Review Article

Immunological effects of graphene family nanomaterials

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ABSTRACT

Graphene and its derivatives are called graphene family nanomaterials (GFNs). Over the past few years, they have been heavily investigated in biomedical arena due to their extraordinary physiochemical properties and potential biomedical applications. However, the biocompatibility of GFNs is becoming important for biomedical applications such as drug and gene delivery, tissue engineering, biosensing and imaging. In this regard, it is crucial to understand the process of interaction of GFNs with immune system, which is also meaningful to manipulate their interaction for safe and efficient applications. Herein, different modalities of GFNs interaction with various components of immune system and the outcome of these interactions are described and evaluated. This review also summarizes different mechanisms involved in immunological effects of GFNs and techniques that are employed for GFNs, to escape the clutches of immune system. We elucidate the intricate balance between immune-stimulation and immune-suppression and expect that understanding of immunological effects of graphene derivatives would help evaluate and estimate the possible biomedical applications as far as immune system is concerned.

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1. Introduction

Nowadays, engineering nanomaterials have been reported to have unique and novel characteristics and hold promises in broad applications in many fields. From the esteemed family of nanomaterials, one shining star is graphene, often called “miracle material”-nicknamed so, for its extraordinary properties. It is a single atom thick sheet of sp^2 -hybridized carbon atoms arranged in a continuous series of hexagons, resembling a honey comb structure. It has become one of the world’s most attractive materials (Lee et al., 2008) with great electrical and thermal conductivity (Schweirz, F., 2010; Baladin et al., 2008) and is impermeable even to an atom as small as helium (Bunch et al., 2008). All of these properties make it very attractive for potentially various applications. Graphene and its derivatives, collectively can be termed as graphene family nanomaterials (GFNs), which include single or few layered graphene (FLG), graphene nanoribbons (GNRs), graphene nanoplatelets (GNPs), graphene oxide (GO), reduced graphene oxide (rGO), graphene quantum dots (GQDs) and a few more of their sort. GFNs have gained promising applications in many fields, such as photonics/plasmonics (Bonaccorso et al., 2010; Grigorenko et al., 2012), electronics (Jang et al., 2016), sensors (Shao et al., 2010), catalysis (Machado et al., 2012), drug delivery (Liu et al., 2013) and DNA sequencing (Heerema et al., 2016). GFNs can be prepared chemically and their properties can be changed by number of synthesis techniques (Sun et al., 2011).

Study of the interaction between GFNs and biological systems has rapidly increased recently in scientific community. As carbon nanomaterials vary in their lateral size, layer number, defect density, surface chemistry, purity and composition, their interaction with biological systems becomes complicated, validating a detailed investigation for their health effects or safety (Zhao et al., 2015). Toxicity profiles of GFNs have been well assessed by researchers over the years and means of biocompatibility have also been elucidated (Guo et al., 2014; Seabra et al., 2014). The most important factor, however, when it comes to using a GFN in human body, is the nature of its interaction with immune system.

Animals, higher in the evolutionary tree, have developed a very sophisticated defense mechanism to ward off any foreign entity. It distinguishes foreign bodies like bacteria, viruses and parasites from body’s tissue and is a layered system with cascade of barriers making it hard for these invaders to affect body’s physiological functions. As a component of immune system, phagocytic cells like macrophages and dendritic cells help clear the foreign bodies with the help of complement system, which is a set of proteins dedicated for better recognition of these foreign bodies by these cells. Moreover, once phagocytic cells are properly stimulated, they can recruit another type of cells in this fight, called lymphocytes – a layered defense system as already mentioned. Nanomaterials are known to be immunostimulant as well as immunosuppressant (Jiao et al., 2014; Hussain et al., 2012) and so are GFNs. That fact makes it complicated to understand their biological outcome after interaction with immune system (Fig.1).

Immunological Effects Of Graphene Family Nanomaterials

Fig. 1. A schematic diagram showing immunological routes followed by GFNs, when they interact with immune system.

Immunomodulation induced by GFNs (immunostimulation or immunosuppression) can, however, be enhanced by biofunctionalization or changing their physiochemical parameters. Specific modulation of immune system by GFNs can also be beneficial as it may lead to development of immunotherapeutic agents and vaccines. Here, in this review, we describe the immunological outcomes of the interaction of GFNs with various immune system components as well as mechanism of immunomodulation.

2. Interaction of GFNs with immune system components

Immune system comprises both innate and adaptive defense part. In innate immunity, macrophages and monocytes are recruited to infection site through production of special class of chemical mediators called cytokines. Then, complement system is activated that ultimately results in phagocytosis of the antigen by phagocytic cells. Adaptive immunity includes recruitment of antigen specific T and B lymphocytes which help in release of cytokines and antibodies. This kind of immunity may exist over a larger time span by remembering the antigen in terms of specific memory of antibodies. In this respect, a detailed insight into the relationship between GFNs and immune system components is importance. It has been known that surface chemistry and dose of carbon nanomaterials, govern its bioaccumulation and toxicity in vivo (Zhao et al., 2015) As most immune system components work together to determine the fate of GFNs, step by step account of the interaction of GFNs with these components is supplied here.

