Review

Carbon Nanotubes Under Scrutiny: Their Toxicity and Utility in Mesothelioma Research

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Abstract: Research on the toxicity of engineered carbon nanotubes (CNT) was initiated by Belgian academic chemists and toxicologists more than 15 years ago. It is now undisputed that some of these attractive nanomaterials induce serious illness such as fibrosis and cancer. The physico-chemical determinants of CNT-induced adverse effects are now elucidated and include shape, nanoscale diameter, and structural defects. Generated in vitro and in vivo data on their inflammogenic and fibrogenic activities were combined and translated in AOP (adverse outcome pathways) available for risk assessment and regulatory policies. The asbestos-like carcinogenic effect of CNT, notably their capacity to induce malignant mesothelioma (MM), remain, however, a cause of concern for public health and strongly curb the craze for CNT in industries. MM still represents a real challenge for clinicians and a highly refractory cancer to existing therapeutic strategies. By comparing mesotheliomagenic CNT (needle-like CNT-N) to non mesotheliomagenic CNT (tangled-like CNT-T), our group generated a relevant animal model that highlights immune pathways specifically associated to the carcinogenic process. Evidence indicates that only CNT-N possess the intrinsic capacity to induce a preferential, rapid, and sustained accumulation of host immunosuppressive cells that subvert immune surveillance and suppress anti-mesothelioma immunity. This new concept offers novel horizons for the clinical management of mesothelioma and represents an additional tool for predicting the mesotheliomagenic activity of newly elaborated CNT or nanoparticles.

Keywords: inflammation; immunosuppression; cancer; mesothelioma; carbon nanotubes; asbestos; nanoparticles; physico-chemical determinants; genotoxicity; animal; human

1. Discovering the CNT

The architect of carbon nanotubes (CNT) is Sumio Iijima, a Japanese physicist awarded with the prestigious Benjamin Franklin Medal in 2002 “for the discovery and elucidation of the atomic structure and helical character of multi-wall and single-wall carbon nanotubes” [1]. The formation of “carbon needles” of few nanometres in diameter suggested that the manufacture of engineered carbon structures should be possible on very large scales. This technical possibility brought a real enthusiasm in chemistry and several academic teams completed this discovery by proposing complementary procedures to elaborate tailored CNT. Professor János B. Nagy and leading colleagues from the Université de Namur in Belgium described in Science an unsuspected extrusion of a carbon tubule from a catalytic particle [2].

These success stories had a huge impact on the rapidly growing material and nanoscale science field. The physico-chemical characteristics (such as the very high thermal, electrical conductivity, tensile strength and stiffness) attracted the attention of scientists of different domains and the interest of
many industrial sectors (mainly in electronics). The production of CNT then emerged from university confinement and was deployed on a larger scale in the early 2000s in spin-offs and newly built industries such as Nanocyl in Belgium. However, this craze was short-lived. The discovery of their toxicity (see below), the difficulty of large-scale synthesis and their high selling price explain slowdown in global demand for CNT during that disillusionment period.

However, industrial production of CNT is now experiencing a revival of interest. Plastic and battery suppliers recently assimilated the major interest of CNT and revived the market. Today, the management of the harmful effects of CNT is no longer be considered as an impassable barrier. Indeed, safe-by-design approaches now offer tailored CNT characterized by weak toxicity and limited health concerns [3,4]. Several recent reports mention that the worldwide market for CNT is estimated to grow from 5 billion U.S. dollars in 2018, to 10 billion by 2023 and 15 billion in 2026. During this forecast period, a CAGR (Compound annual growth rate) of 20% is expected. The Yano Research Institute Ltd. (Tokyo, Japan) released a report predicting a global CNT market of 4000 tons by 2023, representing a compound annual growth of 13% (2250 tons in 2008 when considering the global CNT market) [5].

