A. Lake and B. W. Robinson, "Immunotherapy and chemotherapy—a practical partnership," *Nature Reviews Cancer*, vol. 5, no. 5, pp. 397-405, 2005.
- [189] W. Mu, Q. Chu, Y. Liu, and N. Zhang, "A review on nano-based drug delivery system for cancer chemoimmunotherapy," *Nano-Micro Letters*, vol. 12, no. 1, pp. 1-24, 2020.
- [190] B. Hosnedlova, M. Kepinska, C. Fernandez, Q. Peng, B. Ruttkay - Nedecky, H. Milnerowicz, and R. J. T. C. R. Kizek, "Carbon nanomaterials for targeted cancer therapy drugs: A critical review," *The Chemical Record*, vol. 19, no. 2-3, pp. 502-522, 2019.
- [191] M. Hosseini, M. Haji-Fatahaliha, F. Jadidi-Niaragh, J. Majidi, and M. Yousefi, "The use of nanoparticles as a promising therapeutic approach in cancer immunotherapy," *Artificial cells, nanomedicine, biotechnology*, vol. 44, no. 4, pp. 1051-1061, 2016.
- [192] N. B. Alsaleh and J. M. Brown, "Engineered Nanomaterials and Type I Allergic Hypersensitivity Reactions," *Frontiers in Immunology*, vol. 11, 2020.

- [193] J. N. Larsen, L. Broge, and H. Jacobi, "Allergy immunotherapy: the future of allergy treatment," *Drug discovery today*, vol. 21, no. 1, pp. 26-37, 2016.
- [194] H. Pohlitz, I. Bellinghausen, H. Frey, and J. Saloga, "Recent advances in the use of nanoparticles for allergen - specific immunotherapy," *Allergy*, vol. 72, no. 10, pp. 1461-1474, 2017.
- [195] S. Li, Z. Zhang, W.-F. Lai, L. Cui, and X. Zhu, "How to overcome the side effects of tumor immunotherapy," *Biomedicine & Pharmacotherapy*, vol. 130, p. 110639, 2020.
- [196] M. Bessa-Gonçalves, A. M. Silva, J. P. Brás, H. Helmholtz, B. J. Luthringer-Feyerabend, R. Willumeit-Römer, M. A. Barbosa, and S. G. Santos, "Fibrinogen and magnesium combination biomaterials modulate macrophage phenotype, NF- κ B signaling and crosstalk with mesenchymal stem/stromal cells," *Acta Biomaterialia*, vol. 114, pp. 471-484, 2020.
- [197] S. A. Bustin, V. Benes, J. A. Garson, J. Hellemans, J. Huggett, M. Kubista, R. Mueller, T. Nolan, M. W. Pfaffl, and G. L. Shipley, "The MIQE Guidelines: M inimum I nformation for Publication of Q uantitative Real-Time PCR E xperiments," in *Clinical Chemistry*, vol. 55: Oxford University Press, 2009, pp. 611-622.
- [198] B. Uy, S. R. McGlashan, and S. B. Shaikh, "Measurement of reactive oxygen species in the culture media using Acridan Lumigen PS-3 assay," *Journal of biomolecular techniques: JBT*, vol. 22, no. 3, p. 95, 2011.
- [199] F. Castro, M. L. Pinto, R. Almeida, F. Pereira, A. M. Silva, C. L. Pereira, S. G. Santos, M. A. Barbosa, R. M. Gonçalves, and M. J. Oliveira, "Chitosan/poly (γ -glutamic acid) nanoparticles incorporating IFN- γ for immune response modulation in the context of colorectal cancer," *Biomaterials Science*, vol. 7, no. 8, pp. 3386-3403, 2019.
- [200] M. Danaei, M. Dehghankhold, S. Ataei, F. Hasanzadeh Davarani, R. Javanmard, A. Dokhani, S. Khorasani, and M. Mozafari, "Impact of particle size and polydispersity index on the clinical applications of lipidic nanocarrier systems," *Pharmaceutics*, vol. 10, no. 2, p. 57, 2018.
- [201] E. Joseph and G. Singhvi, "Multifunctional nanocrystals for cancer therapy: a potential nanocarrier," *Nanomaterials for drug delivery therapy*, pp. 91-116, 2019.