2.3. Complement System

Complement system comprises over 40 proteins, which are produced by liver and found in blood circulatory system. It works as a cascade system in which, involved proteins trigger one after another, helping phagocytosis of foreign body by phagocytic cells. This system seals the fate of antigen in three different ways; opsonization, inflammation or/and cytolysis. Three major pathways are followed in this regard, namely; classical pathway, alternative pathway and lectin pathway. In this way complement system is crucial to surveillance and homeostasis of the body (Ricklin et al., 2010). The dynamics of interaction of nanomaterials with complement system is necessary to understand, if nanomaterials are to be employed in nanomedicine (Moghimi et al., 2011). Nanomaterials have been known to interact with complement system in a variety of ways (Thomas et al., 2014), however, only few studies have shed light on the interaction of graphene with complement system. Recent studies of interaction of complement system with CNTs have been widely reported. It has been shown that single walled CNTs

can trigger classical pathway whereas double walled ones can trigger both classical and alternative pathway (Salvador-Morales et al., 2006). Functionalization grants CNTs some protection from complement system (Andersen et al., 2013).

Like CNTs, PEGylation affords graphene some relief from the complement system activation. Tan et al. (2013) showed that that un-functionalized GO, when incubated with human sera, was able to adsorb a large number of serum proteins and induced C3 cleavage. Comparably, PEGylated GO showed much less protein adsorption and greatly reduced the C3 cleavage. About six serum proteins, however, got adsorbed onto PEGylated GO with great specificity, four of which were complement proteins. These complement proteins like C3a/C3a(des-Arg), when adsorbed to PEGylated GO, became unavailable for their receptors, which reduced the complement activation by PEGylated GO. This study showed that functionalization of GO can tune complement activation. Another study showing effect of functionalization on complement system activation was performed by Chowdhury et al (2013), where they used dextran functionalized graphene nanoplatelets (GNP-Dex). Three concentrations of GNP-Dex (1, 7 and 10 mg/mL), were treated with blood samples from two healthy individuals and levels of SC5b-9 were measured as a reflection of complement activation (SC5b-9 is a complement protein produced in classical, alternate and lectin pathways). An increase of 20% in complement activity, as compared to untreated blood, for sample 1 and 12 % for sample 2 was recorded at the concentration of 1mg/mL of GNP-Dex. However, this 20% (474 ng/mL) and 12% (504 ng/mL) increase of SC5b-9 levels still are in normal range (176 to 624 ng/mL). To test whether dextran is responsible for complement activation, the effect of various concentrations of dextran on two blood samples was also evaluated. As dextran comprises about 40% weight of GNP-Dex, three concentrations of dextran (0.4, 2.8 and 4.0 mg/mL) were used for the experiment. An increase in alternate pathway activation (evaluated by measuring levels of Bb, which is a complementary protein special to alternate pathway only) of about 11% and 25% for sample 1 and that of 23% and 37% for sample 2 was observed at two higher concentrations.

In another study by Chowdhury et al. (2015), graphene nanoplatelets (GNPs) were non-covalently functionalized with PEG-DSPE (1, 2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N* [amino (polyethylene glycol)]) and their effect on various components of blood vascular system was investigated. Even at high concentrations (up to 80 µg/mL) of GNP-PEG-DSPE, the activation of complement system was not observed and the activation of alternate pathway was also negligible. From these three studies, it can be inferred that functionalization of GFNs is a hopeful way to make GFNs more biocompatible for immune system.

Furthermore, the effect of oxygen contents of un-functionalized GO on complement system activation was investigated by Wibroe et al (2016). They produced GO with 36%, 29%, and 24% oxygen contents (GO with 36% oxygen contents, that was produced by their methodology was subsequently reduced, resulting in GO with 29%, 24% oxygen contents) and showed that GO with 36% oxygen had a dose dependent SC4d-9 increment (Fig. 2), in accordance with the findings of Tan et al (2013). The level of C4d, which is a byproduct of C4b activation, was significantly increased showing that the classical and/or lectin pathways might have been activated. It was also noted that Sc4d-9 and C5a (a complement protein whose presence signals the terminal phase of complement activation) levels decreased as the oxygen contents of soluble GO dropped from 36% to 24%. They theorized that the lower

levels of surface oxygen may help in forming of irreversible flocculates, thereby decreasing the surface area as compared to that of starting material GO (36% oxygen contents). Comparably, for immobilized GO, the trend was opposite and the levels of complement proteins decreased as the oxygen contents increased. It suggested that the immobilized GO had vastly different physical and surface energy characteristics, however, a clear explanation was not provided for this opposite trend that was seen in case of immobilized GO.

Fig. 2. Complement activation by GO preparations, measured by increases in serum concentration of SC5b-9 and C5a. (A) Concentration effect of the starting GO (36 at% oxygen) solution on rises of serum SC5b-9 levels. (B) Significant increases in C4d levels above background indicates a response initiation from classical and/or lectin pathways. (C&D) The role of surface deoxygenation in complement activation by GO solutions and immobilized GO. Adopted from Wibroe et al (2016)

2.2. Macrophages

Body protects itself from the foreign entities by utilizing an army of specialized cells, which are called phagocytes. This army is divided into different regiments, each dedicated to its own special operations. Macrophages belong to that regiment which operates at the forefront of this war between the body and the antigen. They are actually differentiated monocytes and are professional phagocytes, helping the body clear antigen or dead cells. When activated by reactive oxygen species (ROS) or primed by antigens, macrophages, which trigger an innate immune response through the activation of inflammasome. Nanomaterials are shown to activate macrophage resulting in inflammation (Nicolette et al., 2011; Porter et al., 2009). It must be noted that macrophages are the most studied blood cell type (about 56% of the studies on immune cells) as far as carbon based nanomaterials are concerned (Orecchioni et al., 2014).