Chemical vapor deposition (with or without catalysts) represents the main synthetic technique, and accounts for more than 70% of the global production. Currently, efficient synthesis and declined prices for CNT make these nanomaterials more available and affordable [6]. Multi-walled CNT dominate the market and their increasing applications concern battery additives and plastic parts [7,8]. CNT also hold absorbent potential for organic wastewater treatment or emerging contaminants because of their high specific surface area and mesoporous structure [9–11]. The recent ability to functionalize CNT or control their growth and assembly [12] has given rise to the development of new biomedical applications, including tissue growth and drug delivery [3,13]. For instance, CNT-based materials permit the elaboration of layered cell architectures for embryonic stem cell and spheroid expansion during tissue regeneration [14,15].

2. From Asbestos to CNT: The Toxicological Aspect

2.1. Morphological Similitudes between CNT and Asbestos

Despite the scientific and industrial enthusiasm born from the discovery of CNT, several toxicologists including the Japanese Jun Kano [16] rapidly noticed physical and morphological similarities between CNT and asbestos fibers. Indeed, these particles have a long needle shape (term already used by Iijima) and this morphology (longer than 10 µm) makes them foreign bodies difficult to be removed and therefore interfering with the immune system. This intuitive approach led some toxicologists to test the detrimental effects of CNT in comparison to asbestos.

The prevailing idea prior to these experiments was that CNT (consisting predominantly of carbon) were perfectly biocompatible and non-toxic. However, several groups, including ours in 2005 [17] (Figure 1) reported that CNT injected into the animal lungs were able to induce inflammatory, fibrogenic and carcinogenic responses [16,18–21]. These results, like many others, thereafter, had a very significant global impact (reports widely cited; sometimes more than 1000 times) and initiated extensive in vitro and in vivo investigations focused on CNT toxicity [22]. Altogether, these findings demonstrated that the respiratory diseases induced by CNT (fibrosis and cancer) match precisely to the pathologies observed after asbestos exposure [23].

2.2. Inflammogenic and Fibrogenic Effects of CNT: Mechanisms and Adverse Outcome Pathways (AOP)

Several decades were necessary to define the exact pathogenesis of CNT-induced inflammation and fibrosis (Figure 1). A common scenario is now accepted and can be summarized as follows. Scavenger receptors deployed by sentinel macrophages ensure CNT phagocytosis [24–26]. Internalized cytotoxic CNT destabilize phagolysosomes in macrophages and lysosomal contents released into their cytoplasm activate a sensing cytosolic complex (named inflammasome) which converts immature cytokines (pro IL-1β) into highly inflammatory mediators (active IL-1β). Our group demonstrated that the
permeabilization of lysosomes also induces cell membranolysis and the subsequent release of danger signals (named alarmins) stored in macrophage cytoplasm [25,27,28]. Altogether, these inflammatory mediators orchestrate the persistent accumulation of neutrophils and macrophages. Oxidizing molecules produced by these inflammatory cells strongly damage surrounding tissues [24]. The inflammatory cycles are then followed by a reparative stage, where damaged tissues are renewed. This regeneration involves fibroblast activation by growth factors produced by activated macrophages [29,30]. We have also demonstrated that toxic CNT possess, by themselves, the unique ability to induce directly fibroblast proliferation, differentiation and collagen production [31]. When CNT persist into the tissue, remodeling is therefore permanent and degenerates into uncontrolled scar and fibrosis [24].

Figure 1. Timeline summarizing the findings provided by our laboratory (LTAP, UCLouvain) on CNT toxicity. For this collective and international research work, we used diverse in vivo and in vitro models and CNT possessing different morphological and chemical properties. These findings can be exploited to support fundamental and applied research on mesothelioma.

The global interpretation of these sophisticated mechanisms resulted in the elaboration of several “adverse outcome pathways” (AOP), which detail the exact sequence of molecular and cellular events required for inflammation and fibrosis development after CNT exposure (e.g., AOP #173). AOP are now available to improve current in vitro assays for predicting inflammatory and fibrogenic effects of CNT and to assess health risks for human [29,32,33] (Figure 1).

