

**CHARACTERIZATION OF A NEW INTERMEDIATE MACROPHAGE
SUBPOPULATION: SDC-1-POSITIVE SPM-LIKE MACROPHAGES
POSSESS IMMUNOSUPPRESSIVE FUNCTIONS IN EARLY
MESOTHELIOMAGENIC RESPONSES TO CARBON NANOTUBES**

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ABSTRACT

Malignant mesothelioma is a disease caused by inhalation of needle-like shaped particles and whose pathogenesis is not yet fully elucidated. A long-lasting general inflammation caused by persistent mesotheliomagenic particles is not sufficient to explain mesothelioma onset. Indeed, challenging data have shown that mesothelioma occurs without chronic inflammation.

Our research team previously helped to discover that, beside inflammation, particles such as silica also induce a selective, rapid and sustained accumulation of immunosuppressive cells (regulatory T lymphocytes, myeloid derived suppressive cells and M2/regulatory macrophages) participating to fibrogenesis. To determine whether carcinogenic particles such as asbestos and carbon nanotubes (CNT) also elicit immunosuppressive responses, we investigated the impact of these particles on macrophage turnover, phenotype and immunosuppressive activity.

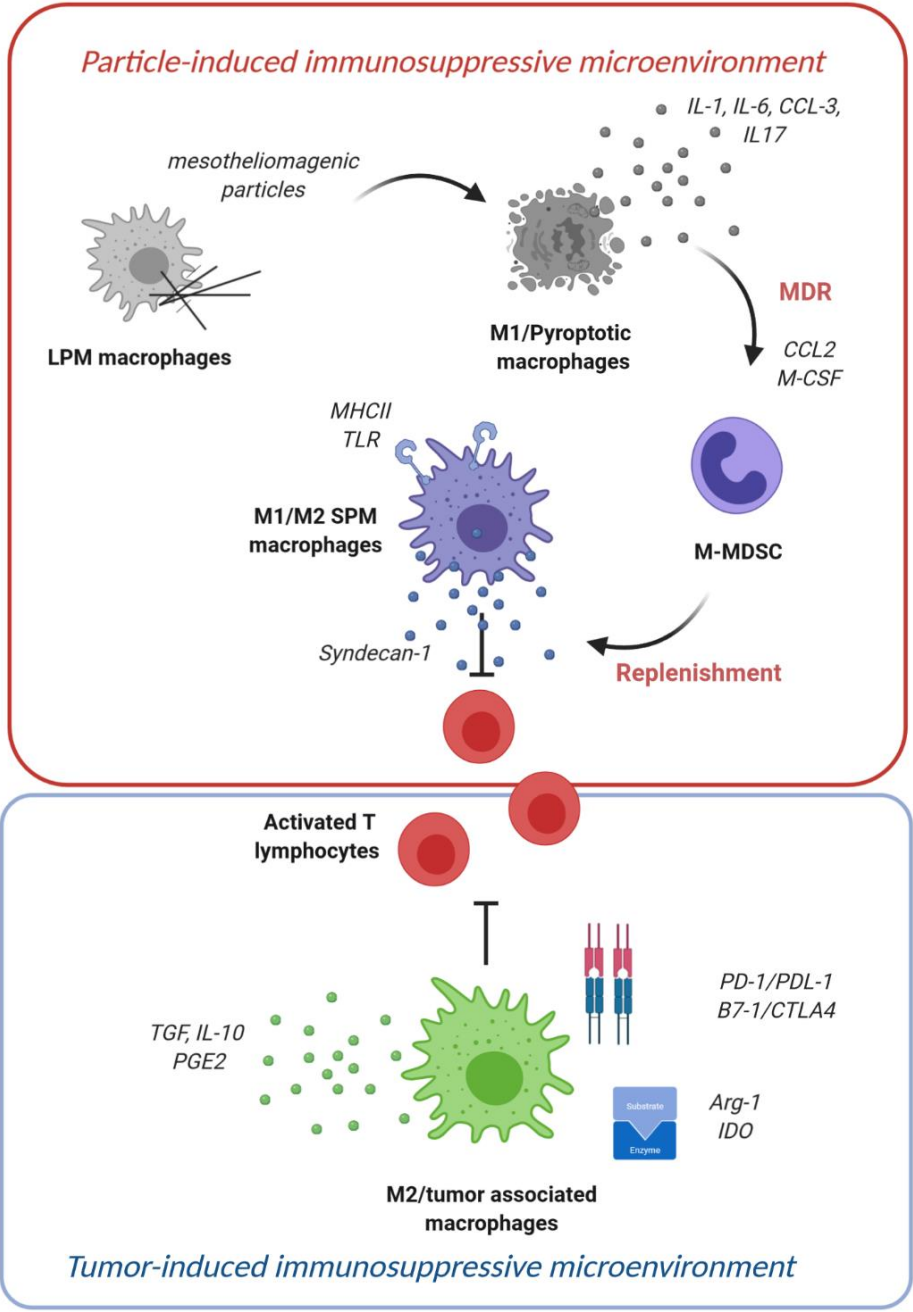
With that purpose, we intraperitoneally injected mesotheliomagenic CNT-7 (needle-like, Mitsui & Co) and non-mesotheliomagenic CNT-T (tangled, Nagoya University) particles in Wistar rats and compared the effects on peritoneal macrophage subpopulations.

We showed that macrophages die very rapidly in the attempt to phagocyte mesotheliomagenic CNT. They are later replenished by monocytic-derived small peritoneal macrophages (SPM-like macrophages) possessing comparable immunosuppressive functions and signatures to Tumor-Associated Macrophages (TAM), which infiltrate mesothelioma and block T cell antitumor activities. Early immunosuppressive SPM-like macrophages express and release the shed form of the immunoregulatory syndecan-1 glycoprotein, which could explain the

Abstract

immunosuppression that they exert on T cells. Non-mesotheliomagenic peritoneal responses induced by CNT-T are, in contrast, characterized by a recruitment of self-proliferating large peritoneal macrophages (LPM-like macrophages) that correspond to homeostatic macrophages without immunosuppressive activity. Our observations indicate that mesotheliomagenic particles elicit a prompt immunosuppressive response involved in an early environment favouring tumoral cell evasion from T cell surveillance.

Since our RNA sequencing data indicated that immunosuppressive SPM-like macrophages have an intermediate polarization state between M1/inflammatory and M2/regulatory macrophages, we suggest that they may represent the gap between the short-term (inflammation) and long-term (tumor immunosuppression) responses to mesotheliomagenic particles. These observations offer new therapeutic targets (SPM or syndecan-1) and *in vivo* bioassays (macrophage replenishment) to anticipate particle hazard and carcinogenicity.



Abstract

Graphical abstract – Resident macrophages die very rapidly (**macrophage disappearance reaction or MDR**) in the attempt to phagocyte needle-like mesotheliomagenic particles and release pyroptosis-related inflammatory cytokines (IL-1 β , IL-6, IL-10 and IL-17A) and chemokines (CCL-3) to alert the immune system. Released M-CSF and CCL2 chemokines also recruit Monocytic–Myeloid Derived Suppressive Cells (M-MDSC), which then differentiate into immunosuppressive **small peritoneal macrophage (SPM)**-like macrophages to **replenish** the empty macrophage niche. SPM-like macrophages are able to arrest T cell proliferation and sustain an early **immunosuppressive** environment probably by releasing the shed form of **syndecan-1** glycoprotein. They resemble Tumor Associate Macrophages (**TAM**) infiltrating mesothelioma for their ability to block T cell activity and share similar immunoregulatory signatures with them. SPM-like macrophages display **an intermediate M1/M2 polarization state**, supporting our hypothesis that they may represent the missing junction between M1 inflammatory and M2 immunosuppressive macrophages during mesothelioma development.

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0. OBJECTIVES

One of the main research areas of LTAP is the assessment of lung toxicity of respirable particles and fibers. *In vitro* and *in vivo* models are employed to elucidate the molecular and cellular pathways involved in lung diseases (i.e. fibrosis and cancer) induced by particles of industrial interest containing lithium, cobalt, silica or indium-tin-oxide.

Our research team initiated toxicological studies on carbon nanotubes (CNT), an innovative material with attractive industrial properties. Because of their needle-like shape, CNT particles trigger, however, the same biological responses as asbestos fibers, known to induce lung fibrosis and mesothelioma. Several years and joint efforts have contributed to clarify that CNT and asbestos mainly affect macrophages, the first line of defense against foreign bodies. In the attempt to phagocyte the particles, macrophages release inflammatory alarmins and cytokines, which promote a long-lasting inflammatory phase leading to transformed mesothelial cells and fibrosis.

This thesis begins with our challenging observations showing that mesotheliomagenic CNT recruit early immunosuppressive monocytes and macrophages in conjunction with inflammatory neutrophils. These findings prompted us to test the hypothesis that the responses to mesotheliomagenic particles comprise an unsuspected and early immunosuppressive environment orchestrated by macrophages and favoring a tumoral cell evasion from T cell surveillance before mesothelioma development.

In this document:

Objectives

- We provide an overview of the current role of macrophages during the different phases of mesothelioma development (Paper 5-Annex 2);
- We highlight the relevance of our *in vivo* model of peritoneal mesothelioma in resolving the enigma of mesotheliomagenic particle-induced immune responses and macrophage polarization (Paper 1);
- We determine the origin and phenotype of macrophages early recruited by mesotheliomagenic particles (Paper 2 and Paper 4-Annex 1);
- We determine their involvement in triggering or dampening inflammation and identify potential immunosuppressive mediators used by macrophages to inhibit T cell activity (Paper 3);
- We suggest the use of our model as a short-term *in vivo* bioassay to predict particle carcinogenicity (General Discussion);
- We highlight that our findings can contribute to the development of new therapeutical strategies for the treatment of mesothelioma (General Discussion).