Pristine graphene has been shown to have catastrophic effects on murine RAW264.7 macrophages in a study by Li et al. (2012). They reported that the cause of graphene induced cytotoxicity was an increase in reactive oxygen species (ROS) and a depletion in mitochondrial membrane potential (MMP). Moreover, apoptosis was observed (Fig. 3) in the cells, due to MAPK and TGF- β signaling pathways activation. The same group, Zhou et al. (2012), also investigated the cytokine profiles of the interaction of graphene with primary murine macrophages and immortalized macrophages and found that sub-cytotoxic concentrations of pristine graphene stimulate the secretion of Th1/Th2 cytokine including IL-1 α , IL-6, IL-10, TNF- α and GM-CSF as well as chemokines such as MCP-1, MIP-1 α , MIP-1 β and RANTES. TLR-mediated and NF- κ B dependent transcription was reasoned as the cause of such cytokine profile.

Fig. 3. TEM images of macrophages incubated with 20 μ g/mL graphene for 48 h. Left: Control, Middle and Right, Graphene exposure. Notice extensive vacuolization. Bar, 2 μ m for Left and Middle images, and 1 μ m for Right image. Adapted from Li et al. (2012).

Interestingly, Elgrabli et al. (2015) showed that CNTs could be degraded by macrophages. In one similar study by Girish et al. (2013), macrophages were also shown to degrade graphene over a period of 3 months in vivo. Mice models were used in this study. Raman confocal imaging showed that intravenously injected graphene appeared to show defects over a period of 8-90 days. Spectral information of these defects were shown as the formation of defective D' band and broadening of D and G bands of graphene.

Effect of graphene oxide (GO), like pristine graphene, on macrophage is also very strong as demonstrated by Chen et al. (2012). They showed that GO was able to induce concentration dependent autophagy as well as inflammatory response from the macrophage cells mediated by TLR-4 and TLR-9 and its downstream adaptor proteins MyD88, TRIF and TRAF6. Necrosis in macrophages was observed in one study by Qu et al. (2013) and was evaluated to be TLR-4 dependent. Cell death was attributed partially to RIP1-RIP3 complex mediated programmed necrosis downstream of TNF- α induction and to the increase in ROS production. Similar cytotoxicity profile for GO derivatives: dodecylamine-GO (DA-GO), reduced GO (rGO), and sodium dodecyl sulfate-rGO (SDS-rGO) were evaluated against RAW 264.7 macrophages by Park et al. (2015). They found that the autophagy was evident in all GO derivatives in a concentration-dependent manner.

GO nanosheets decorated with 1-arm and 6-arm PEG were incubated with murine macrophages and studied for their biocompatibility by Matesanz et al. (2013). They confirmed that GO nanosheets, after internalization, localized on f-actin filaments and induced effects like cell cycle delays, oxidative stress and apoptosis. GO with 1 arm PEG produced more ROS and was easily internalized than GO with 6 arm PEG. The same group, Feito et al. (2014), in another study, using the same kind of GO nanosheets showed that GO with 6 arm PEG significantly increased the secretion of TNF- α by RAW-264.7 macrophages without influence on IL-6 and IL-1 β levels. Duch et al. (2011) demonstrated that GO, compared with pristine graphene, was more potent in generating of ROS in mouse alveolar macrophages (MH-S) and stable nanoscale dispersion was helpful for its biocompatibility in

lungs. Shinwald et al. (2012) studied the effects of graphene nanoplatelets on mouse pleural physiology and found that these nanomaterials stimulate the secretion of MIP-1R, MCP-1, MIP-2, IL-8, and IL-1 β expression in the BAL, the pleural lavage, and cell culture supernatant from THP-1 macrophages. A comparative research about the effects of acid functionalized single walled carbon nanotubes (AF-SWCNTs) and GO on murine peritoneal macrophages was performed by Wan et al. (2013). They established that GO was more potent than AF-SWCNTs in inducing autophagy, lysosomal destabilization, and decreasing cell viability.

Various functionalizations have been adopted to minimize the detrimental effect of graphene derivatives on macrophages. Zhi et al. (2013) showed that PVP modification decreased the phagocytosis of GO by macrophages and showed less cytotoxicity as compared to pristine GO. In another study, Luo et al. (2015) used graphene oxide (GO) and engineered the surface with polyethylene glycol (PEG), bovine serum albumin (BSA), and polyether imide (PEI) to study their interaction with macrophages. PEG and BSA functionalized GO hindered endocytosis and were more biocompatible than PEI-GO. The reason is that, PEI-GO, being positively charged, interacts strongly with plasma membrane and is rapidly internalized. PEI-GO was also found to disrupt mitochondrial functions by eliciting a sharp decrease in membrane potential and structural impairment. Sasidharan et al. (2012), however, demonstrated that if pristine graphene is functionalized with C-O, C-O-C, C=O, and O-C=O groups, the resulting f-graphene (graphene oxide) shows good hemocompatibility with negligible effect on macrophages. The membrane integrity and cell viability remained good in their experiments using f-graphene and they concluded that hydrophilicity of f-graphene is responsible for its better biocompatibility than hydrophobic pristine graphene.

