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PAPER

Graphene, other carbon nanomaterials and the immune system: toward nanoimmunity-by-design

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Abstract

Carbon-based materials (CBMs), such as graphene, nanodiamonds, carbon fibers, and carbon dots, have attracted a great deal scientific attention due to their potential as biomedical tools. Following exposure, particularly intravenous injection, these nanomaterials can be recognized by immune cells. Such interactions could be modulated by the different physicochemical properties of the materials (e.g. structure, size, and chemical functions), by either stimulating or suppressing the immune response. However, a harmonized cutting-edge approach for the classification of these materials based not only on their physicochemical parameters but also their immune properties has been missing. The European Commission-funded G-IMMUNOMICS and CARBO-IMmap projects aimed to fill this gap, developing a functional pipeline for the qualitative and quantitative immune characterization of graphene, graphene-related materials (GRMs), and other CBMs. The goal was to open breakthrough perspectives for the definition of the immune profiles of these materials. Here, we summarize our methodological approach, key results, and the necessary

multidisciplinary expertise ranging across various fields, from material chemistry to engineering, immunology, toxicology, and systems biology. G-IMMUNOMICS, as a partnering project of the Graphene Flagship, the largest scientific research initiative on graphene worldwide, also complemented the studies performed in the Flagship on health and environmental impact of GRMs. Finally, we present the nanoimmunity-by-design concept, developed within the projects, which can be readily applied to other 2D materials. Overall, the G-IMMUNOMICS and CARBO-IMmap projects have provided new insights on the immune impact of GRMs and CBMs, thus laying the foundation for their safe use and future translation in medicine.

1. Introduction

Graphene is one of the most renowned two-dimensional (2D) materials, characterized by a planar sheet of sp^2–carbons arranged in a hexagonal lattice [1–4]. This outstanding material has attracted increasing interest and expectation in the scientific community due to its unique physicochemical properties, including high surface-area-to-volume ratio, mechanical resistance, lightweight, flexibility, chemical inertia with respect to water and organic solvents, reduced atomic thickness, optical transparency, as well as high thermal and electrical conductivity [4, 5]. The Nobel Prize in Physics in 2010 for the ground-breaking isolation and characterization of graphene paved the way for the development of next generation GRMs, including graphene oxide (GO), few-layer graphene (FLG) [6], reduced graphene oxide (rGO), graphene nanoribbons (GNRs), and many others [7]. Beside GRMs, an eclectic family of other carbon-based materials (CBMs) is represented by several interesting materials, including nanodiamonds (NDs) [8], carbon nanotube fibers (CNTfs) [9], and carbon nanodots (CNDs) [10]. GRMs and CBMs, representing the most promising, versatile, economic, and sustainable key enabling materials to favour technological innovation and economic growth [11], are destined to leave an indelible mark in many application areas. Indeed, GBMs and CBMs have been widely investigated for their potential applications in the field of biomedicine and nanotechnology, including energy technology and nanoelectronics (e.g. cosmetics, medical devices, sensors, etc) [12–16]. In particular, due to a multitude of intrinsic exceptional properties, these materials offer new perspectives for the development of advanced applications in nanomedicine, including therapeutic delivery approaches, imaging tools, as well as devices for cancer theranostics, and tissue regeneration or engineering [17–20].

Following exposure to GRMs or CBMs, for instance in the case of biomedical applications, the nanomaterials will immediately be recognized by immune cells, the body's first line of defence against exogenous agents. These heterogeneous materials are characterized by a variety of different physicochemical properties, which could modulate their reactivity and interactions with the immune cells [21]. This makes the assessment of their health and safety risks a challenging field and complicates the implementation in translational approaches [22–24].

Rapid material innovation requires computational modelling approaches to investigate the correlation between the material physicochemical properties and their biological effects, which can then guide their design and highlight the materials that require further evaluations [25]. Understanding whether and how immune cells respond to nanomaterials by immune activation or immunosuppression might allow taking advantage of both of those selected intrinsic immune properties, for example, immunoactivation could be useful to stimulate the immune system against malignant cells in cancer immunotherapy or as vaccine adjuvants. On the other hand, immunosuppression may find applications for overactive inflammation in allergic reactions, chronic inflammation, autoimmune disorders, and organ transplantation. Previous studies, limited to a few immune cell types and characteristics compared at a time, have shed some light on the immune impact of nanomaterials [23, 24, 26–40]. However, thus far, a broad picture on the interactions of distinct immune cell types at single cell level and with a wide variety of well-characterized nanomaterials, particularly in animal models, has remained elusive. In this respect, a method for classification of nanomaterials, based not only on their chemical and physical characteristics but also on their immunological responses and immune properties, was lacking. Going in this direction, two different projects were born: 'Characterization of graphene immune-impacts through omics approaches and genotoxic analysis' (G-IMMUNOMICS), funded by the FLAG-ERA Joint Translational Call (JTC) Graphene 2015, and 'Immune activity mapping of carbon nanomaterials' (CARBO-IMmap), funded by the European Commission Marie Skłodowska-Curie actions (MSCA) Research and Innovation Staff Exchange (RISE) 2016 in the framework of H2020 programme.

In the present technical report, we share the design, methods, and part of the results for these EU-funded projects, as well as our future perspectives for the study of the impact of nanomaterials on the immune

system. We show how the multi- and interdisciplinarity were essential to reach all the projects' objectives. Moreover, we present the concept of 'nanoimmunity-by-design', explaining how this approach can guide future research on nanomaterials towards their safe application in biomedicine.

2. Objectives at a glance: towards nanoimmunity-by-design

In the field of nanotechnology, it has been highlighted that a 'safe-by-design' concept is critical for achieving safe innovation. The aim of this approach consists in obtaining less hazardous products, by a critical design based on chemical and other properties. On the other hand, 'immunity-by-design' is a very well establish concept in biomedicine aimed at providing next generation immune-targeted therapeutics through their rational design, like the antigen design in immunization implementation research for vaccines [41, 42]. We present here the possibility to apply and combine these approaches in the field of nanotechnology, under the new concept of 'nanoimmunity-by-design', not only to enhance their safety but also to fine-tune and exploit the potential immune modulation elicited by the material early in their design process. Therefore, we emphasized that, nowadays, the proper design of new nanomaterials for biomedical applications requires a deep characterization, which cannot be based solely on its physical and chemical properties but requires extending the analysis to its immunological activity. Indeed, the immune system, composed of a balanced network of molecules, cells, tissues, and organs, is the body's defense system from infection, diseases, or other potential harmful influences of the environment, including nanomaterials, while capable of exerting adequate tolerance i.e. to avoid allergy and autoimmune diseases. The immune system governs every aspect of our health. Therefore, evaluating the immune response is a key aspect to evaluate for ensuring a safe application of nanomaterials and developing new therapeutic approaches that allows precise treatment through modulating the ability of the patient's own immune system to fight diseases, by enhancing, restoring or suppressing specific immune pathways. A thorough evaluation of the immune system interaction with nanomaterials is only possible by the adoption of new advanced tools allowing multiplex analysis at a single cell levels in a wide variety of immune cell types, cell activation, and soluble mediators, taking into consideration different sorts of models [43].

The nano-immunity-by-design is going to achieve its maximum potential thanks to the collaboration with a diversified team of chemists that guarantee a homogenous material's characterization in terms of size, functional groups, lateral dimension and impurities. The clusterization of nanomaterials will be achieved combining a standard material characterization with standardized protocols that, following the guidelines proposed by the OECD, will minimize the interference due to the inevitable physicochemical characteristics of the material.

A simplified view of the nanoimmunity-by-design concept is reported in figure 1. We believe that it will be possible to achieve clusterizations of the materials based on the nanoimmunity-by-design concept. The aim is not limited to simply perform a deep screening and characterization of the heterogeneous nanomaterial family, but to propose a new method of material clusterization, by introducing a computational modeling approach to predict the materials' behavior in the biological fluids and their interaction with cells, allowing to envisage the possible activity and, consequently, the related applications and safety profile. Our goal is to perform an integrated analysis of the effects of GBMs and CBMs on human health by contextualizing their action with respect to the immune system. We are going to set up a network of interactions among a large number of biological entities, developing a novel computational tool to infer new knowledge about GBMs and CBMs behavior towards the immune system, contextualizing their mechanism of action in a system biology framework. Indeed, the use of approaches based for instance on proteomics and genomics, allowing a high-throughput functional characterization of nanomaterials, provides a large amount of data requiring a systematic integration with the patterns of molecular alteration of human diseases, drug treatments, and chemical exposures. Such systematic relationship with well-known molecular signatures, could be exploited for positioning nanomaterials in this framework, providing knowledge useful for the identification of adverse outcome pathways or mechanism of actions exploitable for biomedical approaches. We hope that this strategy will reveal new possibilities for achieving fast and accurate read-across evaluation of GBMs and CBMs based on their immune signatures.