LIST OF ABBREVIATIONS

APC, Antigen presenting cells

Arg1, Arginase 1

Asb, Crocidolite asbestos

ATP, Adenosine triphosphate

BrdU, 5-bromo-2'-deoxyuridine

BSA, Bovine serum albumin

CCL, chemokine (C-C motif) ligand

CCR, C-C chemokine receptor

CD, Cluster of differentiation

CNT-7, Multi-walled Mitsui-7 CNT

CNT-M, Muller carbon nanotubes

CNT-T, Tangled carbon nanotubes

CSF1, Colony stimulating factor 1

CTL, Cytotoxic T Lymphocyte

ECM, extra cellular matrix

EGF, Epidermal growth factor

List of Abbreviations

FACS, Fluorescence-activated cell sorting

FOXP3, forkhead box P3

GARP, glycoprotein A repetitions predominant

GM-CSF, Granulocyte-macrophage colony-stimulating factor

HGF, hepatocyte growth factor

HIF, hypoxia-inducible factor

HMGB1, High–Mobility Group Box 1

IDO, Indoleamine-2,3-dioxygenase

IL, interleukin

INF, Interferon

LAP, Latent associated peptide

LPM, Large peritoneal macrophages

LPS, lipopolysaccharide

MCP-1, monocyte chemoattractant protein-1

M-CSF, macrophage colony-stimulating factor

MDR, Macrophage disappearance reaction

MDSC, Myeloid-derived suppressor cells

MHCII, Major histocompatibility complex (MHC) molecules

M-MDSC, Monocytic myeloid-derived suppressor cells

List of Abbreviations

MPM, Malignant Pleural Mesothelioma

MR, Mannose receptor

MWCNT, Multi-wall carbon nanotubes

NF- κ B, nuclear factor kappa B

NK, Natural killer cells

NLRP3, NOD-like receptor family, pyrin domain containing 3

NOS, Nitrogen oxygen species

PBS, phosphate buffered saline

PDGF, Platelet-derived growth factor

PDL-1, Programmed death-ligand 1

PGE₂, Prostaglandine E2

PMN-MDSC, Polymorphonuclear myeloid-derived suppressor cells

qRT-PCR, Quantitative real-time polymerase chain reaction

RELM alpha, Resistin-like alpha

ROS, reactive oxygen species

SDC, syndecan

SiO₂, DQ12 silica

SPF, Specific-pathogen-free

SPM, Small peritoneal macrophages

List of Abbreviations

SR, Scavenger receptor

T reg, regulatory T cells

TAM, Tumor-associated macrophages

TGF, transforming growth factor

Th, T helper

Th, T helper

TLR, Toll-like receptor

TME, Tumor microenvironment

TNF, tumor necrosis factor

VEGF, vascular endothelial growth factor

VLA-4, Very late antigen-4

1. PAPER I

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

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1.1. 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, structural defects and scavenger capacity. Generated *in vitro* and *in vivo* data on their inflammogenic and fibrogenic activities were combined and translated in worldwide accepted 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 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 pathogenic 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 T lymphocyte anti-mesothelioma immunity. This new concept offers novel horizons for clinical management of mesothelioma and represents an additional tool for predicting mesotheliomagenic activity of newly elaborated CNT or nanoparticles.

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

1.2. 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” (Qin et al. 2000). 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. Simultaneously, 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 (Amelinckx et al. 1994).

These success stories had 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 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 (Mohajeri et al. 2018; Simon et al. 2019). 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 4,000 tons by 2023, representing a compound annual growth of 13 % (2,250 tons in 2008 when considering the global CNT market) (2019).

Chemical vapor deposition (with or without catalysts) represents the main synthetic technique, and accounts for more than 70 % of the global production. Currently, efficient production and declined prices for CNT make these nanomaterials more available and affordable (King et al. 2020). Multi-walled CNT dominate the market and their increasing applications concern battery additives and plastic parts (Kong et al. 2019; Luo et al. 2020). CNT also hold absorbent potential for organic wastewater treatment or emerging contaminant because of their high specific surface area and mesoporous structure (Baby et al. 2019; Silva et al. 2020; Yin et al. 2020). The recent ability to functionalize CNT or control their growth and assembly (Venkataraman et al. 2019) gives rise to the development of new biomedical applications, including tissue growth and drug delivery (Maiti et al. 2018; Simon et al. 2019). For instance, CNT-based materials permit the elaboration of layered cell architectures for embryonic stem cell and spheroid expansion during tissue regeneration (Hu et al. 2019; Pei et al. 2019).

1.3. From Asbestos to CNT: The Toxicological Aspect

Morphological similitudes between CNT and asbestos

Despite the scientific and industrial enthusiasm born from the discovery of CNT, several toxicologists including the Japanese Jun Kanno (Takagi et al. 2008) rapidly discerned 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 frustrating for 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 (Muller et al. 2005) (Figure 1) rapidly reported that CNT injected into the lungs of animals were able to induce inflammatory, fibrogenic and carcinogenic responses (Lam et al. 2004; Poland et al. 2008; Shvedova et al. 2003; Shvedova et al. 2005; Takagi et al. 2008). These results, like many others thereafter, had a very significant global impact (papers widely cited; sometimes more than 1000 times) and initiated extensive *in vitro* and *in vivo* investigations focused on CNT toxicity (Donaldson et al. 2013). Altogether, these findings demonstrated that the respiratory diseases induced by CNT (inflammation, fibrosis and cancer) correspond exactly to the pathologies observed after asbestos exposure (Mossman 2018).

Inflammogenic and fibrogenic effects of CNT: mechanisms and Adverse Outcome Pathways (AOP)

Several decades were necessary for defining 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 induce CNT phagocytosis (Huaux 2018a; Huaux 2018b; Pavan et al. 2019). 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-1 β) into highly inflammatory mediators (active IL-1 β). Our group also demonstrated that the permeabilization of lysosomes induces cell membranolysis and the subsequent release of danger signals (named alarmins) stored in macrophage cytoplasm (Huaux 2018b; Rabolli et al. 2014; Rabolli et al. 2016). Altogether, these inflammatory mediators orchestrate the persistent accumulation of neutrophils and macrophages. Oxidizing molecules produced by these inflammatory cells strongly damage surrounding tissues (Huaux 2018a).

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 (Lebrun et al. 2017; Vietti et al. 2016b). We have also demonstrated that toxic CNT possess the unique ability to induce fibroblast proliferation, differentiation and collagen production by themselves (Vietti et al. 2016a). When CNT persist into the tissue, remodeling is therefore permanent and degenerates into uncontrolled scar and fibrosis (Huaux 2018a).

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 predicting inflammatory and fibrogenic effects of CNT and assess health risks for human (Guseva Canu et al. 2020; Nikota et al. 2017; Vietti et al. 2016b) (Figure 1).

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 CNT to stimulate fibroblasts or macrophages, indicating that longer particles are more efficient in inducing inflammation and fibrosis. (Vietti et al. 2013; Vietti et al. 2016b). CNT diameter was also identified as an important factor since thin CNT are more toxic for the lungs compared to thick CNT (Fenoglio et al. 2012). The morphology also determines their capacity to induce inflammation and fibrosis. Tangled CNT are less inflammatory and fibrogenic than straight (needled) CNT (Duke et al. 2018; Murphy et al. 2013). 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 because structural defects introduced in CNT by fracturing procedures increase inflammatory and fibrogenic activity (Fenoglio et al. 2008; Muller et al. 2008). Finally, CNT scavenge free radicals and this quenching capacity governs their toxic potentials *in vitro* and *in vivo* (Fenoglio et al. 2008) (Figure 1).

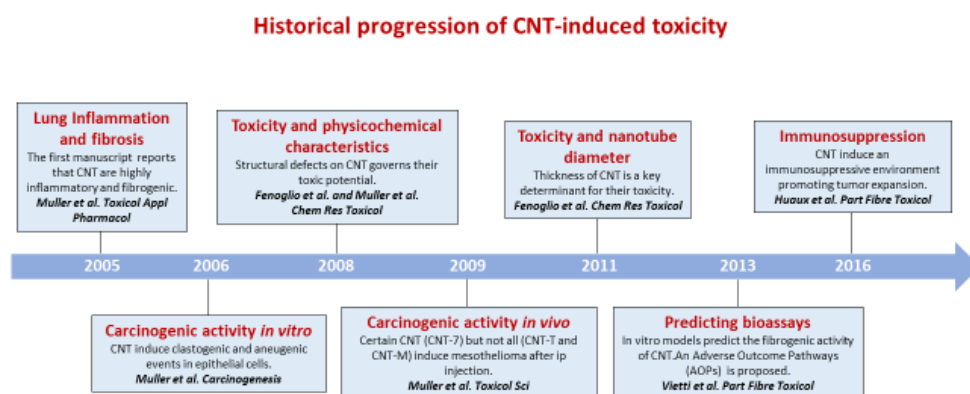


Figure 1

Figure 1: Historical progression of CNT-induced toxicity. Timeline summarizing the discoveries on CNT toxicity obtained by LTAP teams (UCLouvain, Brussels, Belgium) and their scientific collaborators from around the world. For this collective work, we used diverse and relevant *in vivo* and *in vitro* models and CNT possessing diverse morphological and physico-chemical properties.

1.4. The Mesotheliomagenic Activity of CNT: A Remaining Issue

Mesothelioma and particles

Malignant mesothelioma (MM) is a cancer affecting the mesothelium, a layer of squamous cells covering the serous cavities of the body (pleura, peritoneum, pericardium) and protecting the organs they contain (lungs, peritoneal organs, heart). The most commonly affected tissue is the pleura and peritoneum; this is referred to as pleural and peritoneal malignant mesothelioma (Carbone et al. 2019; van Gerwen et al. 2020).

MM is an uncommon cancer and most of the cases are due to asbestos exposure (Poland and Duffin 2019). 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 (Poland and Duffin 2019). These low-degradable fibers trigger MM and other terminal pathologies such as pulmonary fibrosis and bronchial cancer in animal and human (Gaudino et al. 2020).

MM can occur more than 30 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 2030 (Alpert et al. 2020). Unfortunately, patients often reach the final stage of the disease when they are detected making the prognosis of MM very poor (12-15 months) (Fels Elliott and Jones 2020; Kim et al. 2019). 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. Debates also

remain regarding other multimodal approaches as surgery and radiation (Cantini et al. 2020; Disselhorst and Baas 2020).

Some CNT have been incriminated as being responsible for MM because of their physical similarity to asbestos fibers. Several *in vivo* studies then demonstrated that the long and straight multi-walled (MW) CNT-Mitsui-7 are indubitably mesotheliomagenic (see below) (Dong and Ma 2019; Sinis et al. 2018). 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 (2017; Grosse et al. 2014). All other CNT (single-walled or multi-walled nanotubes) were classified in group 3 (not classifiable as to their carcinogenicity to human) (Kane et al. 2018).