Two different groups: Mendes et al. (2015) and Yue et al. (2012) confirmed that lateral size does not affect internalization of GO. The former one used the interaction of two different sized GO i.e. 89 nm and 277 nm with J774.2 macrophages and HeLa cells. The result indicated that both sized GOs were equally internalized regardless of incubation time. However, large sized (277nm) GO was more toxic to both cell lines when the incubation time was short. The latter group evaluated internalization and toxicity of GO with 2 μ m and 350 nm sizes to six cell lines, two of which were murine macrophage J774.1A1 and peritoneal macrophage PM ϕ . They showed that GO of both sizes equally contributed to internalization by these macrophages, however, 2 μ m sized GO showed divergent intracellular locations and induced much stronger inflammation responses. A conflicting study by Russier et al. (2013) showed that smaller lateral sizes of GO contributed to more internalization and induced stronger change in physiological functions of human and murine primary macrophages.

2.3. Dendritic cells

Dendritic cells (DCs) are antigen presenting cells. Upon stimulation, these cells undergo maturation and migration to secondary lymphoid organs where they stimulate native T cells and then activate T cells, B cells, and macrophages. They are like a tether between innate and adaptive immune systems, thus, are critical in establishing immunological memory. DCs are most potent antigen presenting cells that are able to induce T cell-mediated immunity. It is thus important to understand effects of nanomaterials on DCs if novel therapies are to be developed for immune-related diseases. The interaction of nanomaterials with dendritic cells has been investigated over the years (Villiers et al. 2010; Tkach et al. 2011), however, only a few studies have referred to the interaction of graphene with DCs.

GO sheets can be internalized by DCs due to their interaction with cell membrane receptors that are involved in endocytotic pathways as described by Russier et al. (2013). An important study in this regard was performed by Tkach et al. (2013), where they evaluated the effect of GO, C₆₀ fullerenes and C₆₀-TRIS fullerenes on antigen presenting capacity of DCs. The results showed that all three carbonaceous nanoparticles (CNPs) were efficiently uptaken by cells, however, only GO was able to

suppress the antigen presentation ability of DCs to T-lymphocytes. The reason was that the down-regulation of intracellular levels of subunit low molecular mass polypeptide 7 (LMP7) of immunoproteasome responsible for antigens processing in DCs. They tested GO-pretreated DCs for major histocompatibility complex (MHC) class 1 antigen presenting machinery components, however, only LMP7 was down-regulated. Using molecular simulation tool, LMP7 was found to bind to GO due to strong electrostatic attractions.

DCs were treated with GO and PVP-functionalized GO by Zhi et al. (2013) to study their effect on DCs maturation. Their study showed that the treatment of immature DCs for 48 h with 25 mg/mL, 50 mg/mL, and 100 mg/mL GO or PVP-GO induced significant dose-dependent maturation, as was reflected by up-regulation of CD40, CD83, and CD86. In equal concentrations, GO was more potent than PVP-GO in up-regulation of these maturation markers (Fig. 4A). The effect of GO and PVP-GO on cytokines (TNF- α , IL-1 β and IL-6) profile of DCs was also evaluated. The results showed that in contrast to GO treated DCs, PVP-GO treated DCs showed the same levels of TNF- α and IL-1 β as those of control DCs (with no GO or PVP-GO) but IL-6 level was raised in both the GO and PVP-GO treated DCs.

Fig. 4. (A) Cytokines secreted by DCs stimulated with GO and PVP-GO. 5-day DCs (5×10^5 /ml) were treated with 25, 50, and 100 mg/ml GO or PVP-GO for 48 h. Adopted from Zhi et al. (2013). (B) In vitro evaluation of the DC-mediated anti-tumor immune response. DCs were treated with saline, GO, Ag, or GO-Ag. Treated DCs were mixed with PBMCs, which in turn were mixed with the target cells (T98G human glioma cell line) to elicit immune response. Adopted from Wang et al. (2014).

(Note: Above figure needs to be expanded on whole page for better understanding of described concepts)

One important application for graphene is in nano-adjuvants. These adjuvants can greatly enhance the immunogenicity of antigens thereby helping the treatment of infectious diseases, mainly by promoting the cellular uptake of antigens and/or positively modulating the activation of antigen presenting cells such as DCs. Xu et al. (2016) demonstrated that co-functionalizing GO with polyethylene glycol (PEG) and polyethylenimine (PEI) enhanced its effect on maturation of DCs and production of cytokines. None of this effect was seen in case of GO-PEG or GO-PEI. For *in vivo* experiment using Urease B as a model antigen, they found that, as compared to free Urease B and clinically used aluminum-adjuvant based vaccine (Alum-Ure B), GO-PEG-PEI-Ure B induced stronger cellular immunity *via* intradermal administration, suggesting promising applications in cancer immunotherapy. Of course, large surface area and high positive charges on GO-PEG-PEI endows it with high antigen loading capability and intracellular delivery efficiency. Another research in DCs-based immunotherapy was performed by Wang et al. (2014). They studied anti glioma response of GO-antigen peptide with the amino acid sequence as ELTLGEFLKL (GO-Ag) pulsed DCs using T98G human glioma cell line. The DCs were pulsed for 2 h with GO, Ag, and GO-Ag separately and co-incubated for 3 days with cognate peripheral blood mononuclear cells (PBMCs). The results showed that antigen peptide-treated DCs induced a higher anti-tumor response compared to unpulsed DCs.