Therefore, by introducing the nanoimmunity-by-design concept, nanomaterials can be produced by anticipating their possible applications on the basis of physicochemical and immunological parameters. The immunological effects, modulated thanks to the specific design of tuneable properties, such as dimension and functionalization, will allow the clusterization of the materials that will serve as a base to indicate the most suitable application and the safety profile for any specific type of nanomaterial. For example, biomedical nanotools characterized by specific properties that are able to induce immune activation will be useful as vaccine adjuvants or in cancer immunotherapy, where the intrinsic immune modulatory properties, can e.g. either activate the anti-tumour immunity or by-pass the immune-suppression mechanisms put in

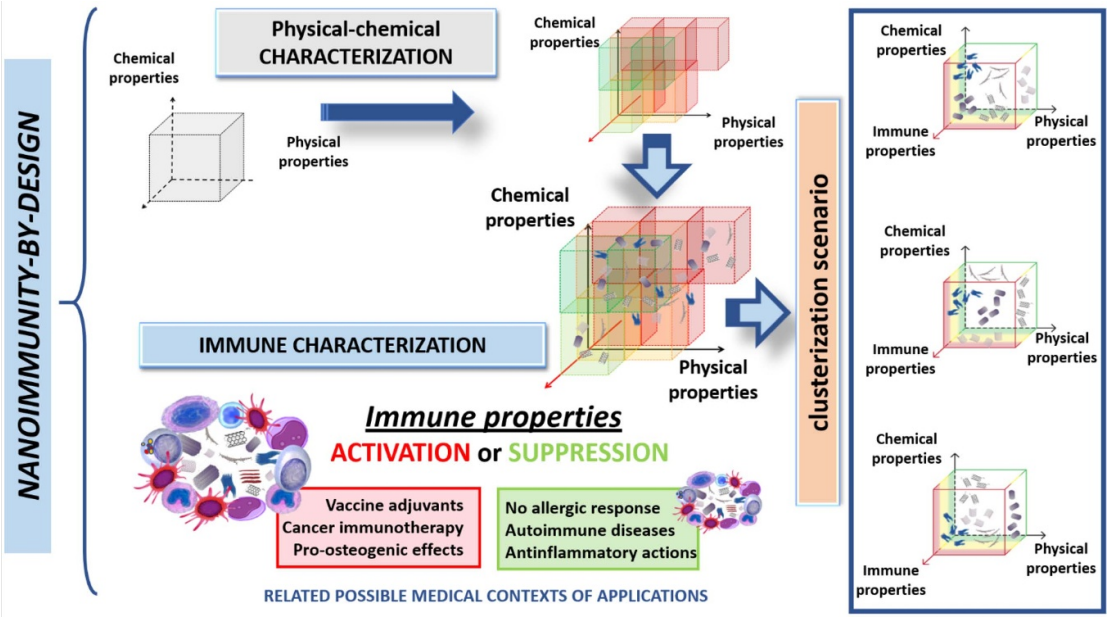

Figure 1. Graphical representation of the nanoimmunity-by-design concept. The design of nanomaterials requires an in-depth characterization process, based not only on the physicochemical properties of the materials (upper part) but also on the analysis of their immunological properties (middle part). The different immune properties may lead to an immune activation or suppression, both potentially useful in a wide variety of biomedical contexts (bottom part). By combining the immune properties with the material physicochemical properties, a clusterization of the nanomaterials can be obtained to serve as a platform for future modelling and systems biology-based approaches (right part).

place by tumor cells [18, 44]. On the other hand, material-induced immunosuppression could be exploited, as an example, for its anti-inflammatory action, or to counteract an allergic response. The choice of GRMs and CBMs as a model for proposing the nanoimmunity-by-design concept, will be of advantage for the engineering of highly specific, novel nanomaterials, with the future aim of turning them into active biomedical next generation nanosystems endowed with immune modulatory properties, which can be tailored by adjusting their physicochemical properties. Moreover, given the huge increase in the production in these nanomaterials, our approach may potentially be expanded in light of the inadvertent exposure to these materials as contaminants or as components of other commercial products. In fact, the precise and accurate definition of the toxicological profile and immune impact of a specific nanomaterial is a prerequisite not only for the development of new and safer biomedical applications but also of new nanotechnology products, in view of the exposure occurring during their industrial or small-scale production, use and waste disposal.

With this in mind, the in-depth physicochemical characterization of these materials is critical to enable the design of safe products and new nanotools, given the tight connection between the microscopic features of the nanomaterials and their biological impact [45].

In this context, the whole family of carbon nanotubes (CNTs), for years compared to asbestos for the hazardous effects shown in terms of lung toxicity [46–48], can be taken as an example. Recently CNTs have been catalogued by the Swedish non-profit organization ChemSec, as chemicals that 'should be restricted or banned in the EU' [49]. The ChemSec generalized evaluation, as highlighted by Heller *et al* [59] and Fadeel and Kostarelos [60] has contributed to create greater confusion regarding the much discussed toxicological profile of this class of nanomaterials [50–56]. Indeed, it has been evidenced that, due to its extreme heterogeneity (e.g. different length, diameter, functionalization, etc) [57, 58], the family of CNTs cannot be considered as one material category [59, 60]. Hence a correct and accurate toxicological classification of CNTs and, more in general, of GRMs and CBMs, on the basis of their physicochemical properties, is of priceless importance for their safety evaluation and, consequently, their applications [61, 62].

Currently, there is a lack of standardized guidelines for establishing regulations aimed at the characterization of nanomaterials, if compared to those used for drugs. Indeed, novel therapeutics drugs are usually subjected to a series of evaluations aimed at certifying their compliance during the entire process that brings them to the market. Moreover, the adoption of the Organisation for Economic Co-operation and Development (OECD) guidelines and the Quality by Design (QbD) procedures allows regulatory bodies, like the Food and Drug Administration (FDA) or the European Medicines Agency (EMA), to guarantee the

science and quality requirements during the development of a pharmaceutical product and its manufacturing process.

In the field of nanomaterials, the Nanomaterial Registry established the Minimal Information About Nanomaterials (MIAN) for physicochemical characteristics [63, 64], in order to address the challenge related to complexity of nanomaterial properties [65]. However, these efforts are still limited to map the knowledge we have on the huge plethora of the nanomaterials currently studied at a scientific level for biomedical and technological purposes. In particular, QbD requires the identification of the Quality Attributes (QA), such as the physicochemical and biological properties of the nanomaterials, which may change during the manufacturing process, therefore representing Critical Quality Attributes (CQA). In this view, our study will facilitate the identification of critical process parameters and materials attributes that could cause the variability of CQA.

Herein, we want to offer a 'horizon' perspective on nanomaterials categorization, the 'nanoimmunity-by-design' concept, that can provide useful information to support the development of guidelines established by regulatory bodies, such as the FDA and EMA, for the classification of nanomaterials for biomedical purposes. It is especially important to address potential immunotoxicities of nanomaterials, as noted by other authors [66].

2.1. The G-IMMUNOMICS objectives

The G-IMMUNOMICS was developed to complement part of the studies on health and environment impact of the Graphene Flagship project, the largest scientific research initiative on graphene worldwide (www.graphene-flagship.eu). The overall objective of the G-IMMUNOMICS project, was to provide new insights on the immune impact of several GRMs on different cell types and model organisms by means of an unexplored approach, based on proteomics and genomics. The specific scientific and technological aims were to:

1. Produce highly stable and dispersible pristine and functionalized GRMs, such as exfoliated graphene and GO, characterized by different lateral size and appropriate functionalization;
2. Characterize through high-throughput functional immunogenomics and proteomics approaches the immune cell response induced by GRMs on different models (e.g. human, swine, mouse, and nematodes);
3. Contribute to the public awareness on graphene advances to citizens, decision makers, and public/private stakeholders.