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 mesotheliomagenic cell lines and animal models (Blanquart et al. 2020; Gaudino et al. 2020).

Direct effects and DNA damages

Carcinogenicity of asbestos fibers 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 (Chew and Toyokuni 2015; Donaldson et al. 2010; Donaldson et al. 2013). Asbestos generate reactive oxygen species (ROS) and induce ROS production by exposed mesothelial cells. ROS trigger genomic instability and mutations by interacting with mesothelial cells (Moller and Jacobsen 2017). These oxidant molecules also activate various signaling

pathways, inducing transformed cell proliferation and survival (Chew and Toyokuni 2015; Donaldson et al. 2010; Donaldson et al. 2013). ROS are therefore involved in the initiation, promotion and progression of cancer, which represent the three stages of the carcinogenic process (Benedetti et al. 2015).

Asbestos fibers are known to penetrate cell membrane and interact with intracellular molecules resulting in direct (or primary) genotoxicity that include DNA strand breaks, mutations and chromosomal aberrations (Moller and Jacobsen 2017). Recent studies showed that CNT induce chromosomal disruptions, fragmentations and translocations (Siegrist et al. 2019; Snyder et al. 2019) and their nuclear deposition results in clear epigenetic alterations (Ghosh et al. 2018; Oner et al. 2018; Oner et al. 2020). The quenching capacity of CNT is also involved in their primary genotoxic effects (Fenoglio et al. 2008) (Figure 1). However, the literature concerning the direct interaction of asbestos or CNT with DNA is contradictory. The non-clastogenicity of the CNT observed by Sasaki suggests that the CNT may not directly interact with DNA (Sasaki et al. 2016).

Inflammatory immune responses as a driver of malignant mesothelioma development

Numerous studies indicate that mesotheliomagenic particles induce, instead, secondary genotoxic damages by promoting inflammation and subsequent free radical release (Linton et al. 2012; Testa and Berns 2020; Westbom et al. 2014) (Figure 2). Resident macrophage present in mesothelial cell-covered tissues, detect, phagocytize and attempt to degrade inhaled fibers (Kagan et al. 2010). Whether these particles are long and resistant to degradation by macrophages, they trigger a phenomenon known as frustrated phagocytosis (Benedetti et al. 2015; Donaldson et al. 2010), which result in the constant and prolonged release of

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highly reactive inflammatory mediators (see above) (Abdul Rahim et al. 2015; Yang et al. 2006) These mediators notably recruit neutrophils and additional macrophages for further particle clearance (Benedetti et al. 2015; Donaldson et al. 2010; Kishimoto et al. 2019; Yang et al. 2006). However, this particle removal process is not efficient for persistent fibers and the inflammation becomes consequently chronic (Benedetti et al. 2015; Donaldson et al. 2010; Sinis et al. 2018).

ROS deriving from frustrated macrophages and neutrophils (Figure 2) cause direct mutations and promote proliferation and invasion of transformed mesothelial cells by modulating cell signaling pathways (Benedetti et al. 2015; Cheng et al. 2020; Chew and Toyokuni 2015). These inflammatory cells also permit mutated mesothelial cells to avoid apoptosis (Acencio et al. 2015). Indeed, cytokines and alarmins such as TNF- α and HMGB1 produced by macrophages and neutrophils activate the NF- κ B (Nuclear Factor Kappa B) signaling pathway in mesothelial cells. This transcription factor then induces the expression of various genes promoting cell survival (Affar and Carbone 2018; Carbone and Yang 2017). These inflammatory mediators also convert mesothelial cells to inflammatory cell partner that release growth and differentiation factors for stem (M- and GM- CSF) (Dammeijer et al. 2017) and endothelial cells (VEGF) (Nowak et al. 2020). Together, these factors increase the survival of transformed mesothelial cells and allow neoplastic cell migration into the tissue (Figure 2).

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 varied pathological profiles among clinical cases. In recent years, a new concept was developed proposing that the immune evasion permits mesothelioma to evade host anti-tumor responses (Chu et al. 2019; Lizotte et al. 2016; Wong et al. 2014).

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 (Wong 2016). Immunosuppressive host cells invading mesothelioma include regulatory T lymphocytes (T regs) (Klampatsa et al. 2019; Noordam et al. 2018), Myeloid-Derived Suppressor Cells (MDSCs) (Salaroglio et al. 2019) and Tumor-Associated Macrophages (TAMs) (Hoves et al. 2018; Lievense et al. 2013). 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 (Chu et al. 2019).

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

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CTLA-4 and PDL-1 neutralizing antibodies) during cytoreduction therapy (chemotherapy, radiotherapy and surgery) (de Gooijer et al. 2020; Forde et al. 2019; Lizotte et al. 2016; Remon et al. 2020; Wong et al. 2014). Tumor immune escapes are also operative in animal models of mesothelioma and innovative therapeutic strategies modifying immunosuppressive monocyte and macrophage differentiation are now successfully obtained (Dammeijer et al. 2017; Veltman et al. 2010b).

The co-existence of early inflammation and immunosuppression after mesotheliomagenic particle-exposure.

Our team was able to demonstrate that immunosuppressive responses were not exclusively generated by tumor cells but also by mesotheliomagenic CNT themselves. Indeed, we demonstrated that mesotheliomagenic CNT induce an early immunosuppressive environment by recruiting immunosuppressive M-MDSCs and macrophages after few days in injected rats (Figures 1 and 2) (Huaux et al. 2016). 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 (Shvedova et al. 2015; Shvedova et al. 2013). 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 (Mitchell et al. 2007; Mitchell et al. 2009). The rapid development of local and systemic immunosuppressive immune responses reduce the number of circulating T lymphocytes and their ability to proliferate (Park et al. 2009; Park et al. 2011a; Park et al. 2011b). This effect is mainly related to IL-10 and TGF- β (Mitchell et al. 2009). Kido and colleagues also noted a rapid increase of IL-10 expression by macrophages after CNT inhalation in rats (Kido et al. 2014). 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 mesothelioma types and long latency period of the disease (Figure 2).

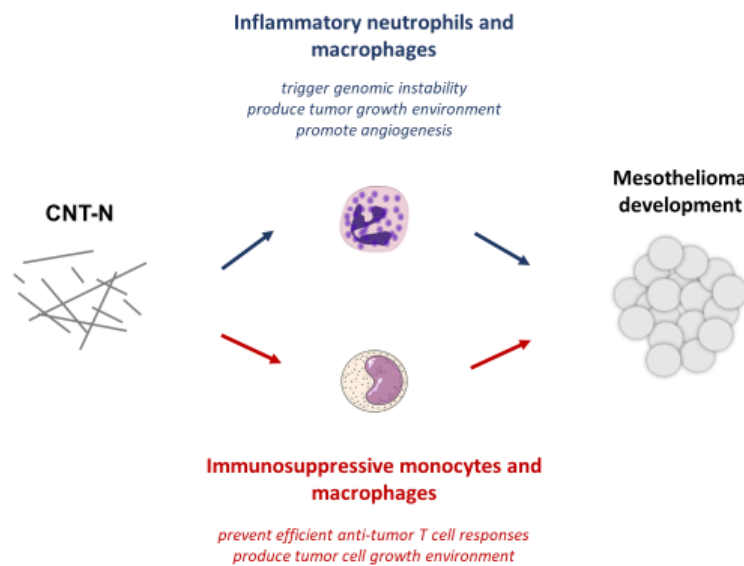


Figure 2

Figure 2: A new pathological pathway governs carcinogenesis induced by mesotheliomagenic needed CNT-N. Persistent inflammation and immunosuppression orchestrate carcinogenesis and mesothelioma. Toxic 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 for transformed mesothelial cells and angiogenesis. An unexpected conjoint immunosuppression (in red) is induced by mesotheliomagenic CNT. This early response to CNT-N is characterized by persistent accumulation of immunosuppressive macrophages and monocytes and a sustained production of regulatory 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.

1.5. An Unexpected Application: The Diversity of CNT to Identify New Pathological Pathways in Malignant Mesothelioma

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 (Donaldson et al. 2010; Donaldson et al. 2013). 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 (Testa and Berns 2020).

The mesotheliomagenic properties of CNT represent an interesting tool for investigating mesothelioma in animals. We have shown that CNT-induced mesothelioma affect all treated rats (only 30 % to 50 % of animals injected with

asbestos) in a limited period (6 months for CNT instead of 2 years for asbestos) (Huaux et al. 2016; Muller et al. 2009). 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 (Huaux et al. 2016; Muller et al. 2009; Nagai et al. 2011; Rittinghausen et al. 2014). 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 (Poland et al. 2012; Poland et al. 2008).

Nanotube geometry is also crucial in the development of inflammation, lymphoid infiltration and granuloma formation in animals. Only CNT-N exposure resulted in neutrophilic inflammation and larger lymphoid and fibrotic granulomas contrary to CNT-T (Duke et al. 2017; Duke et al. 2018). Needled CNT-N were more potent than tangled CNT T to elicit inflammatory effects towards macrophages (Di Cristo et al. 2019; Rydman et al. 2015).

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 (Sasaki et al. 2016). CNT-N elicit a more pronounced primary genotoxicity effect than CNT-T, as assessed by DNA damage and micronuclei formation (Catalan et al. 2016). 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) (Catalan et al. 2016; Huaux et al. 2016; Muller et al. 2009; Nagai et al. 2011; Rittinghausen et al. 2014; Sakamoto et al. 2018).

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 (Hwang et al. 2018).

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 (Huaux et al. 2016) (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 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.