Moreover, GO-Ag-treated DCs induced much higher inhibition of glioma than either Ag-treated or GO-treated DCs (Fig. 4B). IFN- γ levels also serves as the indicator of anticancer immune response of T-Lymphocytes and the results showed that GO-Ag treatment resulted in a significantly higher production of IFN- γ , which indicated that GO-Ag can trigger strong immune response than free antigen peptide or GO alone.

2.4. Lymphocytes

Lymphocytes are immune cells that are involved in antibody-antigen reactions. They have receptors on their surface that are specific to respective antigen and help clear it from the affected body. Lymphocytes can be grouped into three major classes: B lymphocytes, T lymphocytes and natural killer (NK) cells. B lymphocytes are responsible for antibody-driven adaptive immunity (humoral immunity), by secreting antibodies that attach to specific antigens in order to destroy them. T lymphocytes play key role in cytotoxic adaptive immunity, by stimulating phagocytosis and promoting the release of various chemicals in response to an antigen. They do not use antibodies for their immune function. NK cells promote cytotoxic innate immunity by using small granules in their cytoplasm, which contain special proteins to destroy cells. Nanoparticles interact with lymphocytes with various outcomes including ROS production and change in cytokine profiles (Zhornik et al., 2015).

The cytotoxicity of graphene derivatives towards lymphocytes depends on the parameters such as concentration, exposure time, functionalization and type of graphene derivative used. An earlier work on the interaction of pristine graphene and GO with lymphocytes was performed by Sasidharan et al. (2012). Determined by Alamar Blue assay, neither pristine graphene nor GO showed any obvious cytotoxicity even at concentrations up to 75 μ g/ml. At low concentrations, good biocompatibility of GO towards lymphocytes, was confirmed by Ding et al. (2014) in their research on evaluation of cytotoxicity of pristine GO and different functionalizations of GO on T lymphocytes. Using GO, GO-COOH, and GO-PEI, they found that GO and GO-COOH had negligible cytotoxicity below 25 μ g/ml but notable cytotoxicity above 50 μ g/ml. Moreover, they found that GO-PEI was toxic even at 1.6 μ g/ml. It was further evaluated that high concentrations of GO caused ROS-dependent passive apoptosis by inhibiting the ligand binding ability of protein receptors, which led to passive apoptosis through the B-cell lymphoma-2 (Bcl-2) pathway. GO-COOH caused passive apoptosis without any increase in ROS production, probably by passive apoptosis signal to nucleus DNA through an ROS-independent mechanism. GO-PEI, on the other hand, was shown to exhibit severe toxicity towards T lymphocytes by inducing membrane damage.

Similar results were indicated by Cherian et al. (2014) using maura-reduced graphene oxide (MRGO) on human peripheral blood mononuclear cells (PBMCs). PBMCs include different cell populations such as T cells, B cells, monocytes, dendritic cells, and natural killer cells, which are suitable to test wide distribution of immunological effects of nanomaterials. Dose-dependent ROS generation was observed in their study and it was found that ROS induced apoptosis in PBMCs. However, MRGO neither induced genotoxicity nor generated reactive nitrogen species in these cells. GO induced apoptosis in T-lymphocytes was also studied by Zhi et al. (2013). Using GO and PVP-GO sheets of about 250 nm as an average lateral size, they showed that both induced apoptosis of T lymphocytes in a dose-dependent manner. However, the data also revealed that PVP modification delayed the process of apoptosis.

In addition, the effect of lateral size of GO such as sizes at <1.5 μ m and 1-10 nm, on PBMCs was evaluated by Orecchioni et al. (2016). No significant difference in cell viability was observed for two samples of GO, however, at molecular level, the difference was quite significant as was reflected by immune gene expression profiling. The GO with smaller size both enhanced the expression of genes such as IL-1, IL-6, IL-10, TNF- α , CD40, CD80, and CSF2 for the innate response and the expression of genes like TBX21, CD86, and CCL5 for adaptive

immunity. Moreover, the only five genes up-regulated by GO-L were those responsible for innate immune cell response.

Just like dendritic cells, lymphocytes can help make use of nano-adjuvants. In one interesting study, Meng et al. (2016) showed that carosine (an antioxidant) decorated GO (with mean lateral size of 18.2 ± 3.2 nm) proved to be a good adjuvant to enhance humoral and innate immune response in mice model. They found that OVA@GO-Car induced robust OVA-specific antibody response, which lasted for long term. Moreover, it activated CD4⁺ T and CD8⁺ T cells proliferation. The GO-Car also enhanced the antigen-specific adaptive immune response through modulating the expression of cytokines like IL-6, CXCL1, CCL2 and CSF3. As an adjuvant, GO-Car was found to induce no symptoms of allergic response or any physiological anomalies of blood. Cao et al. (2014) also showed that ultra-small GO-supported gold nanoparticles (usGO-Au) could improve immune response. *In vivo* studies demonstrated that usGO-Au@OVA could promote robust response to OVA-specific antibody, proliferation of CD4⁺ T and CD8⁺ T cells, and the secretion of TNF- α and IFN- γ .

2.5. Monocytes, neutrophils, eosinophils, NK cells, and mast cells

These immune cells have been comparatively less studied for their interactions with nanomaterials and only a few of studies are available as far as their interaction with carbon nanomaterials are concerned. NK cells and monocytes are often collectively studied in PBMCs groups. The effect of graphene derivatives on PBMCs has already been already described in lymphocytes section (Cherian et al., 2014; Orecchioni et al., 2016) where they cause ROS-mediated apoptosis and change expression of many genes. Bronchoalveolar lavage fluid (BALF) contains most of these cells and is suitable to study their interaction with graphene.