2.3. The Key Importance of Physico-Chemical Characteristics

The physicochemical features responsible for the inflammogenic and fibrogenic activities of CNT were defined by using chemically and morphologically modified CNT as well as validated in vitro and in vivo models. Size and shape strongly affect CNT toxicity (Figure 1). Our data showed that long CNT were more potent than short CNTs to stimulate fibroblasts or macrophages, indicating that longer particles are more efficient in inducing inflammation and fibrosis [29,34]. CNT diameter was also identified as an important factor since thin CNT are more toxic for the lungs compared to thick CNT [35]. The morphology also determines their capacity to induce inflammation and fibrosis. Tangled CNT are less inflammatory and fibrogenic than straight (needled) CNT [36,37]. Furthermore, the surface reactivity plays a direct role in the ability of CNT to induce inflammatory and fibrotic responses. We demonstrated that the toxic effects of CNT are related to defective sites in the C framework. Structural defects introduced in CNT by fracturing procedures increase inflammatory and fibrogenic activity [38,39]. Oxidative stress caused by toxic particles (i.e., silica and asbestos) and/or inflammatory cells (i.e., neutrophils and macrophages) is implicated in fibrosis and cancer. We proposed, however, that other CNT features than free radical generation govern the toxic potential of CNT. Indeed, CNT exhibit a remarkable radical scavenging capacity and quench rather than generate oxygenated free radicals. This scavenging activity was related to CNT defects and their inflammatory and fibrotic potentials [39] (Figure 1).
3. The Mesotheliomagenic Activity of CNT: A Remaining Issue

3.1. Mesothelioma and Particles

Malignant mesothelioma (MM) is a cancer affecting the mesothelium, a layer of squamous cells covering the serous cavities (pleura, peritoneum, pericardium) and protecting the organs (lungs, peritoneal organs, heart). The most commonly affected tissue is the pleura and peritoneum, and this is referred to as pleural and peritoneal malignant mesothelioma [1,2].

MM is an uncommon cancer and most of the cases are due to asbestos exposure [3]. In 1997, all asbestos fibers were classified as carcinogenic to human by IARC (International Agency for Research on Cancer, monograph 100C) because they are inhalable, poorly soluble and can migrate from the lungs to the pleura or peritoneum, directly or through the lymphatic system [3]. These low-degradable fibers trigger MM and other terminal pathologies such as pulmonary fibrosis and bronchial cancer in animal and human [4].

MM can occur more than 40 years after exposure. Considering the use of asbestos until the 1980s and the long latency period of the disease, a peak incidence is expected for 2020 [5]. Unfortunately, patients often reach the final stage of the disease when they are detected making the prognosis of MM very poor (12–15 months) [6,7]. Treatment with conventional therapies is not effective. The classical clinical management is chemotherapy based on platinum salts (alkylating agents) and Pemetrexed (anti-metabolite), which prolongs the patient survival by only 15 months [8]. Debates also remain regarding other multimodal approaches as surgery and radiation [8,9].

Several in vivo studies then demonstrated that the long and straight CNT-Mitsui-7 are indubitably mesotheliomagenic as asbestos fibers (see below) [10,11]. In 2014, IARC debated on the carcinogenicity of CNTs (monograph 111) and the consulted experts classified CNT-7 in Group 2b, i.e. as possibly carcinogenic to human [12,13]. All other CNTs (single-walled or multi-walled nanotubes) were classified in group 3 (not classifiable as to their carcinogenicity to human) [14].

3.2. Advances in Understanding Mesothelioma Development

The precise cellular and molecular mechanisms explaining asbestos- or CNT-induced MM are difficult to investigate. Indeed, mesothelial cells progressively acquire features common to cancer and tissues representing non-advanced stages of the disease are difficult to obtain. Nevertheless, several pathological mechanisms have already been identified by using human mesothelioma cell lines and biopsies as well as animal models [4,15].