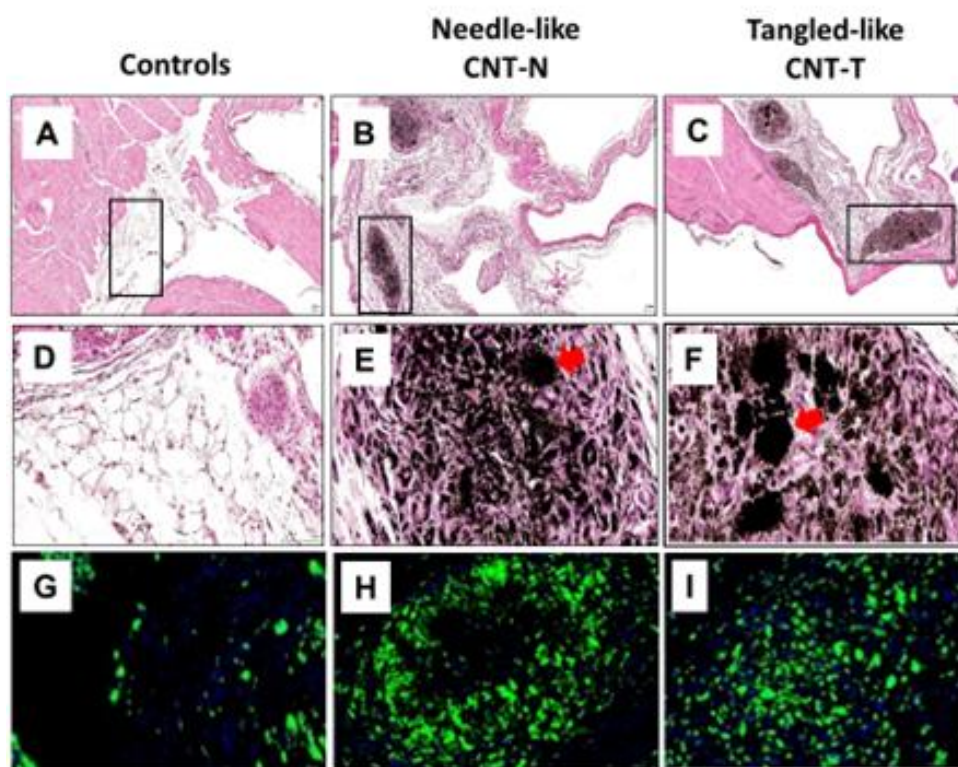


Figure 3: Mesotheliomagenic CNT-N and non-mesotheliomagenic CNT-T induce comparable peritoneal lesions and macrophage accumulation in rats. Wistar rats untreated or injected (i.p) with CNT-N or CNT-T (2 mg) were sacrificed (day 15) and peritoneal tissues (diaphragm) were harvested, fixed in paraformaldehyde and embedded in paraffin. 5- μ m sections were stained with classical H&E coloration (A-D-G for controls, B-E-H for CNT-N and C-F-I for CNT-T, magnification x4 first line, x40 second line). The red arrows indicate granulomas containing CNT crystalline structures within the connective tissue (selected from the frame of A-B-C panels). Granulomas mainly comprise macrophages around nanotube aggregates (G-H-I). For macrophage identification, 5- μ m sections were incubated with mouse anti-rat CD68 antibody (Abcam monoclonal) and secondary antibody donkey anti-mouse (Jackson ImmunoResearch) coupled with HRP. After incubation with AlexaFluor Tyramide 488, a counterstaining with Hoechst44432 dye was

performed. Stained slides were digitalized using a Pannoramic 250 FlashIII scanner (3DHistech) at x20 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 (Doran et al. 2020). 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 (Guilliams et al. 2020). The macrophage polarization diversity and our histological observations suggest that the difference between the two comparative models (with and without mesothelioma) is not simply related to the presence or absence of macrophages in affected tissues but probably reside in the ability of macrophages to adopt contrasting immune profiles. These differentially polarized macrophages can explain the inappropriate immune responses leading to mesothelioma development.

We applied NGS-gene profiling methods to our model to reveal macrophage profile unambiguously related to mesothelioma (Figure 4). Peritoneal macrophages collected from CNT-N or CNT-T-treated rats were purified using flow cytometry cell sorting (CD68-positive cells). RNA was isolated from bulk macrophage populations and libraries were prepared and sequenced using an Illumina platform. Bioinformatics tools analyzed RNA sequences, generated comparative tables or barcode plots and associated immune and carcinogenic responses for each macrophage subpopulation (Figure 4). By applying this strategy, we already supported the hypothesis that a specific and early immunosuppressive response characterized by TGF-, integrin-, or CD9-related signaling pathways are induced in

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macrophages after mesotheliomagenic CNT-N injection. Our sequencing data also revealed new molecular axis implicating arachidonic acid metabolites and cell cycle. Futures studies are now needed to determine whether these new possible pathogenic pathways are major in mesothelioma development in animal and human.

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 (Schyns et al. 2019). The proteomic resources are also concerned by innovative technologies. New generation of mass spectrometry quantitatively analyze protein networks at single cell resolution (Bogaert et al. 2020; Bottek et al. 2020). Interestingly, recent bioinformatic tools integrate conjoint analyses of the transcriptome (RNA) and proteome (protein) in purified macrophages, boosting their characterization and classification (Li et al. 2020). These new technical platforms and experimental strategies greatly help basic science and medical applications and, if used in CNT toxicology, may open new exciting horizons in the physiopathology and for the therapy of mesothelioma.

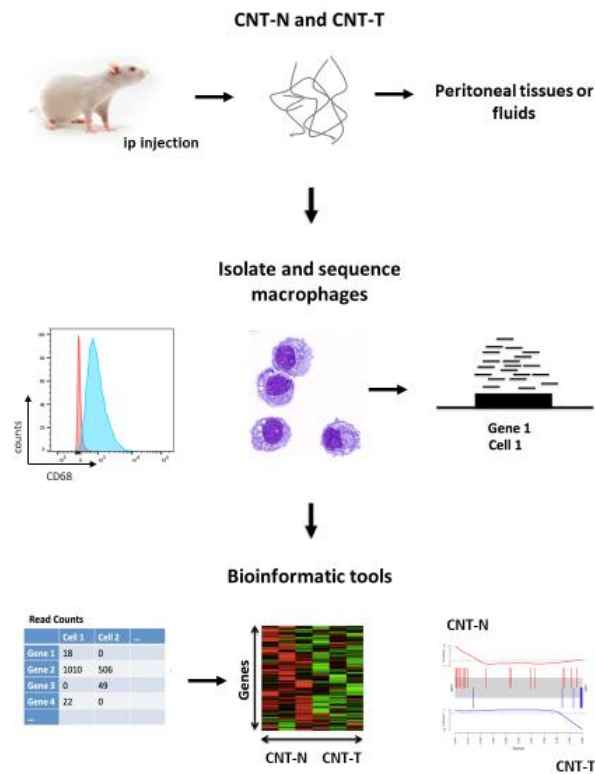


Figure 4

Figure 4: New opportunities to use CNT for delineating specific macrophage immune pathways specifically associated to malignant mesothelioma. Cellular and molecular characterization of macrophage subpopulations by using next-generation sequencing (NGS) technologies. Peritoneal macrophages from CNT-N or CNT-T-treated rats (day 15) were isolated from peritoneal cell suspensions using flow cytometry cell sorting (FACSARIA III, BD Biosciences) and APC-antibodies specific for CD68 (mouse anti-rat CD68 antibody, Abcam monoclonal). Cytoentrifuge preparations of purified macrophages were stained with Diff-Quick. RNA was isolated using Qiagen kits and libraries were prepared and sequenced using the Illumina platform. The gene count matrix was transformed in fold-change-related tables or barcode plots.

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Author Contributions

FH, CH, RL and MO were involved in the conception of the review and wrote the manuscript.

Conflicts of Interest

The authors declare that they have no competing interests.

2. PAPER II

Monocytic Ontogeny of Regenerated Macrophages Characterizes the Mesotheliomagenic Responses to Carbon Nanotubes

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2.1. Abstract

Macrophages not only derive from circulating blood monocytes, but also expand by proliferation. This dichotomic origin has an impact on macrophage functions in steady state and pathological conditions. Macrophages predominantly infiltrate fiber-induced mesothelioma tumors and contribute to cancer development. Here, we revealed the ontogeny of macrophage populations by comparing the response to needle-like mesotheliomagenic carbon nanotubes (CNT-7) with tangled-like non-mesotheliomagenic CNT-T.

In a rat peritoneal cavity model of mesothelioma, both CNT induced a rapid and complete macrophage disappearance reaction (MDR) of MHCII^{low} resident macrophages generating an empty niche available for macrophage repopulation. Macrophage depletion after mesotheliomagenic CNT-7 was followed by a substantial inflammatory reaction, and macrophage replenishment completed after 7 days. Fifteen days after non-mesotheliomagenic CNT-T, macrophage repopulation was incomplete and accompanied by a limited inflammatory reaction. Cell depletion experiments, flow cytometry and RNA-seq analysis demonstrated that, after mesotheliomagenic CNT-7, resident macrophages were mainly replaced by an influx of monocytes, which differentiated locally into MHCII^{high} inflammatory macrophages. In contrast, the low inflammatory response induced by CNT-T was linked to the activation of self-renewing MHCII^{low} macrophages.

In conclusion, the mesotheliomagenic response to CNT specifically relies on macrophage niche recolonization by monocytic progenitors. In contrast, the homeostasis returns after exposure to non-mesotheliomagenic CNT involves a macrophage regeneration by proliferation. Macrophage depletion and repopulation are thus decisive events characterizing the carcinogenic activity of particles and fibers.

2.2. Introduction

Macrophages are the main immune cells recruited to clean up the body from foreign materials. They also contribute to the adverse effects of inhaled particles, and their fundamental role in particle-induced diseases is widely recognized (Ma 2020). In the present work, we focus on the macrophage response to carbon nanotubes (CNT) in a rat peritoneal model largely used to investigate the development of mesothelioma (Poland et al. 2008).

As for asbestos, the first macrophage response to mesotheliomagenic CNT is recognition via the mannose (MR), toll-like (TLR) and scavenger receptors (SR), followed by internalization via phagocytosis (Mukherjee et al. 2018). Downstream events comprise lysosome membrane permeabilization, reactive oxygen species secretion and activation of the NF- κ B signaling pathway, pivotal for the release of neutrophil- (TNF- α and IL-6) and monocyte- (CCL2) chemotactic cytokines (Dong and Ma 2019). Moreover, particle endocytosis results in the specific release by macrophages of active IL-1 β via the NLRP3-inflammasome machinery (Rabolli et al. 2016). The inflammatory neutrophils recruited in CNT-induced damaged and inflammatory tissues represent an important driver of carcinogenesis by triggering genomic instability in mesothelial cells (Murphy et al. 2013).