2.2. The CARBO-IMmap objectives

The CARBO-IMmap project, funded under the Marie Sklodowska-Curie program, had three major objectives:

1. Research objective: to develop, optimize and apply a functional pipeline based on computational model and high-throughput techniques to describe the immune activity of CBMs.
2. Training objective: to provide scientists having specific expertise in molecular nanotechnology, computational modelling, immune cell-biology, genetics and translational medicine with the opportunity to develop and apply a novel qualitative and quantitative approach to assess the impact on immune cells of a wide variety of carbon-based nanomaterials.
3. Collaborative objective: to establish and support a network of international collaborations of excellence, enabling a collaborative scientific team to effectively use a diversity of approaches to address one of the most important challenges in nanotechnology, which can open new horizons in the everyday medical practice and in the CBM production and applications.

The CARBO-IMmap project intends to overcome the limits of nanotoxicology approaches in order to achieve an inclusive and quantitative picture of the immune-activity and behaviour of CBMs in relation to their physicochemical properties.

Going beyond the conventional methods, the proposed CARBO-IMmap pipeline is integrating the expertise coming from a multidisciplinary approach: materials chemistry, computational modelling, immune functional phenotyping and genotyping.

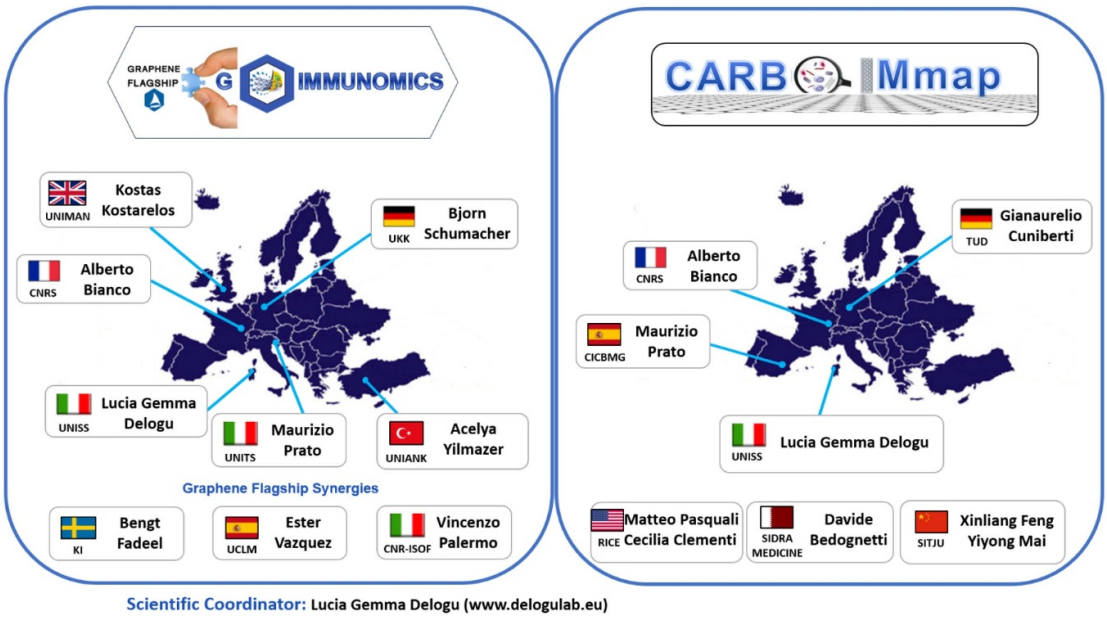

Figure 2. The G-IMMUNOMICS and CARBO-IMmap consortia.

3. The G-IMMUNOMICS and CARBO-IMmap consortia

G-IMMUNOMICS and CARBO-IMmap Consortia are reported in figure 2. The G-IMMUNOMICS project is one of the 13 partnering projects funded by the first competitive Joint Translational Call (JTC) FLAG-ERA in 2015 in the framework of the Graphene Flagship. The G-IMMUNOMICS Consortium gathers six European Research Institutions coming from five different EU Member States (Italy, France, United Kingdom, Germany and Turkey). Only the European level dimension of the engaged consortium team ensures the integration of different complementary backgrounds and expertise allowing to achieve the holistic approach here envisaged. To respond to the research challenges, G-IMMUNOMICS has been thought as a highly multidisciplinary project collecting deep expertise in chemistry, nanomaterials, biochemistry, immunology and molecular and cellular biology. The Consortium promotes the European women-friendly process ensuring a gender balance in the research team and in the management structures, where the project coordinator and one of the WP leaders are leading female scientists.

The CARBO-IMmap project was supported by the European Commission MSCA RISE in the framework of the H2020 programme. The Consortium involves four European Countries (Germany, Italy, France, and Spain) and three key non-EU countries (USA, China, and Qatar). With this project, European researchers have the opportunity to build new knowledge in international laboratories that carry out cutting-edge research in different fields. The project is enabling long-term, transformative research collaborations.

3.1. CARBO-IMmap: an opportunity of training in leading extra-EU institutions

This project will serve to establish the basis of a long-lasting collaboration between European and international partner institutions, which will also create novel career opportunities for Early-Stage Researchers at the European and International level and taking into consideration the women-friendly process in Europe.

Figure 3 shows the three extra-EU Partners of CARBO-IMmap project: Rice University (Houston, USA), Sidra Medicine (Doha, Qatar), and Jiao Tong University (Shanghai, China).

Rice University is a leading institution in nanoscale science and technology research. As the birthplace of carbon nanotechnology, Rice University has produced many leading researchers in the field, including Nobel Laureates Robert Curl and Richard Smalley. It is equipped with cutting edge instruments for analysing, modelling, and processing nano-structures.

Sidra Medicine is one of the top medical centers in the world for pioneering clinical and translational biomedical research. Founded by an impressive cash endowment of 7.9 billion, Sidra Medicine hosts internationally renowned scientists (mainly coming from prestigious USA Universities) and ultramodern infrastructures serving as a hub for biomedical research in Qatar.

Figure 3. Extra-EU partners of the CARBO-IMmap project.

Shanghai Jiao Tong University's School of Chemistry and Chemical Engineering (SCCE) was founded in 1928 as the Department of Chemistry. Nowadays, SCCE has three departments including Department of Polymer Science and Technology, Department of Chemistry, and Department of Chemical Engineering. The school has a qualified faculty of 110 full-time faculty members and staffs with over a half in developing new and functional materials for biological technology, catalysis, energy storage and conversion, and organic optoelectronic devices, among others. According to the data published by ESI, the subject of chemistry at SJTU ranks in top 1% in the world. In the 2014 QS World University Rankings, the subject of chemistry and the subject of chemical engineering rank in global top100.

4. G-IMMUNOMICS and CARBO-IMmap: a cutting-edge approach

Hand in hand with the nanotechnological and chemical development, the exposure to carbon nanomaterials, whether accidental, occupational or through biomedical applications, is a topic that has been widely discussed during the past decade or more [67, 68]. The route of exposure or administration is particularly relevant for their biological impact and can modulate the immune response elicited by the nanomaterials, their half-life, the metabolism and excretion from the human body [69].

Therefore, in our studies, with the aim to shed light on this aspect, we used different *in vitro*, ex vivo and *in vivo* models, allowing to explore different exposure routes. In particular, we focused on the intravenous and intraperitoneal injections, representing the most likely and effective administration routes in biomedicine, enabling approaches where nanomaterials can act as carriers favouring the distribution of drugs, or being themselves therapeutic agents as well as imaging dyes [17, 70].

The collective approach of the G-IMMUNOMICS and CARBO-IMmap projects, from a biological point of view, is based on high-throughput technologies, omics sciences and system biology enabling a comprehensive immune profiling, as suggested previously for other engineered nanomaterials [62].

4.1. G-IMMUNOMICS design and methods

The G-IMMUNOMICS project was based on the use of different models, as indicated in figure 4, each of them with specific particular advantages.