3.2.1. Direct Effects and DNA Damages

The carcinogenicity of asbestos is related to their physical and chemical properties. Once fibers reach the pleural or peritoneal cavity, longer particles accumulate and are directly in contact with mesothelial cells [16–18]. Asbestos fibers generate reactive oxygen species (ROS) and induce ROS production by exposed mesothelial cells. ROS trigger genomic instability and mutations when interacting with mesothelial cells [19]. These oxidant molecules also activate various signalling pathways, inducing transformed cell proliferation and survival [16–18]. ROS are therefore involved in the initiation, promotion, and progression of cancer, which represent the three stages of the carcinogenic process [20].

Asbestos fibers are known to penetrate cell membrane of mesothelial cells and interact with intracellular molecules resulting in direct (or primary) genotoxicity that include DNA strand breaks, mutations and chromosomal aberrations [19]. Recent studies show that CNT induce chromosomal disruptions, fragmentations, and translocations [21,22] and their nuclear deposition results in clear epigenetic alterations [23–25]. The quenching capacity of CNT is involved in their primary genotoxic effects [26] (Figure 1). However, the literature regarding the direct interaction of asbestos or CNT with DNA of mesothelial cells is contradictory. The team of Mossman [27] states that this process does not
exist, unlike most other chemical carcinogens. The non-clastogenicity of the CNT observed by Sasaki suggest that the CNT may not directly interact with DNA [28].

3.2.2. Inflammatory Responses as a Driver of Malignant Mesothelioma Development

Numerous studies indicate that mesotheliomagenic particles induce secondary genotoxic damages by promoting inflammation and subsequent free radical release [29–31] (Figure 2). Resident macrophages present in mesothelial cell-covered tissues, detect, phagocytize and attempt to degrade inhaled fibers [32]. When these particles are long and resistant to degradation by macrophages, they trigger a phenomenon known as frustrated phagocytosis [17,20], which result in the constant and prolonged release of highly reactive inflammatory mediators [33,34]. These mediators notably recruit neutrophils and additional macrophages for further particle clearance [17,20,34,35]. However, this particle removal process is not efficient for persistent fibers and the inflammation becomes consequently chronic [11,17,20].

**Figure 2.** Persistent inflammation and immunosuppression conjointly orchestrate carcinogenesis and mesothelioma. Mesotheliomagenic needled-like CNT-N induce an inflammatory cascade (in blue) resulting in the influx of inflammatory macrophages and neutrophils. Sustained production of free radicals by these activated immune cells induces irreversible DNA damage. Pro-inflammatory cytokines are also considered as potent polypeptide growth factors to induce mesothelial cell transformation and angiogenesis. An unexpected conjoint immunosuppression (in red) is additionally induced by mesotheliomagenic CNT. This early responses to CNT-N is characterized by a persistent accumulation of immunosuppressive macrophages and monocytes and a sustained production of anti-inflammatory cytokines (i.e., IL-10 and TGF-β). These immunoregulatory components are incriminated in carcinogenesis by preventing host immune responses directed against transformed cells and favoring tumor growth.

ROS deriving from frustrated macrophages and neutrophils (Figure 2) cause direct mutations and promote proliferation and invasion of transformed mesothelial cells by modulating cell signalling pathways (see above) [16,20,36]. These inflammatory cells also permit mutated mesothelial cells to avoid apoptosis [37]. Indeed, cytokines and alarmins such as TNF-α and HMGB1 produced by macrophages and neutrophils activate the NF-κB (Nuclear Factor Kappa B) signalling pathway in...
mesothelial cells. This transcription factor then induces the expression of various genes promoting cell survival [38,39]. These inflammatory mediators also convert mesothelial cells to inflammatory cell partner that release growth and differentiation factors for stem (M- and GMSCF) [40] and endothelial cells (VEGF) [41]. Together, these factors increase the survival of transformed mesothelial cells and allow angiogenesis and neoplastic cell migration into the tissue (Figure 2).