Chronic exposure combined with impaired clearance of CNT cause their accumulation in the lung and mesothelial cavities (mainly the pleura, but also the peritoneal or pericardial spaces), where accumulated macrophages switch from a pro-inflammatory M1 to a pro-tumoral M2 phenotype (Ma 2020). The skewing into M2 phenotype causes the dampening of T cell activity against cancer cells by producing immunosuppressive mediators (Arginase 1, TGF- β and IL-10), and the

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development of malignant responses (Chu et al. 2019). As with asbestos fibers, the intensity of mesothelioma responses to CNT is related to their physico-chemical properties: rigid and needle-like CNT impair macrophage clearance and trigger a pronounced and persistent release of inflammatory cytokines and immunosuppressive mediators (Dong and Ma 2019; Ma 2020).

The exact origin and expansion pathways of macrophage populations after CNT exposure and during mesothelioma development is, however, almost unknown. While it has long been accepted that macrophages originate from bone marrow-derived circulating blood monocytes, new studies have extended this paradigm. Tissue macrophage subsets can also be self-renewing cells, independent of hematopoietic progenitors (Ginhoux and Guilliams 2016).

Animal studies showed that the peritoneal cavity, in steady state conditions, houses these two distinct macrophage subsets (Bain and Jenkins 2018). The majority of resident macrophages (90 %) are large peritoneal macrophages (LPM), self-renewing cells arising from embryo-derived precursors and characterized, in the mouse, by the expression of F4/80, CD11b and CD163 but not MHCII. These cells maintain the homeostasis of the cavity (Cassado Ados et al. 2015). Upon homeostatic imbalance following an inflammatory stimulus, MHCII^{low} LPM macrophages disappear from the peritoneum (macrophage disappearance reaction or MDR), leaving empty spaces in the pattern. The repopulation of this empty niche is triggered by the sudden lack of proximity between the cells and by a higher disposability of growth factors for monocyte engraftment or macrophage proliferation (Guilliams et al. 2020). During the macrophage disappearance reaction, MHCII^{low} LPM macrophages can easily migrate from the peritoneal cavity to the near omentum, a visceral adipose tissue where they restore an already existing pool of proliferating macrophage progenitors (Meza-Perez and Randall

2017). In contrast, small peritoneal macrophages (SPM) represent 10 % of the population in steady state conditions and are MHCII^{high} short-lived cells continuously replaced by CCR2^{pos} monocytes (Bain and Jenkins 2018). During an inflammatory reaction, these cells are recruited from circulating monocytes and become the predominant population of the peritoneal cavity. SPM have higher phagocytic capacity than LPM for particles or bacteria (Bain and Jenkins 2018).

The aim of this study is to determine the exact origin (monocyte- or embryonic-derived) and the phenotype of macrophage populations in response to mesotheliomagenic particles. We used a relevant rat peritoneal model of mesothelioma to define the phenotype and origin (MHCII^{high} SPM versus MHCII^{low} LPM signature) of macrophage populations in response to needle-like mesotheliomagenic CNT-7 and tangled-like non-mesotheliomagenic CNT-T (Huaux et al. 2016). This original comparative model is not available by using natural asbestos fibers where all forms are carcinogenic.

Our results reveal, for the first time, a specific macrophage ontogeny during the early events of mesothelioma development. We show that monocyte-derived MHCII^{high} SPM-like macrophages dominates the early peritoneal responses to mesotheliomagenic particles.

2.3. Materials and Methods

Carbon nanotubes

CNT-7 are multi-walled carbon nanotubes MWCNT-XNRI-7 from Mitsui & co (Ltd., Tokyo, Japan, Lot # 05072001 K28) sub-sampled at Norwegian Research Centre for the Working Environment (NRCWE) with the code NRCWE-006. CNT-T (tangled,

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kindly provided by prof S. Toyokuni, Nagoya university, Japan) and their physico-chemical characteristics have been documented in details previously (Muller et al. 2009). All particles were first treated at 200 °C for 2 h to remove any possible trace of endotoxin and suspensions were prepared immediately before administration by sonication and manual vortexing in sterile phosphate-buffered saline (PBS) containing 1.4 mg bovine serum albumin/ml.

Animals

Specific pathogen free (SPF) female Wistar rats (8 weeks old, 200 g) (RGD ID: 13792727) were purchased from Janvier SAS (St.-Berthevin, France). The animals were housed at the local animal facility under SPF conditions, with a 12-h light–dark cycle and controlled temperature and humidity. All animal experiments were approved by the local committee for animal research at the Université Catholique de Louvain, Comité d’Ethique pour l’Expérimentation Animale, Secteur des Sciences de la Santé, Brussels, Belgium (No LA1230312). International recommendations for doses administered, controls and group size were followed (Cullen et al. 2002). The animals were injected intraperitoneally with the different particles suspended in a volume of 1 ml of PBS-BSA. They were given tap water and sterilized pellets ad libitum. Groups of 4 to 5 Wistar rats were injected with 2 mg of test material and sacrificed after 3 hours and 1, 3, 7 and 15 days. The peritoneal cavity was opened, washed with 15 ml of saline and the cells present in the peritoneal fluid were collected.

Cell analysis and purification

Leukocyte counts were determined on a Burker cell chamber. Cytocentrifuge preparations of peritoneal cells were stained with Diff-Quick (Baxter, Lessines, Belgium). Rat peritoneal cell suspensions were also stained using antibodies specific

for CD11b/c-APC (Cat. # 201809, RRID: AB_313995) obtained from BioLegend or CD11b/c-BV421 (Cat. # 743977, RRID: AB_2741898) from BD Biosciences, His48-FITC (Cat. # 11057082, RRID: AB_465100) from ThermoFisher or His48-PE (Cat. # sc-19613PE, RRID: AB_627686) from SantaCruz Biotechnology, MHCII-FITC (Cat. # 11-0920-82, RRID: AB_465159) obtained from Thermo Fisher Scientific and CD163-PerCP (Cat. # NBP2-39099PCP, RRID: AB_2861402) from Novus Biologicals. Samples were acquired on a FACSCantoII (BD Biosciences) and analyzed using FlowJo software (RRID:SCR_008520). At least 10,000 events were recorded. Rat peritoneal leukocytes were separated and isolated using flow cytometry cell sorter (FACS Aria III, BD Biosciences). The purity of the obtained leukocyte cell preparations was routinely > 93 % as assessed by Diff-Quick staining.

In vitro cytotoxicity test

Peritoneal leukocytes were obtained from untreated rats and added in culture in a 96-well plate (2×10^5 cells/well). After 24 hours of cell culture at 37 °C, to promote macrophage adhesion, cells were exposed for 24 hours to 4 different concentrations of CNT-7 or CNT-T (20, 10, 5 and 3 $\mu\text{g}/\text{cm}^2$). The luminescent signal (CellTiter-Glo® Reagent, Cat. # G7570 from Promega), proportional to cellular ATP, was measured by luminometer after exposure. Each value is relative to the control condition (cells not exposed to CNT).

Monocyte depletion

Busulfan (Cat. # B2635-10G) from Sigma Aldrich was dissolved in Dimethyl sulfoxide (DMSO) Hybri-Max™ (Cat. # D2650) and injected at a dose of 15 mg/kg twice subcutaneously in rat, 3 days before and 3 days after intraperitoneal injection of CNT (Peake et al. 2015).

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BioPlex cytokine analysis

Levels of soluble IL-1 β , IL-6, IL-17A, CCL3, CCL2 and M-CSF in peritoneal fluid supernatants were analyzed using the Bio-Plex Pro™ Rat Cytokine Assay (Cat. # 171K1001M, RRID: AB_2861403) from Bio-Rad.

RNA sequencing

Peritoneal macrophages from CNT-7 or CNT-T-treated rats sacrificed after 15 days, were separated and isolated using flow cytometry cell sorting (FACS Aria III, BD Biosciences). RNA was isolated using RNeasy mini kit (Cat. # 74104) from Qiagen. RNA quantity was measured with Qubit™ RNA HS Assay Kit (Cat. # Q32852) from Thermofisher. RNA integrity and quality were assessed using an Agilent Bioanalyzer using the Agilent RNA 6000 Nano kit (Cat. # 5067-1511). All RNA used had an RNA Integrity Number between 9 and 10. Libraries were prepared starting from 100 ng of total RNA using the Illumina® TruSeq® Stranded mRNA Sample Preparation Kits following the standard protocol. Libraries were sequenced using an Illumina HiSeq1500 platform. Approximately 50 million paired end reads (2x125bp) per sample were obtained. All sequencing data were analyzed using the Automated Reproducible MODular workflow for preprocessing and differential analysis of RNA-seq data (ARMOR) pipeline (Orjuela et al. 2019). In this pipeline, reads underwent a quality check using FastQC (RRID:SCR_014583) (Andrews 2010). Quantification and quality control results were summarized in a MultiQC report (RRID:SCR_014982) (Ewels et al. 2016) before being mapped using Salmon (RRID:SCR_017036) (Patro et al. 2017) to the transcriptome index which was built using all Ensembl cDNA sequences obtained in the *Rattus_norvegicus*.Rnor_6.0.cdna.all.fa file (Cunningham et al. 2019). Then, estimated transcript abundances from Salmon were imported into R using the

tximeta package (Love et al. 2019) and analyzed for differential gene expression with edgeR (RRID:SCR_012802) (Robinson et al. 2010). Multiple testing correction of the p-value was performed within edgeR by the Benjamini-Hochberg procedure in order to control the false discovery rate (FDR) at a level of 0.10. Accordingly, genes associated with an adjusted p-values < 0.10 were considered significant and were therefore expected to include 10 % of false positive result. A downstream analysis was performed in order to interpret the differential expression results by considering a global biological context. In that context, pathway enrichment analysis was performed by using the WebGestaltR bioconductor package and the KEGG database (RRID:SCR_012773). Barcode gene enrichment plots were generated with limma bioconductor package (RRID:SCR_010943).

Both raw and processed RNA-seq data were deposited on the Gene Expression Omnibus (GEO) and made publicly available (GSE157487).