(1) *Sus scrofa (S. scrofa). S. scrofa* has highly conserved genetic mechanism regulating the immune response and excellent anatomical similarities with human [71–73]. Moreover, it has an excellent compatibility

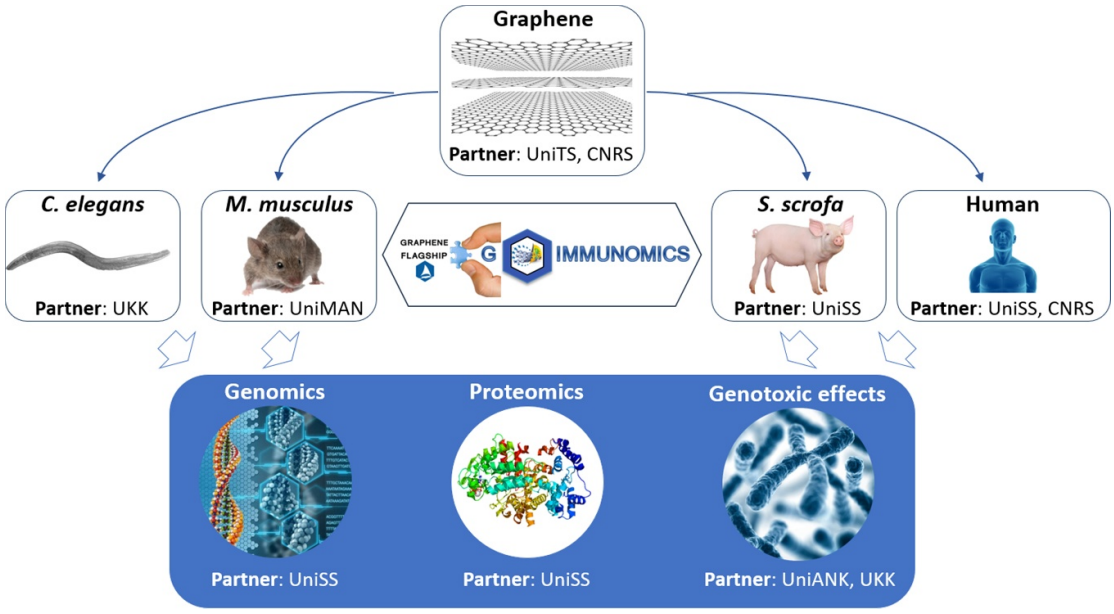

Figure 4. Overview of the G-IMMUNOMICS research models and efforts.

with human ultrasound probes and waves and magnetic resonance imaging systems [74], which makes it suitable for testing and developing graphene-based imaging diagnostic tools.

(2) *Mus musculus (M. musculus). M. musculus* is characterized by a remarkable consistency between gene expression profiles and transcriptional regulators in the mouse and human immune systems (conservatively estimated at 80%) [75–79]. Moreover, due to its exceptional easiness to produce cancer models in comparison to other species, it represents an ideal model in view of future validation of graphene-based therapeutic approaches.

(3) *Caenorhabditis elegans* (*C. elegans*). The well-defined innate immune system response of *C. elegans* is initiated by conserved MAP kinase signaling cascades [80]. The DNA damage response program induces a systemic innate immune response [81], the activation of DAF-16/FOXO-mediated stress responses [82, 83] and its fast regeneration time made it highly suitable for our purpose and its fast cycle of reproduction well applied to our purpose.

(4) *Human primary immune cells (ex vivo)*. To evaluate whether GRMs cause immune responses relevant to humans, it was essential to assess the *ex vivo* effects using human primary cells. The donors gave their explicit and written consent to the research activities. It is important to note that a buffy coat contains the white blood cells and is a waste product after the red blood cells have been used for blood transfusions. The identity of the blood donors remained unknown to the researchers. The processing and the storage of the biological samples were held in accordance with the relevant approved international procedures. Peripheral blood mononuclear cells (PBMCs) were treated with different GRMs, indeed they represent the complex mix of circulating immune cells into the blood therefore offering a close-to- human *in vivo* suitable model.

The inclusion of *in vivo* models in our study resulted beneficial since it did not only improve the knowledge on any potential interaction and possible toxicity in living organisms, but also contributed to new scientific information, allowing the assessment of the biocompatibility of functionalized materials for future translation into clinical applications.

Despite the effort in developing the science and technology to replace animals wherever possible, animals cannot yet be completely replaced. However, the research design and the description of procedures involving animal use were carefully addressed and all procedures were applied keeping in mind Russell and Burch's Three R's: Reduce, Refine, and Replace. In particular, animal experiments have been replaced with alternatives when possible. Suffering by animals was avoided or kept to a minimum. Number of animals was minimized and data maximized using cultured systems to accomplish the project objectives. Vertebrate animals have been replaced by less sentient animals. Unnecessary duplication with prior research was avoided by the reference to previously published work. Only the minimum number of animals, sufficient for statistically significant results, has been used in order to validate an experiment. All animal manipulations were performed by authorized persons and respecting ethical standards in the EU and legal requirements for

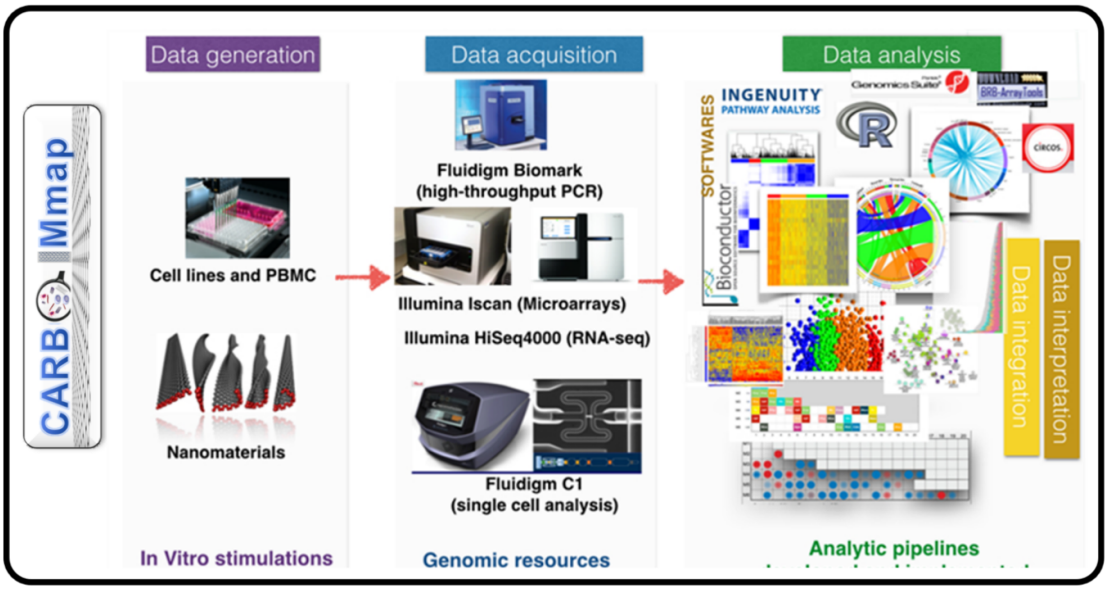

Figure 5. Overview of the transcriptomic pipeline for the CARBO-IMmap project.

keeping and handling laboratory animals in each country where mice and swine experiments were performed.

Use of cell lines. In order to avoid unnecessary animal usage, a cell-cultured based pre-screening has been carried out to characterize the impact of the materials on different cell types involved in adaptive and innate immunity (e.g. Jurkat cells, THP-1 cells, JAWS II cells, NK92 cells, etc). The use of immortalized cell lines from human or animal does not require ethical approval, as these cell lines were created outside the body and are completely anonymized.

4.2. CARBO-IMmap design and methods

As for the G-IMMUNOMICS project, also for the CARBO-IMmap project the characterization of the immune activity of the different materials went through a pervasive exploitation of *in vitro* cell lines. The impact of CBMs was initially assessed on different human cell types involved in adaptive and innate immunity (e.g. Jurkat cells, AHH1 cells, THP-1 cells, JAWS II cells, NK92 cells, etc). The obtained results will be then validated in primary cells following an *ex vivo* approach allowing to recapitulate the complex reactions occurring among the different cell subpopulations. To this end, PBMCs will be obtained from informed healthy donors (20 to 50 years old).

In the CARBO-IMmap project, the characterization of the bio- and immuno-compatibility of the materials is performed through multi-parametric flow cytometry, allowing the simultaneous analysis of diverse cell parameters. This aim is achieved thanks to the design and application of large flow cytometry panels, using specific fluorescently labelled monoclonal antibodies to detect the different PBMC immune cell subpopulations according to the expression of specific clusters of differentiation (CD) cell surface markers.

This strategy allows conducting detailed cytotoxic analysis, evaluating the impact of differently functionalized materials on cell viability, apoptosis, and proliferation of the different PBMC immune cell subpopulations. Cell activation is investigated looking at the expression of several activation markers in the different cell populations. Well-known activation molecules are used as positive controls and immune stimuli. Further analysis on the cellular uptake of CBMs are conducted for more sophisticated cell population analysis. A graphical overview of the transcriptomic approach, which is a core part of the project, is represented in figure 5.