In a study by Sydlík et al. (2015), subcutaneous implantation of GO and reduced GO through transcutaneous injection was performed on mice. After exposure for 3 days, the amount of monocytes present at the interface significantly increased for the reduced GO(R-GO) as compared to GO, which suggested that R-GO was more potent than GO in attracting monocytes. They also injected GO and R-GO through intra-peritoneal route, at the same dose as was administered subcutaneously and found that GO resulted in increased accumulation of monocytes and an enhanced pro-inflammatory environment, while R-GO showed less chronic inflammation. The reason, why R-GO exhibited less inflammation, was possibly due to high clearance rates from the site of injection.

To evaluate the effect of GO on airway immune response in murine model of ovalbumin (OVA)-induced allergic asthma, Shurin et al., (2014) showed that GO exposure at the time of OVA sensitization reduced eosinophil counts compared to OVA/ALL treated mice. It must be noted that a large accumulation of eosinophils in BALF was observed on OVA sensitization in OVA/ALL mice before GO administration. Similar study was performed by Roberts et al. (2016), where mice were exposed by pharyngeal aspiration to 3 lateral sizes of graphite nanoplates (20 μ m (Gr20), 5 μ m (Gr5), and <2 μ m (Gr1) at doses of 4 or 40 μ g/mouse. At 4 h, 1 day, 7 days, 1 month, and 2 months after exposure, bronchoalveolar fluid was collected and cells were used for analysis of lung injury and inflammation. BAL analysis showed that neutrophil influx was greatest in the carbon black CB exposed group relative to all other groups at 1 day post-exposure, but was not increased in the CB group at any other time point. Gr1 also had an increase in neutrophil influx only at 1 day post-exposure. Exposure to the larger sizes of graphite nanoplates (Gr20 and Gr5) at the high dose significantly increased neutrophils in the lungs up to 1 month post-exposure, peaking at the 4 h time point and decreasing over time relative to control and to the low dose groups. Similar pattern was observed for neutrophil influx, which peaked on 1 day post-exposure for the Gr5 and Gr20 groups, however, no increase in eosinophils was found in the CB or Gr1 groups.

Moreover, a study, using a nose-only inhalation system for male Sprague-Dawley rats, was performed for 5 day repeated inhalation toxicity of graphene by Shin et al. (2015). The study was divided into three groups: control group (ambient air), low concentration one (0.68 ± 0.14 mg/m³ graphene), and high concentration one (3.86 ± 0.94 mg/m³ graphene). After the 5-day exposure and 1-day recovery, the results of the blood tests revealed notable changes in the neutrophil, lymphocyte, and monocyte counts for the high-dose group when compared with the control. After 3-day recovery period, the number of white blood cells and lymphocytes decreased significantly in the high dose group when compared with the control. However, after 28-day recovery period, only the mean corpuscular hemoglobin concentration was significantly elevated. Their study showed that graphene at a high dose may trigger immunological responses in the blood.

Furthermore, Ma-Hock et al. (2013) compared the inhalation toxicity of the various carbon-based nanomaterials on male Wistar rats. They used concentrations of 0.1, 0.5, or 2.5 mg/m³ for MWCNTs and 0.5, 2.5, or 10 mg/m³ for graphene, graphite nanoplatelets, and low-surface carbon black. Significant differences in toxicity were observed after short term inhalation exposure of four carbon-based nanomaterials, which showed that there was negligible toxicity for carbon black and graphite nanoplatelets, whereas MWCNT and graphene induced obvious lung toxicity. They observed that the toxicity of MWCNT at 2.5 mg/m³ was more than that of graphene even at 10 mg/m³.

With respect to neutrophil and graphene interaction, a study showed that GO can be biologically degraded by neutrophil-derived myeloperoxidase in presence of low concentration of hydrogen peroxide, which was reported by Kurapati et al. (2015). They used three types of GO samples with different levels of dispersability. The results showed that the degradation of various GO dispersions was proportional to the percentage of their carboxylic groups and to their aqueous colloidal stability. The degradation of CNTs by neutrophil-derived myeloperoxidase and eosinophil peroxidase has also been reported in other studies (Kagan et al., 2010; Andón et al., 2013).

As far as mast cells are concerned, Chowdhury et al. (2015) used graphene nanoribbons (GNR) noncovalently functionalized with PEG-DSPE (1, 2-distearoyl-sn-glycero-3-phosphoethanolamine-N [amino (polyethylene glycol)]) for their effects on blood vascular system components. RBL-2H3 rat mast cells treated with DNP-BSA (for inducing histamine release) produced an approximately 40% increase in histamine release compared with the activated but GNR-unexposed cells. In contrast, cells treated with GNR-PEG-DSPE or PEG-DSPE alone did not show significant increase in histamine release. Their study showed that PEG-DSPE functionalization makes graphene more biocompatible to immune system than pristine ones.