BrdU incorporation

Total peritoneal cells from untreated and CNT-7 or CNT-T-treated rats sacrificed after 7 days, were seeded on polypropylene tubes and incubated for 24 h in RPMI 1640 Medium (Cat. # 21875-034) from Thermofisher supplemented with 10 % of decompemented Fetal Bovine Serum (FBS) (Cat. # 10270-106) (Thermofisher) and 1 % antibiotic (Cat. # 15240-062) from Thermofisher. The BrdU was added to the culture (10 μ M) 24 hours at 37 °C. The cells were stained with CD11b/c and HIS48 antibodies and fixed and permeabilized using the Cytofix/Cytoperm Buffer provided with the FITC BrdU Flow Kit (Cat. # 559619, RRID: AB_2617060) from BD Biosciences, according to the protocol. After permeabilization, cells were stained using anti-BrdU antibody provided with the kit. The samples were then analyzed by flow cytometry.

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Ki-67 staining

Peritoneal macrophages from untreated and CNT-7 or CNT-T-treated rats (7 days), were sorted out (FACS Aria III), fixed and permeabilized with a 3:1 ice cold methanol-acetone mixture for 30 minutes. After the staining with anti-ki67 antibody (BD Pharmingen) (Cat. # 556026, RRID: AB_396302) samples were analyzed by flow cytometry.

qRT-PCR

Total RNA extraction was performed using TriPure Isolation Reagent (Cat. # 11667165001) from Roche Diagnostics. Reverse transcription was carried out from 1 µg of extracted RNA resuspended in Hyclone water, using the M-MLV Reverse Transcriptase (Cat. # 28025013) from Thermofisher and random hexamers from Eurogentec. The resulting cDNA was used as template in subsequent polymerase chain reactions (PCR). Sequences of interest were amplified by PCR using the following forward primers from Invitrogen: TGCCTGTTTGAAGGAGTATTGA (Mki67), CGGCTACCACATCCAAGGAA (h18sRNA) and reverse primers: TTTATAATAGTGCCATTTACTTGAGTTGGAT (Mki67), ATACGCTATTGGAGCTGGAATTACC (h18sRNA). PCR was performed with AmpliTaq Gold polymerase (Invitrogen) according to the manufacturer's instructions with the following temperature program: 10 min 95 °C, (15 s 95 °C, 1 min 60 °C) ×40 cycles. Amplified DNA fragments were purified from a 2 % agarose gel using PCR clean-up Gel extraction kit(s) (Macherey-Nagel, Düren, Germany) and then serially diluted to serve as standards in real-time PCR. Reverse transcribed mRNAs were finally quantified by real-time PCR using SYBR Green technology on an ABI Prism 7000 Sequence Detection System (Applied Biosystems) according to the following program: 2 min 50 °C, 10 min 95 °C, (15 s 95 °C, 1 min 60 °C) ×40 cycles. In order to

verify specific amplification, a dissociation curve was obtained by increasing temperatures up to 95 °C over a 20-minute period. Results were calculated as a ratio of gene expression to the expression of the reference gene, RNA 18s.

Immunohistochemistry

Omentum tissues from untreated and treated rats were harvested, fixed for 48h at 4 °C in 4 % paraformaldehyde and embedded in paraffin. Five-µm sections were incubated with mouse anti-rat CD68 antibody (Abcam monoclonal, Cat. # ab31630, RRID: AB_1141557) 90 minutes at RT, followed by 40-min of incubation with secondary antibody donkey anti-mouse IgG (Cat. # 715035151, RRID: AB_2340771) from Jackson ImmunoResearch, coupled with HRP. The samples were then incubated for 10 minutes with AlexaFluor Tyramide 488 at RT. A counterstaining to detect the nuclei was performed with Hoechst44432 dye (Cat # 14533) from Sigma Aldrich. Stained slides were digitalized using a Pannoramic 250 FlashIII scanner (3DHistech) at x20 magnification. Whole tissue sections were then analyzed using Visiopharm software (v2017.2). Tissue was first manually delineated at low magnification and large empty spaces and artifacts were automatically discarded. Cells were then detected at high resolution (20×) with a nuclear-based cell classification relying on the Hoechst staining. Following segmentation, post processing steps were applied to separate CD68-stained and unstained cells. The same parameters were kept constant for all slides. Results were expressed as percentage of stained cells.

Statistics

Data were evaluated by one-way analysis of variance (ANOVA) using the Dunnett's Multiple Comparison Test when appropriate. Statistical significance was considered

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at $p < 0.05$. Data were evaluated by an unpaired t test, when appropriate. Statistical significance was considered at $p < 0.05$.

2.4. Results

Rapid macrophage disappearance reaction and repopulation after injection of mesotheliomagenic CNT-7

We first assessed the number of macrophages in the peritoneal cavity after CNT treatments. Wistar rats were injected intraperitoneally with a reference and mesotheliomagenic dose of CNT-7 or CNT-T (Poland et al. 2008), and macrophage population was analyzed by flow cytometry in peritoneal lavage. Figure 1 shows the percentage and number of macrophages (CD11b^{high} and His48^{dim} cells, Figure 1 A, frame a) accumulated at 0, 3 hours, 1, 3, 7 and 15 days (Figure 1 B–E). Resident macrophages were depleted in the peritoneal cavity fluid from 3 hours to 3 days after treatment with both CNT in comparison with controls (Figure 1 B–E), reflecting a macrophage disappearance reaction (MDR). This MDR was followed by a replenishment phase with a different macrophage repopulation according to the type of CNT. Indeed, macrophage regeneration in CNT-7-treated rats was already complete after 7 days (Figure 1 B–C) while the renewal reaction was still incomplete after 15 days in CNT-T-treated rats (Figure 1 D–E). These data suggested that the macrophage replenishment phenomenon arising after early MDR is more rapid and pronounced in mesotheliomagenic response.

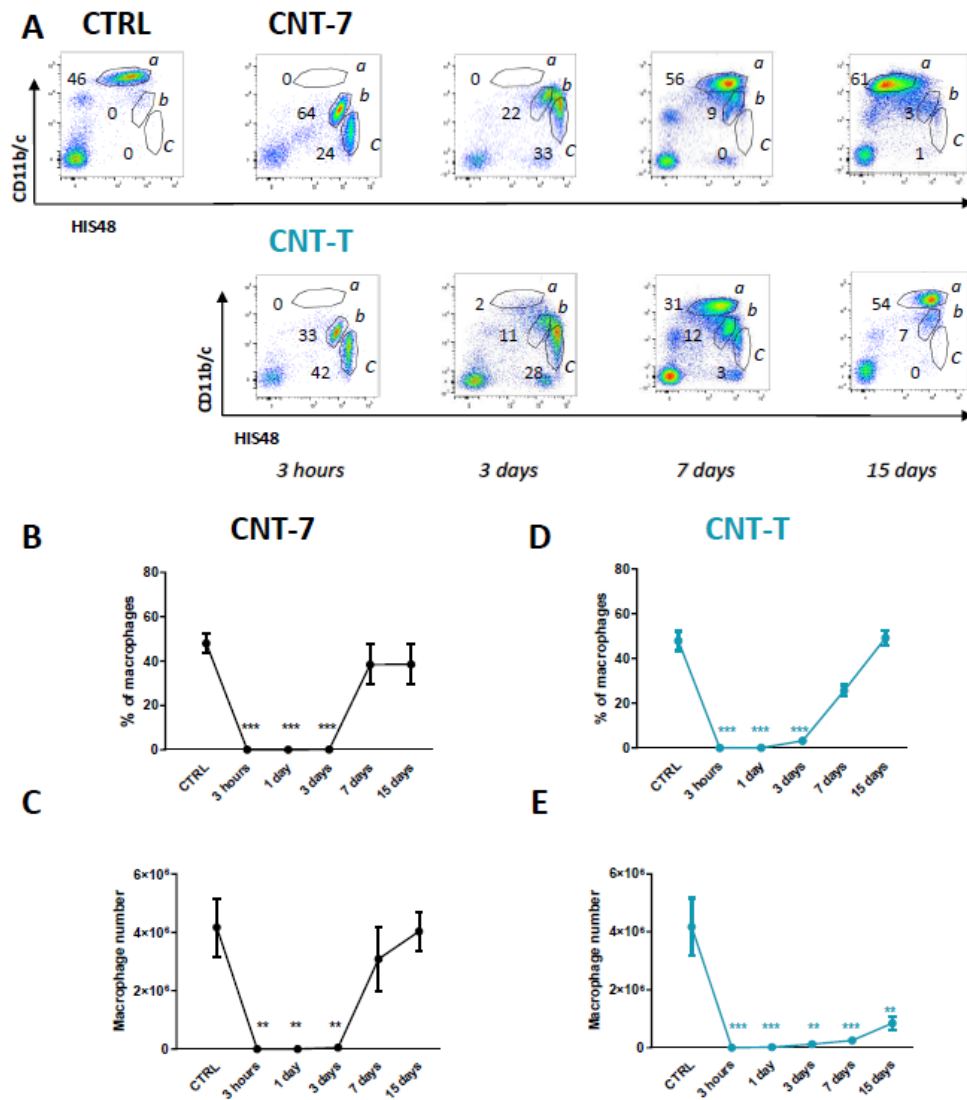


Figure 1: Disappearance reaction and repopulation of peritoneal macrophages after exposure to CNT-7 and CNT-T. (A) Flow cytometry dot plots (CD11b/c, His48) of peritoneal cells (a = macrophages, b = neutrophils, c = monocytes) of CTRL (untreated rats sacrificed at 3 hours) or CNT-treated Wistar rats (2 mg, i.p.) (3 hours, 3, 7 and 15 days). Percentage (B and D) and number (C and E) of peritoneal macrophages in response to mesotheliomagenic

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CNT-7 and non-mesotheliomagenic CNT-T (day 0-15). Each point represents the mean of 4 samples $\pm$ SEM. The results were statistically analyzed using ANOVA test followed by Dunnett's test. ** = $p < 0.01$, *** = $p < 0.001$ indicates a statistically significant difference with controls.