5. Depicting the immune properties of GRMs and other CBMs

The innovative characteristics of the proposed projects, as well as the strong expertise of the partners, made possible to publish the first results of the research carried out in high impact-factors journals. In the following sections, we summarize selected publications as outcome of our research.

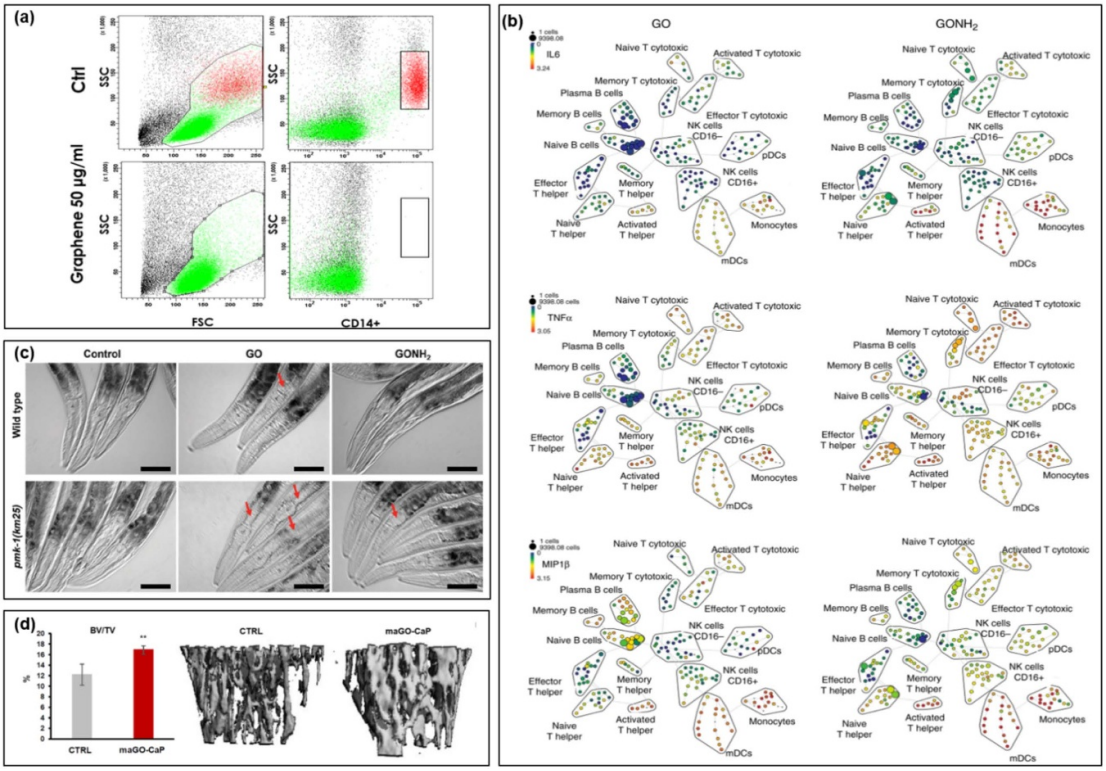

Figure 6. (a) Impact of FLG on different immune cell populations. Relative percentage of the different immune cells incubated for 24 h with 50 mg ml^{-1} FLG or left untreated. Adapted with permission from [44], copyright 2017 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim. (b) Assessment of the immunological impact of graphene using single-cell mass cytometry. SPADE (spanning tree progression analysis of density-normalized events) clustering algorithm analysis of significantly secreted cytokines. The tree plots show the different immune cell subpopulations, and the size of each cluster in the tree indicates the relative frequency of cells that fall within the dimensional confines of the node boundaries. Node color is scaled to the median intensity of marker expression of the cells within each node, expressed as a percentage of the maximum value in the data set. a: IL-6; b: TNF-α, and c: MIP-1β, for GO (left) and GO-NH_2 (right). Adapted with permission from [24], copyright 2017 Springer Nature. (c) Characteristic images of the *Caenorhabditis elegans* head region after chronic treatment at 25 °C. Red arrows show morphological abnormalities in the proximal intestine and pharynx. Size bars correspond to 50 μm. Adapted with permission from [86], copyright 2019 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim. (d) *In vivo* bone formation. Mice were injected intratibially with maGO-Cap or PBS, as a negative control. The tibias were analyzed by means of μCT. On the left, the histogram displays the ratio between trabecular bone volume and total volume (BV/TV). On the right, μCT images of untreated tibia (CTRL) and one month after treatment with maGO-CaP. Adapted with permission from [87], copyright 2019 Royal Society of Chemistry.

5.1. G-IMMUNOMICS project results

One of the key publication of the G-IMMUNOMICS is on to the FLG developed in the group of Professor. Ester Vazquez at the University of Castilla la Mancha [6]. Our study has highlighted that FLG dispersions were capable of a specific killer action towards monocytes, displaying neither toxic nor activation effects on the other immune cells [44] (figure 6(a)). FLG was able to specifically target and boost the necrosis of monocytic cancer cells. We challenged the intrinsic property of FLG to eliminate the monocytes when they are in their tumor form and without affecting the viability and functionality of other immune cell types. Myelomonocytic leukemia is an aggressive type of cancer where the monocytes are in their malignant form. FLG was able to kill *ex vivo* the tumor cells coming from myelomonocytic leukemia patients, without impacting the healthy cells. Moreover, FLG compared to etoposide, a common chemotherapeutic drug, showed a higher specificity and toxicity to the tumor cells *ex vivo*.

To deepen the understanding and the characterization of the biomolecular interactions between graphene and human immune cells, Orecchioni *et al* [24, 84, 85] applied for the first time single cell mass cytometry in this context. Compared to conventional flow cytometry, mass cytometry employs element-tagged probes that enable the discrimination of metals according to their mass/charge ratio, with minimal overlap and background cellular signal. The approach is enabling high-dimensional cytometry experiments that would not be possible otherwise and is a revolution in cell biology and immunology. In this study, single-cell mass cytometry was used to dissect the effects of graphene oxide (GO) and GO functionalized with amino groups ($GONH_2$) on 15 different immune cell populations, interrogating 30 markers at the single-cell level. Single-cell mass cytometry was integrated with genome-wide transcriptome analysis, showing that the amine groups contribute to reduce the perturbations caused by GO on cellular metabolism and increase biocompatibility. Furthermore, $GONH_2$ was proved to activate specifically T cells, DCs, and monocytes,

which were polarized to sustain a M1/Th1 immune response (figure 6(b)). This pilot study has paved the way for an innovative experimental pipeline, using single-cell mass cytometry for the assessment and deep characterization of the immune responses to any type of nanomaterials exploitable for biomedical purposes.

In order to improve the $GONH_2$ biocompatibility awareness, Rive *et al* [86] described the effects of this functionalization during *C. elegans* development and ageing upon acute or chronic exposure. Rive *et al* demonstrated that the animals require the PMK-1/p38-mediated triggering of an innate immune response when exposed to graphene oxide and this could be greatly ameliorated when the graphene oxide was amino-functionalized. In direct contrast to its pristine form, $GONH_2$ exposure did not cause detrimental effects in the wild type or in *pmk-1/p38* mutants, inducing a considerably less pronounced innate immune response. This work was able to assess the enhanced biocompatibility of $GONH_2$ in a whole organism, underlining its potential as a biomedical nanomaterial (figure 6(c)).

Graphene interaction with immune cells can be also of advantage in tissue engineering. Bordoni *et al* [87] studied the intricate process requiring the mutual interplay between immune and skeletal cells. Monocytes activation is a promising strategy to improve bone regeneration based on their ability to produce targeted pro-osteogenic signals to mesenchymal stem cells, promoting osteogenesis. A biocompatible and immune-characterized [26] nanomaterial called maGO-CaP (monocytes activator GO complexed with calcium phosphate, CaP) was designed in order to induce monocytes activation and contribute to the bone regeneration prompted by graphene and calcium phosphate, a pro-osteogenic molecule. Studying the mechanisms of action, an upregulation of Wnt and BMP signaling, two key osteogenic pathways and monocyte activation with an over-production of Oncostatin M, a pro-osteogenic factor were detected. Finally, the pro-osteogenic effects of maGO-CaP were tested *in vivo*. The graphene-based nanotool was injected into the tibia of mice enhancing local bone mass and bone formation rate, suggesting that maGO-CaP is able to activate monocytes in order to enhance the osteogenesis *ex vivo* and *in vivo* (figure 6(d)).