- [202] A. Angelopoulou, E. Voulgari, E. Diamanti, D. Gournis, and K. Avgoustakis, "Graphene oxide stabilized by PLA-PEG copolymers for the controlled delivery of paclitaxel," *European Journal of Pharmaceutics Biopharmaceutics*, vol. 93, pp. 18-26, 2015.
- [203] A. F. Rodrigues, L. Newman, N. Lozano, S. P. Mukherjee, B. Fadeel, C. Bussy, and K. J. D. M. Kostarelos, "A blueprint for the synthesis and characterisation of thin graphene oxide with controlled lateral dimensions for biomedicine," *2D Materials*, vol. 5, no. 3, p. 035020, 2018.
- [204] D. R. Dreyer, A. D. Todd, and C. W. Bielawski, "Harnessing the chemistry of graphene oxide," *Chemical Society Reviews*, vol. 43, no. 15, pp. 5288-5301, 2014.
- [205] K. Yang, L. Feng, H. Hong, W. Cai, and Z. Liu, "Preparation and functionalization of graphene nanocomposites for biomedical applications," *Nature protocols*, vol. 8, no. 12, pp. 2392-2403, 2013.

- [206] J. Yokota and S. Kyotani, "Influence of nanoparticle size on the skin penetration, skin retention and anti-inflammatory activity of non-steroidal anti-inflammatory drugs," *Journal of the Chinese Medical Association*, vol. 81, no. 6, pp. 511-519, 2018.
- [207] F. A. Silva, R. Costa-Almeida, L. Timochenko, S. I. Amaral, S. Pinto, I. C. Gonçalves, J. R. Fernandes, F. D. Magalhães, B. Sarmiento, and A. M. Pinto, "Graphene Oxide Topical Administration: Skin Permeability Studies," *Materials*, vol. 14, no. 11, p. 2810, 2021.
- [208] S. N. Alam, N. Sharma, and L. Kumar, "Synthesis of graphene oxide (GO) by modified hummers method and its thermal reduction to obtain reduced graphene oxide (rGO)," *Graphene*, vol. 6, no. 1, pp. 1-18, 2017.
- [209] Q. Lai, S. Zhu, X. Luo, M. Zou, and S. Huang, "Ultraviolet-visible spectroscopy of graphene oxides," *Aip Advances*, vol. 2, no. 3, p. 032146, 2012.
- [210] Y. Ding, P. Zhang, Q. Zhuo, H. Ren, Z. Yang, and Y. Jiang, "A green approach to the synthesis of reduced graphene oxide nanosheets under UV irradiation," *Nanotechnology*, vol. 22, no. 21, p. 215601, 2011.
- [211] D. N. Syed, F. Afaq, and H. Mukhtar, "Differential activation of signaling pathways by UVA and UVB radiation in normal human epidermal keratinocytes," *Photochemistry photobiology*, vol. 88, no. 5, pp. 1184-1190, 2012.
- [212] R. Li, L. M. Guiney, C. H. Chang, N. D. Mansukhani, Z. Ji, X. Wang, Y.-P. Liao, W. Jiang, B. Sun, and M. C. Hersam, "Surface oxidation of graphene oxide determines membrane damage, lipid peroxidation, and cytotoxicity in macrophages in a pulmonary toxicity model," *ACS nano*, vol. 12, no. 2, pp. 1390-1402, 2018.
- [213] B. Wang, X. Su, J. Liang, L. Yang, Q. Hu, X. Shan, J. Wan, and Z. Hu, "Synthesis of polymer-functionalized nanoscale graphene oxide with different surface charge and its cellular uptake, biosafety and immune responses in Raw264. 7 macrophages," *Materials Science Engineering: C*, vol. 90, pp. 514-522, 2018.
- [214] S. P. Mukherjee, M. Bottini, and B. Fadeel, "Graphene and the immune system: a romance of many dimensions," *Frontiers in immunology*, vol. 8, p. 673, 2017.
- [215] H. Lim, N. Huang, and C. Loo, "Facile preparation of graphene-based chitosan films: Enhanced thermal, mechanical and antibacterial properties," *Journal of Non-Crystalline Solids*, vol. 358, no. 3, pp. 525-530, 2012.
- [216] I. Sengupta, P. Bhattacharya, M. Talukdar, S. Neogi, S. K. Pal, and S. Chakraborty, "Bactericidal effect of graphene oxide and reduced graphene oxide: Influence of shape of bacteria," *Colloid Interface Science Communications*, vol. 28, pp. 60-68, 2019.