Mesotheliomagenic CNT-7-induced MDR results from cytotoxicity and is concomitant to neutrophilic inflammation

The mechanism explaining CNT-induced MDR was investigated. We excluded a migration of macrophages from peritoneum to omentum. Indeed, no CD68-positive macrophage accumulation was observed in the omentum after CNT injection in comparison to control tissue (Figure 2 A). We next considered the possibility that the cytotoxic activity of CNT could explain macrophage disappearance. We exposed peritoneal macrophages from untreated rats to different doses of CNT *in vitro* and assessed cell viability by measuring ATP cellular contents. CNT-7 and CNT-T both exerted a dose-dependent cytotoxicity, with a higher effect at low doses for CNT-7 (Figure 2 B), suggesting that the *in vivo* MDR resulted from the intrinsic cytotoxicity of CNT-7 and CNT-T to resident macrophages.

We also noticed that, after CNT-7, MDR and macrophage death were strongly related to an influx of neutrophils (Figure 2 C; gating strategy see Figure 1 A, frame b) and to a broad release of pyroptosis-associated IL-1 β and cytokines (IL-6, IL-17A and CCL3) (Figure 2 E–H) in the peritoneal cavity. In contrast, limited inflammatory responses in time and amplitude were observed after CNT-T injection (Figure 2 D–H). These data indicated that MDR is concomitant with a pyroptotic and inflammatory phase, which is faster and more pronounced for mesotheliomagenic CNT-7 than for non-mesotheliomagenic CNT-T.

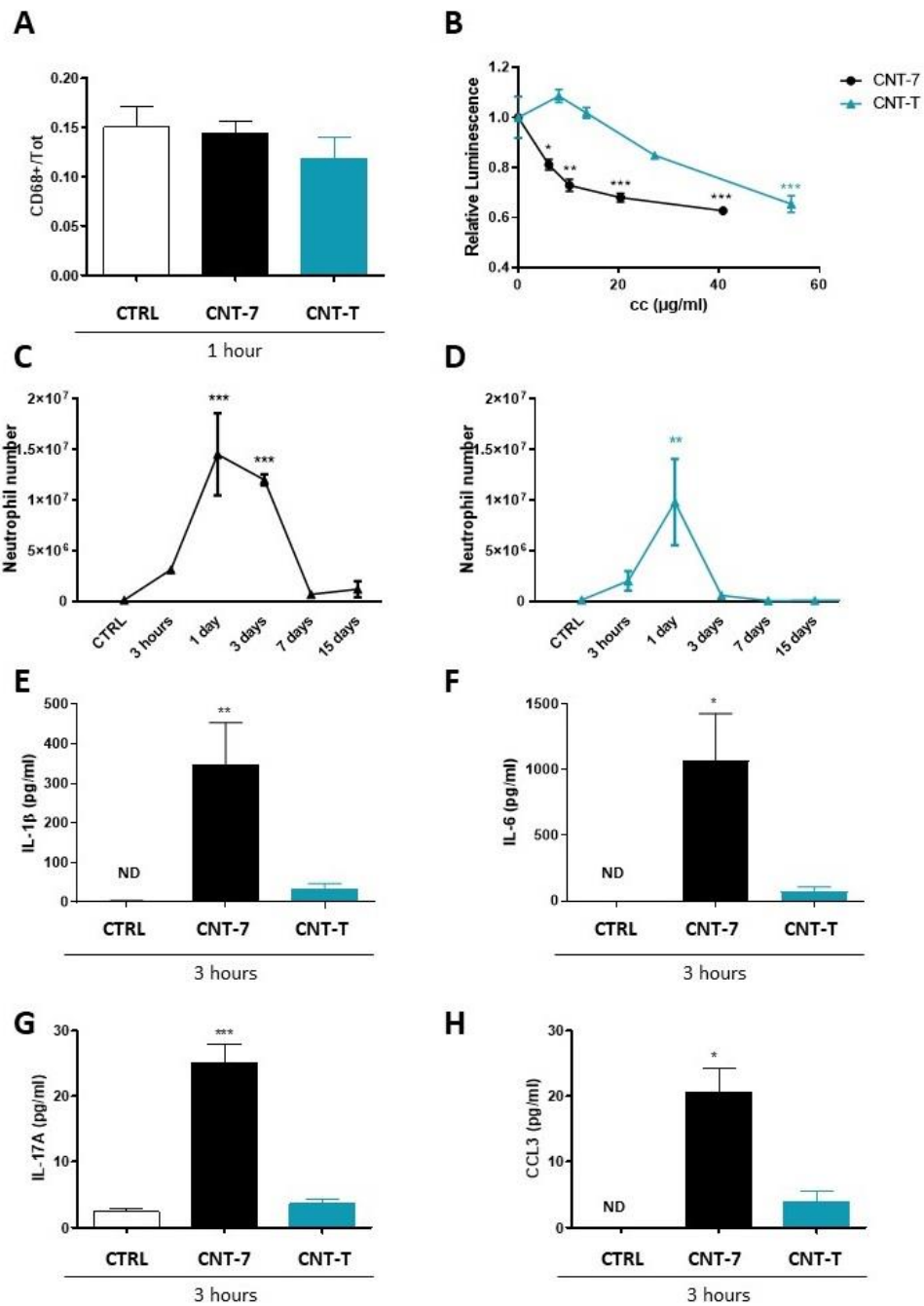


Figure 2: An early cytotoxicity but not a relocation in the omentum explains the CNT-induced disappearance reaction (MDR) and inflammatory responses. (A) Quantification of CD68-positive cells of the omentum of untreated and CNT-7 or CNT-T-treated rats (2 mg, I.P.) sacrificed after 1 hour. The immunofluorescent staining was performed with anti-rat CD68 antibody. (B) Cellular ATP levels measured 24 hours after *in vitro* exposure of naïve peritoneal macrophages to 20, 10, 5 and 3 µg/ml of mesotheliomagenic CNT-7 or non-mesotheliomagenic CNT-T. ATP concentration was measured with CellTiter-Glo® Luminescent Cell Viability Assay. Each point represents the mean of 3 replicates ± SEM. (C-D) Number of peritoneal neutrophils (see Figure 1 A, frame b) in response to CNT-7 and CNT-T (2 mg, I.P.) (day 0-15). CTRL are untreated rats sacrificed at 3 hours. Each point represents the mean of 4 samples ± SEM. (E-H) IL-1β, IL-6, IL-17A and CCL3 released in untreated and CNT-7 or CNT-T-treated rat (2 mg, I.P.) peritoneal fluids were tested using Bio-Plex multiplex assay. The results were statistically analyzed using ANOVA test followed by Dunnett's test. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ indicates a statistically significant difference with controls.

Mesotheliomagenic CNT-7 specifically induce MHCII^{high} small peritoneal macrophage expansion

We next studied the replenishment phase after MDR by characterizing the phenotype of regenerated macrophages after CNT-7 or CNT-T injection. We performed RNA-sequencing of replenished peritoneal macrophages to explore the gene expression profile characterizing MHCII^{high} SPM (Small Peritoneal Macrophages) and MHCII^{low} LPM (Large Peritoneal Macrophages) (see Table 1). Then, we coupled RNA-sequencing data with flow cytometry analysis to characterize the peritoneal macrophage phenotype after regeneration.

As illustrated in the barcode plot (Figure 3 A, bottom panel), there was a strong enrichment of MHCII^{high} SPM gene signatures in the CNT-7 versus CNT-T macrophages. In agreement with these results, we observed that macrophages

recruited after CNT-7 markedly expressed MHC-II (but not CD163) in comparison to controls and CNT-T treatment (Figure 3 B–C). Our data thus suggested that mesotheliomagenic CNT-7 specifically regenerated macrophages with an MHC-II^{high} SPM-like phenotype. In contrast, macrophages collected from CNT-T-treated rats expressed genes related to the MHCII^{low} LPM phenotype (Barcode data, Figure 3 A, top panel). As appreciated by flow cytometry, these macrophages were mainly CD163-positive and MHC-II-negative, and thus corresponded to the macrophage phenotype in homeostatic conditions (Figure 3 B–C).

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Table 1: LPM/SPM genes and functions. From references (Accarias et al. 2016; Bain and Jenkins 2018; Kim et al. 2016; Sobecki et al. 2016).

LPM (genes and functions)		SPM (genes and functions)	
Alox15	Lipid uptake	CSF1R	M-CSF receptors
Fabp4	Lipid uptake	CX3CR1	CX3CL1 receptor
Cav1	Lipid uptake	DNAM1 (CD226)	Cell adhesion
Cd36	Lipid uptake	Mrc1 (CD206)	Mannose receptor
Icam2 (CD102)	Intercellular adhesion molecule	Retnla (RELMalfa)	immunoregulatory cytokine
Adgre1 (F4/80)	adhesion G protein-coupled receptor E1	Aldh1a2 (Raldh2)	retinaldehyde dehydrogenase A2
MerTK	Mer tyrosine kinase	CD80	costimulatory protein
Nt5e (CD73)	5'-nucleotidase	RT1-Bb	MHCII protein
Cd40	costimulatory protein	LOC688090	MHCII protein
Tlr4	Toll like receptor	Ciita	MHCII protein
CXCL13	Chemokine for serous cavity homing	RT1-Db1	MHCII protein
CD86	costimulatory signals	RT1-Da	MHCII protein
CD163	Scavenger receptor	Cd74	MHCII protein
Itgam (CD11b)	Integrin alphaM	Klrc2	MHCII protein
Gata6	Transcription factors	Klrc3	MHCII protein
Klf2	Transcription factors	RT1-Ba	MHCII protein
Klf4	Transcription factors	IRF4	Transcription factors
Cebpb	enhancer-binding protein		
Mki67	nuclear protein		