With respect to carbon-based nanomaterials, important steps material great strides have been made by the group of Yilmazer *et al* [88] thanks to the study of graphitic carbon nitride (g-C_3N_4) for theranostic applications. The overall results suggested that visible light photoexcitation of g-C_3N_4 could be used effectively in a PDT protocol for cancer therapy without using any other nanocarrier, additional PS or a chemotherapeutic drug. This can be considered the first study evaluating the efficacy of this g-C_3N_4 based system for *in vitro* and *in vivo* PDT by supporting the observed phenomenon through molecular mechanisms obtained via both transcriptomic and proteomic analysis.

5.2. CARBO-IMmap results

Under the CARBO-IMmap project, we will be able to immune-characterize CBMs using several high-throughput approaches.

In our previous work, funded by the G-IMMUNOMICS project, we have demonstrated that the amino-functionalization of GO was able to drastically modulate the impact on human immune cells, improving the biocompatibility [24, 86]. Hence, with the CARBO-IMmap project Fusco *et al* aimed to confirm the immune impact of nanodiamonds (NDs), a promising class of CBMs, characterized by two different types of functionalization: carboxylic acid modified NDs (NDs-COOH) and amino-functionalized NDs (NDs-NH2) by testing them on PBMC subpopulations [89]. The obtained results displayed an increased hemocompatibility by the NDs-NH_2. Both functionalizations modulated the PBMC immune response. However, the NDs-COOH caused a more pronounced reduction of cell viability and an increased regulation of the immune-modulatory transcripts, with the inflection of signalling involving type-I interferon, T cell lineage differentiation toward a T helper 2 and T helper 17 polarization, and monocytes activation. Moreover, cytokine analysis corroborated gene expression data for the proinflammatory cytokines related to the innate response (figure 7(a)). Overall, our results confirmed the increase of the immune compatibility induced by the amino functionalization.

Within the CARBO-IMmap, McCauley *et al* [9] studied the mechanical properties of carbon nanotube fibers (CNTfs) as suture materials to restore the myocardial conduction. The obtained results show that in sheep, CNTfs sewn across epicardial scar are able to acutely improve conduction. Moreover, CNTfs were capable of maintaining the conduction for 1 month after atrioventricular nodal ablation in rats in the absence of inflammatory or toxic conditions, but only in the paced condition. CNTfs resulted to facilitate local, downstream myocardial activation. Thanks to their conductivity and biocompatibility CNTfs were chosen to restore electrical conduction in diseased myocardium, allowing potential long-term therapeutic solutions in pathologies that cause, in electrically excitable tissues, the interruption of the electrical transduction. Indeed, all the large animal studies, together with the rodent ones, demonstrated an improvement in hearth conduction due to the presence of the CNTf (figures 7(b)–(f)).

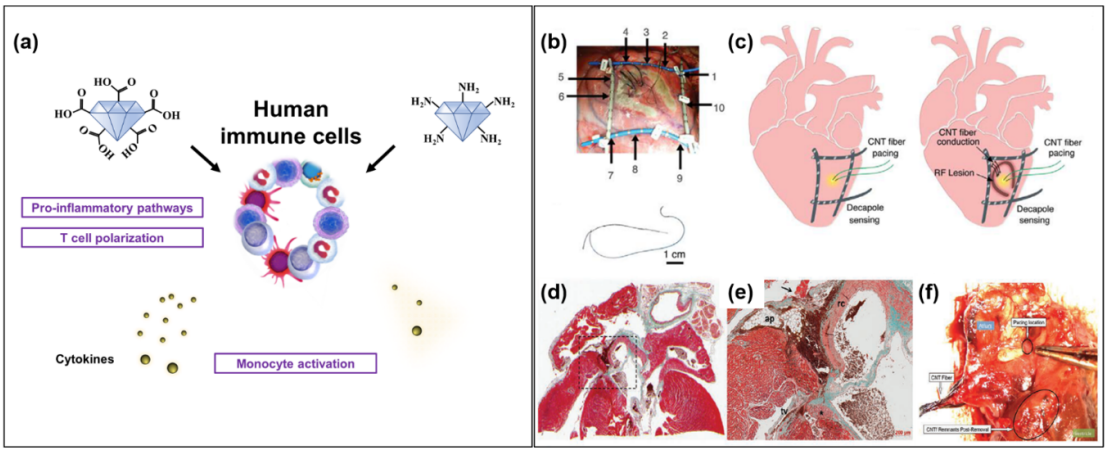

Figure 7. (a) Graphical overview of the impact of NDs on human immune cells. Adapted with permission from [89]. (b) Carbon nanotube fibers (CNTfs) enhancing electrical conduction over acutely scarred myocardial tissue. Photograph of epicardially implanted CNTf in a sheep heart. (c) Schematics of CNTf pacing and 4 decapolar sensing catheter setup before and after scar formation by radiofrequency ablation. (d) Chronic atrioventricular (AV)-bridging carbon nanotube fibers (CNTf) implants in rats at 30 d. Coronal section of a rat heart receiving 70% ethanol ablation of the AV node, stained with Masson trichrome. The dotted rectangle denotes an area of higher magnification demonstrated at the site of injection, (e) magnification of the injection site. The epicardial adipose pad (ap) was used as a landmark for the ethanol injections and shows a needle entrance wound site plugged by acute thrombus (arrow). Acute hemorrhage infiltrates the area and extends to the surroundings of the AV node (asterisk), which shows acute injury. (f) Photograph of an excised rat heart with delineation of atrium, ventricle, sites of CNTf placement, and site of remote atrial pacing. Adapted with permission from [9], copyright 2019, Wolters Kluwer Health.

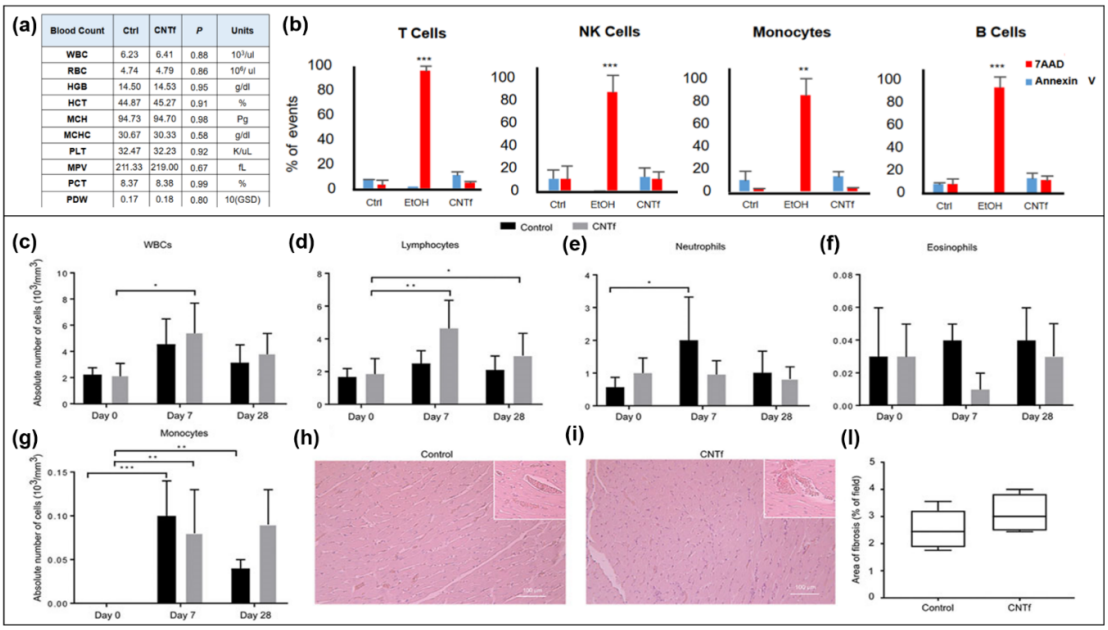

Figure 8. *Ex vivo* and *in vivo* CNTf biocompatibility (a) Human whole blood and immune cell counts after 24 h exposure. (b) Necrosis and apoptosis assessment of immune cells trough amine-reactive dye (7AAD) and Annexin V staining protocols. Ethanol was chosen as positive control. All the experiments were performed at least in triplicate. Statistical differences: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$ (Two-tailed Student's t-test).