3.2.3. Tolerant Microenvironment and Immunosuppressive Cells within Mesothelial Tumors

Overall, these convincing data therefore demonstrate that chronic inflammation contributes to the initiation, promotion, and progression of MM. However, several recent studies have suggested additional mechanisms to explain the various pathological profiles among clinical cases. In recent years, a new concept was developed proposing that the immune evasion allows mesothelioma to evade host anti-tumor responses [42–44].

It is now admitted that hematopoietic and lymphocytic immune cells infiltrating MM are reprogrammed by their new microenvironment and play a critical role in the maintenance and progression of cancer. These immunosuppressive or immunoregulatory cells create a tolerant environment by blocking T lymphocytes (dedicated to recognize and eliminate mutated cells) and stimulate tumor growth by promoting angiogenesis, stroma deposition and metastatic tumor formation [45]. Immunosuppressive host cells invading mesothelioma include regulatory T lymphocytes (T regs) [46,47], Myeloid-Derived Suppressor Cells (MDSCs) [48] and Tumor-Associated Macrophages (TAMs) [49,50]. Soluble factors (IL-10, TGF-β and PGE2) and immune checkpoint ligands (PD-1 and CTLA-4) represent the main elements contributing to the establishment of an immunosuppressive microenvironment [42].

The discovery of this tolerant tumor environment resulted in new clinical approaches to control mesothelioma. Immunotherapy aims to boost immunity and block immunosuppressive capacities of tumor cells. The current clinical option to reverse immunosuppressive mechanisms is to inhibit immune checkpoints (anti CTLA-4 and PDL-1 neutralizing antibodies) during cytoreduction therapy (chemotherapy, radiotherapy and surgery) [43,44,51–53]. Tumor immune escapes are also operative in animal models of mesothelioma and innovative therapeutic strategies modifying immunosuppressive monocyte and macrophage differentiation are now successfully implemented [40,54].

3.2.4. The Co-Existence of Early Inflammation and Immunosuppression after Mesotheliomagenic Particle-Exposure.

Our team demonstrated that immunosuppressive responses were not exclusively generated by tumor cells but also by mesotheliomagenic CNT themselves. Indeed, these CNT induce an early immunosuppressive environment by recruiting immunosuppressive M-MDSCs and macrophages after few days in injected rats (Figures 1 and 2) [55]. This effect is associated with the acute neutrophilic inflammation already well described (see above). The early presence of MDSC was later confirmed by other teams [56,57]. Increased expression of immunosuppressive mediators such as IL-10, TGF-β, NADPH oxidase and prostaglandin synthase was also noted in murine lungs after exposure to mesotheliomagenic CNT [58,59]. The rapid development of local and systemic immunosuppressive immune responses reduce the number of circulating T lymphocytes and their ability to proliferate [60–62]. This effect is mainly related to IL-10 and TGF-β [59]. Kido and colleagues also noted a rapid increase of IL-10 expression by macrophages after CNT inhalation in rats [63]. IL-10 release by macrophages results in IL-2 deficiency (an essential factor for T cell proliferation) and reduces antitumor activity of T lymphocytes.

Altogether, these results indicate that CNT rapidly induce an immunosuppressive environment, affect T lymphocyte activity and expand transformed mesothelial cells generated by conjoint inflammatory elements (Figure 2). The requirement of these dual environments can explain the diversity of clinical situations and long latency period of mesothelioma (Figure 2).
4. An Unexpected Application: The Diversity of CNT to Identify New Pathological Pathways in Malignant Mesothelioma

4.1. Toxic Needled-Like (CNT-N) Versus Non-Toxic Tangled-Like (CNT-T) Carbon Nanotubes

Several animal models have been established to study mesothelioma by using asbestos fibers. Intraperitoneal injection is the preferred administration route because long, straight, fibrous and solid fibers directly reach mesothelial cells of the peritoneal cavity. In addition, the clearance mechanisms of this cavity are similar to those of pleural cavity, which is the predilection site for malignant mesothelioma [17,18]. Not all rodents are equally sensitive to mesotheliomagenic fibers. Indeed, the incidence in rats is higher than in mice, which therefore appears to be more resistant to mesothelioma development [31].