3. Mechanisms of GFN-induced immunomodulation

Pathophysiological mechanisms of biomedical nanomaterials have been well investigated in the past decade (Wang et al., 2016). GFNs have been shown to induce ROS and cause apoptosis or autophagy in many types of immune cells. A detailed insight into the mechanisms leading to these outcomes is crucial for understanding the immunological effects of GFNs. The signaling pathways that produce these toxic effects have been carefully researched over the years. Here, we give an overview of these immunomodulatory mechanisms that would help reveal the interaction dynamics of graphene with immune system components.

Autophagy is a finely orchestrated process, in which cells undergo a controllable destruction of old and damaged cellular components, misfolded proteins, and intracellular pathogens. Sometimes, autophagy is one kind of cellular response to stress. Nanomaterials interact with cell physiology and may trigger many defense mechanisms including autophagy. Nanomaterials' autophagy induced by nanomaterials seems to follow distinct course with prominent vacuolization and mitochondria mediated cytotoxicity (Khan et al., 2012; Wu et al., 2011). They may suppress Akt-mTOR signaling, alter autophagy related gene/protein

expression (e.g., Atg5), induce oxidative stress-dependent signaling (e.g., ER and mitochondria stress)(Stern et al., 2012). Similar to other nanomaterials, graphene has also been known to cause autophagy in macrophages, as was reported by Chen et al. (2012), where they show that TLR4/TLR9 signaling pathways and their downstream adaptors MyD88, TRAF6 and NF- κ B played pivotal roles in GO-induced autophagy (Fig. 5A). TLRs are generally considered to be sensors of biological molecules such as nucleic acids, proteins and polysaccharides but GO can also activate these receptors and this triggers the release of cytokines such as IL-2, IFN- γ , IL-10 and TNF- α . Beclin 1 is a protein that plays a critical role in the regulation of both autophagy and cell death. TLRs activation of downstream adaptor proteins MyD88 or TRIF, TRAF6 activates Beclin 1 to initiate have shown that e the autophagic processes. However, Wan et al. (2013) GO is able to induce significant accumulation of autophagosomes and lysosomal dysfunction, which leads to autophagy blockade in macrophages. Their analysis on the autophagic degradation target p62, showed that there was a decrease in autophagic degradation, suggesting impaired autolysosomal fusion. Significant lysosome impairment was observed which could be due to overload of lysosomes by GO. Qin et al. (2015) has shown that in GQDs induced autophagy, both beclin 1 expression and LC3-II/LC3-I conversion ratio were enhanced, confirming the accumulation of autophagosomes in macrophages exposed to GQDs. They further showed that both p38 MAPK and NF- κ B pathways were involved in GQD-induced autophagy in macrophages.

Fig. 5. A pictorial description of the different cellular pathways followed by GFNs for immunomodulation. (A) GO induced cytokine response and autophagy mediated by the TLR4/TLR9 signaling pathway. Adopted from Chen et al. (2012). (B) An elucidating of the mechanisms of programmed necrosis by GO, mediated by TLRs. Adopted from Qu et al. (2013). (C) Overview of signaling pathways involved in pristine graphene-induced cell apoptosis. Adopted from Li et al. (2012).

(Note: Above figure needs to be expanded on whole page for better understanding of described concepts)

Apoptosis is one type of programmed cell death, marked by various changes inside the cells, including nuclear fragmentation, chromatin condensation, mRNA decay, and blebbing. It is regulated by both intrinsic and extrinsic pathways, which include the activation of proteases, called caspases to degrade proteins, and execute apoptotic cell death. It is one important manifestation of nanotoxicity, which is triggered either by mitochondrial dysfunction or by activation of death receptors on cell membrane. The mitochondrial pathway often relates to ROS production by nanomaterials, which decreases mitochondrial membrane potential, promoting the release of cytochrome c, and the activation of caspase 3. Nanomaterials have been reported to induce apoptotic response (Liang et al., 2016; Wang et al., 2015). Pristine graphene decreases mitochondrial membrane potential (MMP), which induces the generation of ROS and final apoptosis in macrophages (Li et al., 2012). Graphene affects mitochondrial membrane integrity, which disrupts MMP. As a result, ROS production increases and the

redistribution of pro-apoptotic mitochondrial factors occurs. Activation of pro-apoptotic members of Bcl-2 family and mitogen activated protein kinase (MAPK) cascades induce apoptosis. The MAPKs signal pathway includes three pathways: extracellular signal-regulated kinase (ERK, components of the MAPK cascades), the p38 mitogen-activated protein kinases (p38), and c-Jun N-terminal kinase (JNK) signal pathways. In their study, all three MAPKs pathways were activated to lead to apoptosis. Moreover, TGF- β and its downstream proteins SMADs participate in apoptosis process. Both TGF-beta receptors I and II were shown to be activated along with R-SMADs (SMAD 2/3) and Co-SMAD (SMAD4) mRNA levels. As a result, pro-apoptotic members of the Bcl-2 family, Bim, and Bax got activated, which caused relocation of mitochondrial pro-apoptotic factors into the cytosol (Fig. 5B).

Similar mechanism was involved in mitochondrion-related toxicity of graphene oxide.

GO was reported to impair mitochondria, followed by a fluctuation in intracellular ROS and the release of Cyt c from mitochondria (Luo et al., 2015). Cyt c translocation then initiated activation of caspases and induced the apoptosis of macrophages. Apoptosis can occur either in active form or in passive form. Passive apoptosis is inhibited by anti-apoptotic protein Bcl-2, while active apoptosis requires TCR stimulation and death cytokines such as Fas ligand (FasL). When pristine and COOH-functionalized GO were used to expose to T lymphocytes, these cells were induced to passive apoptosis (Ding et al., 2014).