Biocompatibility was assessed by characterizing systemic and local foreign body reaction to CNTf chronic implants both *ex vivo* and *in vivo*. CNTf exposure for 24 h did not induce any significant immune response in human whole blood from healthy donors (figure 8(a)); moreover, no evidence of apoptosis and necrosis has been observed in PBMCs (figure 8(b)). To determine whether CNTf elicited any toxicity within whole organs, whole-animal toxicity studies in mice were performed, without assessing significant muscle or organ toxicity after CNTf implantation at 30 and 90 d.

In addition, the chronic biocompatibility of the CNTf implants has been evaluated *in vivo* in terms of immune response and fibrosis induced on day 0, day 7, and day 28, with no significant difference between control and CNTf group. (figures 8(c)–(l))

CNTf (n = 5) and prolene (control, n = 5) sutures were implanted in rat hearts for 4 weeks, while leukogram analysis was performed on day 0, 7, and 28. Data were analyzed using Two-way ANOVA with multiple comparisons. At the end of the 4-week study period, no significant differences were assessed between CNTf and control groups in the number of (c) white blood cells (WBCs), (d) lymphocytes, (e) neutrophils, (f) eosinophils, or (g) monocytes. By the histological analysis of hematoxylin and eosin in the tissues surrounding the implant sites, it was shown heart tissue preservation in both (h) control and (i) CNTf groups. Fibrosis was quantified by ImageJ, using at least 10 images per animal, data were analyzed by unpaired t-test. (l) No statistical difference was reported between fibrotic areas of control and CNTf groups. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

Within the CARBO-IMmap project, another group led by Professor Xinliang Feng managed to obtain defect-free GNRs grafted with flexible poly(ethylene oxide) (PEO) chains through a bottom-up solution synthesis method [90]. The GNRs, characterised by an armchair edge structure with a width of 1.0–1.7 nm and mean lengths of 15–60 nm, enable near-infrared absorption and a low bandgap of 1.3 eV. Moreover, the PEO grafting renders the GNRs highly dispersible in common organic solvents, with a maximum concentration of $\sim$1 mg ml^{-1} (for GNR backbone), higher than that (<0.01 mg ml^{-1}) of previously reported GNRs. The PEO-functionalized GNRs are readily dispersed in water, accompanying with supramolecular helical nanowire formation. The scanning probe microscopy analysis identified raft-like self-assembled monolayers of uniform GNRs on graphite substrates. Besides being promising application in electronic devices, these GNRs are being studied for the determination of the polymer functionalized GNRs in electronic devices.

5.3. Synergies with the graphene flagship

The structure of G-IMMUNOMICS is interlinked and integrated with the Graphene Flagship [91], as demonstrated by the number of studies of the Graphene Flagship aimed at evaluating the impact of nanomaterials on health. This work, within the Graphene Flagship, builds, in part, on previous research conducted in the FP7 project NANOMMUNE in which it was shown that single-walled CNTs are susceptible to degradation by neutrophils [92] and eosinophils [93]. For example, it has been demonstrated in the Flagship that GO sheets of different lateral dimensions were effectively degraded by primary human neutrophils without signs of toxicity induced by the biodegraded GO at the pulmonary level [94]. It has also been shown that primary human monocyte-derived macrophages have the ability to internalize GO without signs of cytotoxicity, with consequent NLRP3 inflammasome activation independent of the lateral dimensions of the GO sheets [36]. In other studies of the Graphene Flagship matching and complementing the work of the G-IMMUNOMICS project, single-layer FLG was found to undergo degradation when incubated with neutrophil myeloperoxidase [95]. Moreover, GO was shown to trigger so-called neutrophil extracellular traps or NETs in primary human neutrophils leading to the entrapment of GO, suggesting that GO elicits conserved responses resembling those triggered by pathogens [96]. The surface reactivity and the lateral dimension of GO were found to be key parameters able to change the biological outcome *in vivo* in mice, with a greater recruitment of monocytic cells to the peritoneal cavity induced by small GO (<1 μm) compared to large GO (1–20 μm) [21].

5.4. Dissemination: NanoBioMed Sardinia international workshop

Within the G-IMMUNOMICS and the CARBO-IMmap projects, our commitment has been constant in particular as regards the dissemination at the international level at congresses and through invited seminars in leading institutions. The knowledge derived from our research and from these experiences was then widely shared and complemented by interventions in research institutes of international importance, such as University of Cambridge, Karolinska Institutet, Italian Institute of Technology, Houston Methodist Hospital and Research Centre, just to mention some of them. Among all the activities, aimed at boosting the transfer of knowledge achieved on graphene, it is worth to emphasize the first Workshop 'NanoBioMed Sardinia' (figure 9), organized by the University of Sassari (Sassari, Sardinia, Italy) having Dr Lucia Gemma Delogu as Chairman. NanoBioMed Sardinia aimed at providing a forum to the large community of scientists working on nanotechnology for biomedical applications. This event offered the opportunity to reflect and discuss on the most pioneering advances in nanomedicine (www.nanobiomedsardinia.eu). The workshop took place in Alghero (Sardinia, Italy) on June 24–27, 2017. The meeting was divided into several sessions based on specific themes, covered areas related to different nanomaterials, in particular CBMs, such as carbon nanotubes and graphene. International and National high-profile speakers, have reported their most recent and innovative research published in top journals including Nature, Cell and Science. Among the speakers were eight ERC grantees, more than 200 people from all over the world including USA, Germany, Qatar,

Figure 9. NanoBioMed Sardinia workshop held in Alghero, Italy, in 2017 (http://www.nanobiomedsardinia.eu/).

Spain, France, United Kingdom, Switzerland, Turkey, and China. The sessions included a total of 46 oral scientific presentations, of which nine plenary conferences, seven keynote speakers and 30 other oral communications and, moreover, numerous presentations in the form of posters. The organizers took the gender balance as a serious prerequisite in selecting the speakers, oral and poster presentations. The conspicuous program, with interventions of high scientific value, showed the enormous interest in nanobiomedicine. Many areas were touched in the workshop, ranging from the synthesis and characterization of materials, nanotoxicology, neuroscience, cardiology, cancer, and bone regeneration. During the workshop, the new consortium of the CARBO-IMmap project was presented, while some of the pertinent results obtained in the G-IMMUNOMICS project were illustrated.

6. G-IMMUNOMICS and CARBO-IMmap: what have we learned

Over the last decade, nanotechnologies have aroused increased academic and industrial interest due to the research explosion concerning new nanomaterials, attracting public growing attention and awareness [97]. In particular, graphene, GRMs, and other 2D materials have been extensively studied *in vitro* and *in vivo* as promising tools for several applications in the biomedical field. For example, these outstanding materials can serve as nanoplatforms for the development of new advanced and targeted strategies for cancer detection, diagnosis, treatment, and prevention. Moreover, the ability showed by graphene and other 2D materials, such as MXenes, to combine imaging and diagnostic modalities with different treatments into one single nanosystem makes them promising candidates for theranostic applications [17]. In this contest, we have reviewed the most recent developments of GRMs and other new 2D materials for cancer theranostics based on photodynamic therapy together with imaging, photothermal therapy, or drug and gene delivery. All the retrieved publications from 2008 to January 2019 were analyzed and reported, underlining the different applications, models, and types of cancer used (figure 10(a)). Moreover, in our recent, comprehensive review [70] we stressed how the study of 2D materials in theranostics has evolved over the years (figure 10(b)). The analysis of the most important imaging methods and the application of nanomaterials to help a non-invasive, early detection of lesions, combined with cancer therapy, provides a useful example of how nanotechnology can be exploited to overcome the current barriers in clinical practice, and how it will build a future revolutionizing pipeline of treatments, prevention, and diagnosis of cancer [98] (figure 10(c)). These papers will help scientists to engineer new non-toxic GRMs for future preclinical or clinical studies, new theranostic protocols, and non-invasive technologies, e.g. in the fight against cancer.