The mesotheliomagenic properties of CNT represent an additional interesting tool for investigating mesothelioma in animals. We have shown that CNT-induced mesothelioma affects all treated rats (only 30–50% of animals injected with asbestos) in a limited period (six months for CNT instead of two years for asbestos) [55,64]. The other key advantage of CNT compared to natural asbestos is the existence of a very wide range of manufactured CNT. These particles can be categorized from a structural and toxicological point of view by using their morphology (tangled CNT T versus needled/straight/rod CNT N). CNT-T are thin enough to fold and self-assemble into short, tangled aggregates, while straight CNT-N are fibrous, resistant and long. Unlike asbestos fibers, which are all considered carcinogenic, several studies demonstrate that only CNT-N are associated with the development of chronic pathologies (including mesothelioma) unlike CNT-T, which are poorly reactive and toxic [55,64–66]. The exact reasons for this discrepancy have not yet been fully elucidated. The greater bio-persistence of CNT-N (longer and therefore more difficult to clear) is one possible explanation.

In that respect, Poland and co-authors reported frustrated phagocytosis by macrophages in rodents treated with CNT-N. Accordingly, these needled CNT-N are not completely covered by phagocytes and remain biopersistent in the tissue. In contrast, tangled CNT-T are entirely phagocytosed and taken up by macrophages resulting in tissue translocation, particle biodegradation and accelerated rate of clearance [67,68]. Nanotube geometry is also crucial in the development of inflammation and granuloma formation in animals. Only CNT-N exposure resulted in neutrophil accumulation and fibrotic granulomas contrary to CNT-T [69,70]. Needled CNT-N were more potent than tangled CNT-T to elicit inflammatory effects towards macrophages [71,72].

For Sasaki and colleagues, the shape- and length-related structural determinants of CNT are also crucial factors for their potential carcinogenic activity. Straight and fibrous CNT-N were the strongest inducers of chromosomal aberrations in cell cultures [28]. CNT-N elicit a more pronounced primary genotoxicity effect than CNT-T, as assessed by DNA damage and micronuclei formation [73]. These in vitro observations are in accordance with data obtained in vivo. CNT-N cause secondary genotoxicity and mesothelioma in rodents (absolute incidences of 100%) contrary to CNT-T (no genotoxicity and tumor) [55,64–66,73,74].

4.2. A New Strategy to Identify Pathogenic Immune Pathways: Gene or Protein Expression by Purified Macrophages after Needled and Tangled CNT Exposure

The paradigm associating physicochemical characteristics and carcinogenicity of CNT represents a major asset for risk management and predictive toxicology. However, these data do not entirely explain the reasons why some CNT induce or not mesothelioma, and do not offer specific mechanisms or targets for clinical management or drug development against mesothelioma. RNA-related profiling methods and next-generation sequencing (NGS) technologies were elaborated to cartography genes expressed by purified cell populations. Bioinformatic analysis of these (big) data often reveal unexpected pathological axis at the level of molecular and cell biology [75].

We used this new strategy to shed light on the immune events that specifically regulate mesothelioma development by comparing the effects of CNT on macrophages (Figures 3 and 4). We
used our model of early responses (induced by particles and not tumor cells) in rat (sensitive species), after a single intraperitoneal injection of mesotheliomagenic (CNT-N) and non-mesotheliomagenic (CNT-T) CNT [55] (Figure 3). Through histological analysis, we showed that exposure to both types of CNT similarly induces granuloma formation in the connective tissue bordering the peritoneal cavity. CNT-N and CNT-T are assembled in granuloma center and form compact crystalline structures (red arrow, Figure 3). Under both exposure conditions (CNT-T and CNT-N), numerous CD68 positive cells infiltrate damaged tissue and surround CNT. Most of the cells constituting granuloma are thus macrophages in a greater proportion in tissues exposed to CNT-N and CNT-T compared to controls (Figure 3). Therefore, mesotheliomagenic CNT-N and non-mesotheliomagenic CNT-T alter peritoneal tissues and induce macrophage accumulation in a comparable manner.