If apoptosis is a suicide, necrosis would be a train wreck. Cellular necrosis refers to non-programmed cell death due to external factors i.e. injury, infection, cancer, toxins, and inflammation, with a destiny of unregulated digestion of cellular components. Necrosis, unlike apoptosis, is always detrimental to the cell and could result from lack of supply of cellular energy sources and oxygen, depletion in ATP contents, DNA damage, followed by cellular ionic imbalance, mitochondrial dysfunctions and poly(ADP-Ribose) polymerase activation. Recently, necroptosis has been proposed to a regulated form of necrosis. Studies have shown that graphene may result in necrotic effects on various cell lines (Pan et al., 2009; Jaworski et al., 2013; Zhang et al., 2014; Begum and Fujetsu, 2013). ROS and autocrine TNF- α were involved in TLR4- dependent necrosis (Qu et al., 2013), which shows that the activation of TLR4 by GO increased TNF- α secretion and resulted in programmed necrosis in macrophages through initiating the RIP1/RIP3 centered signaling (Fig. 5C).

Fig.6. Signaling pathway of macrophage activation stimulated by graphene nanosheets. Graphene may be recognized by certain types of TLRs, thus activating kinase cascades by a MyD88-dependent mechanism. Activation of IKK initiates the phosphorylation and consequent degradation of I κ B, resulting in the release of NF- κ B subunits, and their translocation into the nucleus. NF- κ B binds to the promoter regions of its effector genes, and initiates the transcription of multiple proinflammatory genes and the secretion of various proinflammatory factors. Adopted from Zhou et al. (2012)

Moreover, graphene can also interact with plasma membrane, resulting in changed structure and disturbing its functions, even trigger inflammation. Li et al. (2013) have shown through their molecular dynamics study that the entry into cells is initiated at corners/asperities that are abundant along the irregular edges of graphene materials and local piercing by these sharp protrusions initiates membrane propagation along the extended graphene edge. This mechanism allows cellular uptake of large multilayer sheets of graphene in primary human keratinocytes, human lung epithelial cells, and murine macrophages. The disruption of plasma membrane of red blood cells (RBCs) was investigated by Liao et al. (2011) and their result indicate that the disruption of the RBC membrane is due to the strong electrostatic interactions between negatively charged oxygen groups on GO surface and positively charged phosphatidylcholine lipids which are present on the RBCs outer membrane. The same phenomenon involving disruption of plasma membrane by GO was reported by Luo et al. (2015) for macrophages, where, more positively charged functional groups on GO were more disruptive towards plasma membrane than those that were less positively charged.

Immunomodulation can be activated through interaction of graphene with cell membrane toll-like receptors. Zhou, H. et al. (2012) found that pristine graphene could interact with particular TLRs and its sub-cytotoxic concentrations are shown give a unique cytokine profile. Cytokines like Th1/Th2 including IL-1 α , IL-6, IL-10, TNF- α and GM-CSF as well as chemokines such as MCP-1, MIP-1 α , MIP-1 β and RANTES were shown to increase when sub-cytotoxic concentrations of graphene were applied. Krishnamoorthy et al. (2012) reported that graphene nanosheets and the graphene oxide (GO) could interact with bacteria outer membrane and the produced reactive oxygen free radical induced lipid peroxidation of membrane, which finally resulted in bacteria death. After interaction with plasma membrane, graphene induces lipid peroxidation which finally trigger NLRP3 inflammasome activation. lipid peroxidation due to interaction of graphene and membrane lipid may trigger stronger oxidation stress for cells. Pyrin domain-containing 3 (NLRP3) inflammasome is a complex consisting of multiple proteins that activates caspase 1, induces the processing and secretion of the pro-inflammatory cytokines such as IL-1 β and IL-18. NLRP3 inflammasome activation usually results from the lysosome rupture model and release of cathepsin B, K⁺ efflux, and ROS. ROS can induce NLRP3 inflammasome activation through the release of the ROS-sensitive NLRP3 ligand thioredoxin-interacting protein (TXNIP) from its inhibitor thioredoxin (TRX) (Tschopp et al. 2010). Ma et al. (2015) reported that GO with larger size are easily adsorbed on cell membrane and induce ROS generation, which caused lipid peroxidation. As a result, the adsorbed GO and TLRs on plasma membrane may trigger downstream of NF- κ B signaling pathways, which promoted the polarization of monocytes and enhanced the production of cytokines such as IL-6, IL-1 β , and TNF α . toll-like receptors.

4. Conclusion and Perspectives

The research account of GFNs interaction with immune system shows that there is a fundamental polarity associated with this interaction. On one hand GFNs may activate certain immune cells and on the other hand, cause suppression of maturation of others. This interaction also depends on numerous variables like lateral dimensions, oxidation, functionalization, layer number and purity of GFNs. These variables may decide whether the immune interaction of GFNs would tilt in favor of immune-suppression or immune-stimulation. This two-way immunomodulation can be very helpful in developing new vaccine adjuvants, novel drug delivery mechanisms and advanced biosensors.

The unpredictability about the outcome of the interaction of GFNs with immune system, can however be averted by careful research in this field. The extra ordinary properties of GFNs would be promising for rational use in biomedical applications in coming years.

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Graphical Abstract

Immunological Effects Of Graphene Family Nanomaterials