To enable the translation of nanotechnology to clinical practice, nanomaterials can be designed to meet the most fervid objectives of modern medicine. However, before novel materials are translated into the clinic, their potential toxicity and genotoxicity need to be thoroughly investigated, and that was one of the tasks within our G-IMMUNOMICS project. The main mechanism responsible of graphene cytotoxicity is thought

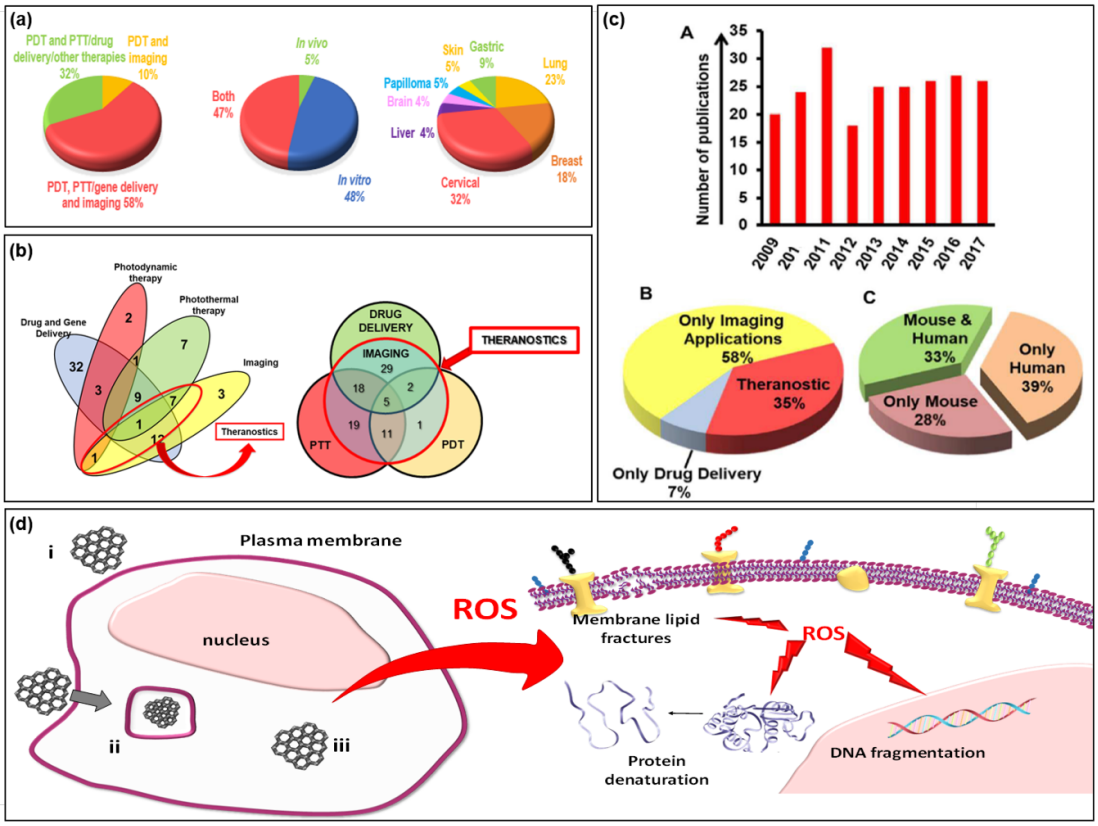

Figure 10. (a) Overview of graphene-based PDT use in theranostics. Percentages represent different type of applications combined with PDT, model used for the study (*in vivo* or *in vitro*), type of cancer studied. Adapted with permission from [17], copyright 2019 Gazzi, Fusco, Khan, Bedognetti, Zavan, Vitale, Yilmazer and Delogu. (b) Evolution of theranostic literature over the years, from [18] to [70]. Adapted with permission from [70]. (c) Status of the applications used in breast cancer studies. Analysis of publications in between 2009 and 2017. Percentages of publications on imaging, theranostic and drug delivery applications. Model adopted for each publication (human, mouse and combination of human and mouse). Adapted with permission from [98], copyright 2018 The Royal Society of Chemistry. (d) Graphene-induced reactive oxygen species (ROS) production and consequent mechanism of toxicity and genotoxicity. Adapted with permission from [99], copyright 2019 The Author(s). Published by IOP Publishing Ltd.

to be represented by reactive oxygen species (ROS) production, and ROS, in turn, are able to interact with various biomolecules, including DNA, inducing severe damages (figure 9(d)).

In the recent review by Gurcan *et al* [99] different genotoxicity studies, performed with GRMs, have been dissected with a specific focus on different cell types and conditions.

Nanomaterials can enter into the body following different exposures routes, including possible intravenous injection during biomedical applications, therefore encountering the immune cells, representing the first line of defence against exogenous agents. Therefore, independently from the final purpose, a crucial phase for future translational applications of nanomaterials is represented by the assessment of their impact and biocompatibility on the immune system [18, 22, 23, 40]. GRMs and CBMs do not represent a single entity, therefore it is necessary to clarify the structure-immune-activity relationship for this heterogeneous class of materials, each of them with specific characteristics [100]. In this regard, G-IMMUNOMICS and CARBO-IMmap expanded the knowledge horizon in the field of nanomaterial immune compatibility and modulation, as demonstrated by the resulting papers revealing how different types of GRMs can either stimulate or suppress the immune response based on their size, lateral dimension, shape variation and surface chemistry [9, 24, 26, 44, 86], the same concept can be applied to a wide variety of other nanomaterials. Moreover, the understanding of the biomolecular interactions between GRMs and human immune cells is a prerequisite for their safe exploitation for applications outside of biomedicine. It is essential to assess GRM and CBM human hazard using *in vitro* and *in vivo* models with the aim to understand the mechanisms that underlie the biological effects. Deciphering the interactions of engineered nanomaterials with the immune system has a considerable toxicological relevance. Inflammation consists in a complex biochemical response, requiring cells and soluble factors, that arises in tissues as direct response to adverse stimuli: pathogens, toxicants, or dead cells. This process normally leads to a temporary damage followed by recovery and healing. However, inflammation can also induce chronic tissue damages and even cause a neoplastic transformation. Numerous studies were focused on nanomaterial interactions with macrophages,

Table 1. Take home messages for successful and safe applications of nanomaterials.

Heterogeneity of nanomaterials
Nanomaterials are characterized by a variety of different physicochemical properties (e.g. size, number of layers, functionalization, etc), making them different from each other. Therefore, it is crucial to deeply characterize the nanomaterials and investigate the relationship between the structure and the activity of the materials. The specific nanostructure can be designed to modulate different physicochemical properties and the consequent biological and immunological impacts suitable for specific biomedical applications.
Multi-interdisciplinary approach
To fully clarify the biological impact of nanomaterials the scientific community is becoming aware that a multi-interdisciplinary approach is required, involving the cooperation among scientists whose efforts rely on different fields such as material science, physics, engineering, chemistry, biology, immunology and toxicology.
Interactions with the immune system
The impact of nanomaterials, including graphene on human health, still need to be fully elucidated. A key aspect to ensure a safe use of nanomaterials in our everyday life and their successful applications in biomedicine is represented by the study of interaction with the complex entities of the blood immune cells. Two are the main routes of exposure that may lead to the recognition of blood immune cells: inhalation (i.e. in workplaces) and intravenous injection (i.e. for biomedical applications). In both cases, the study of immune interactions through high-throughput screening and a wide variety of models (e.g. *C. elegans*, mice, and swine) is required to capture the emergent biological properties of graphene and other carbon materials.

less attention was paid to neutrophils, despite neutrophils, as key factors in inflammation, are the most abundant population among white blood cells. With this in mind, Keshavan *et al* [101] dissected the impact of engineered nanomaterials on neutrophils describing how these cells, in turn, may digest certain CBMs (carbon nanotubes and GO). Indeed, the discrimination between acute and chronic inflammation is considered a fundamental step in toxicology.

6.1. Conclusions and future perspectives

Overall, our results and critical view of the literature is expanding the awareness of the importance of nanomaterial immune interactions and the potential implications for safe future development in many contexts, including in biomedicine. The take-home messages for successful and safe applications of nanomaterials are summarized in table 1.

Thanks to the concept of nanoimmunity-by-design, where the production and use of nanomaterials is not solely based on their physicochemical properties but also shaped by their predictable immune properties, we are encouraged to further investigate the immune impact of emerging 2D materials and other advanced materials. The final goal is to lay the basis for a safe technology exploitation by defining the key features of highly immune-compatible materials for biomedical applications. We also believe that our wide-ranging study and the proposed method will be able, in the following years, to ensure the reduction of research and production times in order to encourage a faster and safer advancement of nanotechnologies aimed at a purely nanobiomedical use. In this sense, we addressed the advancements obtained during the two projects G-IMMUNOMICS and CARBO-IMmap; we are confident that those can help the research to bridge the gap of knowledge on nanomaterials by opening the doors to a new nanomedicine.

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