**Figure 3.** Mesotheliomagenic needled-like CNT-N and non mesotheliomagenic tangled-like CNT-T induce comparable peritoneal lesions and macrophage accumulation in rats. Wistar rats untreated or injected (i.p) with CNT-N or CNT-T and diaphragms were stained with H&E (A-D-G for controls, B-E-H for CNT-N and C-F-I for CNT-T, magnification x4 first line, x 40 second line). The red arrows indicate granulomas containing CNT crystalline structures within the connective tissue (selected from the frame of A-B-C panels). CNT-N and CNT-T-induced granulomas mainly comprise comparable accumulation of macrophages (G-H-I) identified by using anti-rat CD68 antibody (Abcam monoclonal), secondary donkey antibody coupled with HRP (Jackson ImmunoReserch) and Alexa Fluor Tyramide 488. Stained slides were digitalized using a Pannoramic 250 FlashIII scanner (3DHistech) at × 20 magnification.

Macrophages are crucial immune cells in response to particles (see above). Their versatility is well recognized by immunologists and various distinct functional phenotypes (termed macrophage polarization) directed by specific microenvironmental stimuli and signals have been established and associated to allergic, parasitic and autoimmune diseases for instance [76]. Their cellular origin has been revisited by recent observations, showing that macrophages derive from circulating blood monocytes but also originate from embryonic progenitors and proliferate [77]. The macrophage polarization diversity and our histological observations suggest that the difference between the two
comparative models (with and without mesothelioma) is not solely related to the presence or absence of macrophages in affected tissues but probably resides in the ability of macrophages to adopt contrasting immune profiles. These differentially polarized macrophages can explain the inappropriate immune responses leading to mesothelioma development.

NGS-gene profiling methods can be applied to the comparative model for revealing macrophage profile unambiguously related to mesothelioma (Figure 4). Peritoneal macrophages collected from CNT-N or CNT-T-treated rats are purified using flow cytometry cell sorting (CD68 positive cells). RNA is isolated from bulk macrophage populations and libraries are prepared and sequenced using an Illumina platform. Bioinformatics tools analyze RNA sequences, generate comparative tables or barcode plots, and associate immune and carcinogenic responses for each macrophage subpopulation (Figure 4).

Figure 4. New opportunities to use CNT for identifying immune pathways specifically associated to malignant mesothelioma. Peritoneal macrophages from CNT-N or CNT-T-treated rats are purified from peritoneal cell suspensions using flow cytometry cell sorting and APC-antibodies specific for CD68. Cytocentrifuge preparations of collected macrophages are stained with Diff-Quick. Cellular and molecular characterization of macrophage subpopulations is achieved by using next-generation sequencing (NGS) technologies. RNA is isolated using Qiagen kits and libraries are prepared and sequenced using the Illumina platform. The gene count matrix is transformed in fold-change-related tables or barcode plots.
NGS technologies have evolved dramatically in recent years, making individual cell analysis possible. Single-cell RNA sequencing now reveals new characteristics of macrophage subpopulations and is directly relevant for studying and tracing distinct macrophage lineages and polarizations in chronic diseases [78]. The proteomic resources are also concerned by innovative technologies. New generation of mass spectrometry quantitatively analyze protein networks at single cell resolution [79,80]. Interestingly, recent bioinformatic tools integrate conjoint analyses of the transcriptome (RNA) and proteome (protein) in purified macrophages, boosting their characterization and classification [81]. These new technical platforms and experimental strategies greatly help basic sciences and medical applications and, if used in CNT toxicology, may open new and exciting horizons in the physiopathology and for the therapy of mesothelioma.

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