- [217] J. Tur, T. Vico, J. Lloberas, A. Zorzano, and A. Celada, "Macrophages and mitochondria: a critical interplay between metabolism, signaling, and the functional activity," *Advances in immunology*, vol. 133, pp. 1-36, 2017.
- [218] I. I. Alsaedi, Z. J. Taqi, A. M. A. Hussien, G. M. Sulaiman, and M. S. Jabir, "Graphene nanoparticles induces apoptosis in MCF-7 cells through mitochondrial damage and NF-KB pathway," *Materials research express*, vol. 6, no. 9, p. 095413, 2019.

- [219] M. J. Feito, R. Diez-Orejas, M. Cicuéndez, L. Casarrubios, J. M. Rojo, and M. T. Portolés, "Characterization of M1 and M2 polarization phenotypes in peritoneal macrophages after treatment with graphene oxide nanosheets," *Colloids and Surfaces B: Biointerfaces*, vol. 176, pp. 96-105, 2019.
- [220] J. Ma, R. Liu, X. Wang, Q. Liu, Y. Chen, R. P. Valle, Y. Y. Zuo, T. Xia, and S. Liu, "Crucial role of lateral size for graphene oxide in activating macrophages and stimulating pro-inflammatory responses in cells and animals," *ACS nano*, vol. 9, no. 10, pp. 10498-10515, 2015.
- [221] H. Zhou, K. Zhao, W. Li, N. Yang, Y. Liu, C. Chen, and T. Wei, "The interactions between pristine graphene and macrophages and the production of cytokines/chemokines via TLR- and NF- κ B-related signaling pathways," *Biomaterials*, vol. 33, no. 29, pp. 6933-6942, 2012.
- [222] E. Toichi, K. Q. Lu, A. R. Swick, T. S. McCormick, and K. D. Cooper, "Skin-infiltrating monocytes/macrophages migrate to draining lymph nodes and produce IL-10 after contact sensitizer exposure to UV-irradiated skin," *Journal of Investigative Dermatology*, vol. 128, no. 11, pp. 2705-2715, 2008.
- [223] V. Shreedhar, T. Giese, V. W. Sung, and S. E. Ullrich, "A cytokine cascade including prostaglandin E₂, IL-4, and IL-10 is responsible for UV-induced systemic immune suppression," *The Journal of Immunology*, vol. 160, no. 8, pp. 3783-3789, 1998.
- [224] Y. Li, Y. Liu, Y. Fu, T. Wei, L. Le Guyader, G. Gao, R.-S. Liu, Y.-Z. Chang, and C. Chen, "The triggering of apoptosis in macrophages by pristine graphene through the MAPK and TGF- β signaling pathways," *Biomaterials*, vol. 33, no. 2, pp. 402-411, 2012.
- [225] G. Qu, S. Liu, S. Zhang, L. Wang, X. Wang, B. Sun, N. Yin, X. Gao, T. Xia, and J.-J. Chen, "Graphene oxide induces toll-like receptor 4 (TLR4)-dependent necrosis in macrophages," *ACS nano*, vol. 7, no. 7, pp. 5732-5745, 2013.
- [226] C. Zhang, S. Chen, P. J. Alvarez, and W. Chen, "Reduced graphene oxide enhances horseradish peroxidase stability by serving as radical scavenger and redox mediator," *Carbon*, vol. 94, pp. 531-538, 2015.
- [227] C. Foged, B. Brodin, S. Frokjaer, and A. Sundblad, "Particle size and surface charge affect particle uptake by human dendritic cells in an in vitro model," *International journal of pharmaceutics*, vol. 298, no. 2, pp. 315-322, 2005.
- [228] X. Zhi, H. Fang, C. Bao, G. Shen, J. Zhang, K. Wang, S. Guo, T. Wan, and D. Cui, "The immunotoxicity of graphene oxides and the effect of PVP-coating," *Biomaterials*, vol. 34, no. 21, pp. 5254-5261, 2013.
- [229] M. R. Shurin, N. Yanamala, E. R. Kisin, A. V. Tkach, G. V. Shurin, A. R. Murray, H. D. Leonard, J. S. Reynolds, D. W. Gutkin, and A. Star, "Graphene oxide attenuates Th2-type immune responses, but augments airway remodeling and hyperresponsiveness in a murine model of asthma," *ACS nano*, vol. 8, no. 6, pp. 5585-5599, 2014.