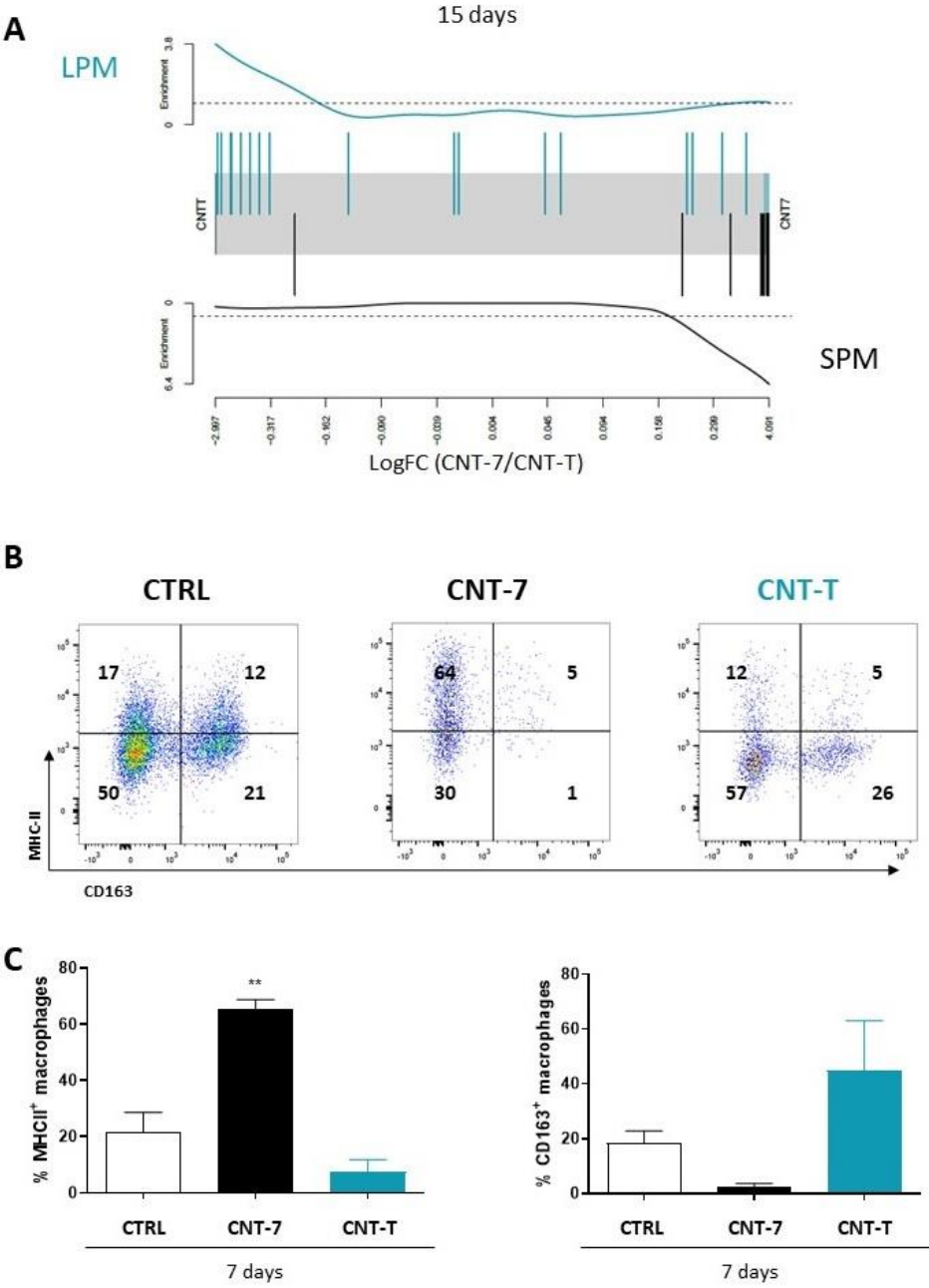


Figure 3: CNT-7 and CNT-T exposure induces MHCII^{high} SPM and MHCII^{low} LPM macrophage development, respectively. (A) Barcode plot showing enrichment of MHCII^{low} (LPM-like)

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(top panel) and MHCII^{high} (SPM-like) (bottom panel) signatures in the CNT-T vs CNT-7 macrophage comparison. Macrophages were purified from the peritoneal cavity of rats treated with CNT-7 or CNT-T (2 mg I.P.) and sacrificed after 15 days. Black bars show up MHCII^{high} signature genes, blue bars show up MHCII^{low} signature genes. The worms show relative enrichment. (B) Flow cytometry dot plots (MHCII-CD163) of CD11b^{high} and HIS48^{dim} macrophages from untreated and mesotheliomagenic CNT-7- or non-mesotheliomagenic CNT-T-treated Wistar rats (2 mg, I.P.) at 7 days. (C) Percentage of MHCII and CD163-positive macrophages from untreated or CNT-7/CNT-T-treated rats sacrificed 7 days after. Each bar represents the mean of 3 replicates $\pm$ SEM. The results were statistically analyzed using ANOVA test followed by Dunnett's test. ** = $p < 0.01$ indicates a statistically significant difference with controls.

MHCII^{high} macrophage replenishment during mesotheliomagenic responses depends on monocyte-lineage progenitors

Our phenotypic data suggested that regenerated MHCII^{high} SPM macrophages after mesotheliomagenic CNT-7 derived from monocytic progenitors. We next assessed monocyte composition of peritoneal lavages and found that CNT-7 induced a clear accumulation of monocytes (CD11b/c^{int} His48^{high}, gating strategy see Figure 1 A, frame C) starting at 3 hours to reach a peak at 3 days, just before macrophage repopulation (Figure 4 A). This reaction was less important and limited to day 1 after CNT-T (Figure 4 A). Moreover, high levels of CCL2 and M-CSF, essential for monocyte chemo-attraction, survival and differentiation were detected at 3 hours in peritoneal fluid of CNT-7-treated rats in comparison to CNT-T and untreated rats (Figure 4 B). These data additionally supported a monocytic ontogeny for MHCII^{high} macrophages. Finally, we also depleted the source of monocytes by depleting the bone marrow progenitors with Busulfan and appreciated macrophage regeneration in CNT-treated rats. Peritoneal macrophage replenishment was completely abrogated in monocyte-depleted animals treated with CNT-7 (Figure 4 C).

Altogether, these results demonstrated that the replenishment of the peritoneal cavity by MHCII^{high} SPM relies on a monocytic source after mesotheliomagenic CNT-7-induced MDR.

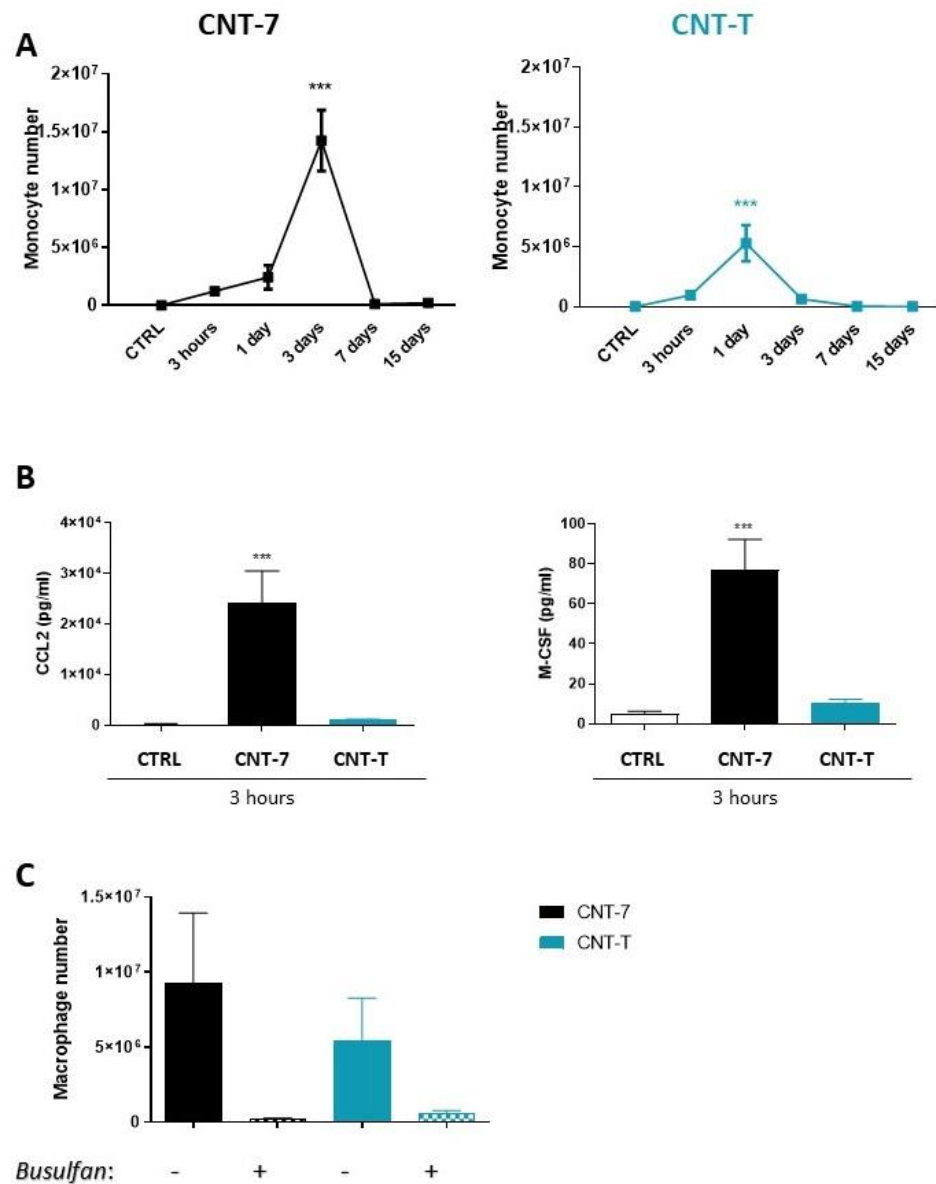


Figure 4: Macrophage regeneration after CNT-7 treatment depends on blood monocytes.

(A) Number of peritoneal monocytes in response to mesotheliomagenic CNT-7 and non-mesotheliomagenic CNT-T (2 mg, I.P.) (day 0-15). CTRL are untreated rats sacrificed at 3 hours. Each point represents the mean of 4 samples $\pm$ SEM. (B) CCL2 and M-CSF released in

untreated and CNT-7 and CNT-T-treated rat (2 mg, I.P.) peritoneal fluid were tested using Bio-Plex multiplex assay. M-CSF and CCL2 were found to be abundantly produced at 3 hours by CNT-7-treated rats. (C) Percentage of peritoneal macrophages (CD11b^{high} HIS48^{dim}) after S.C. injection of double dose of Busulfan (15 mg/kg) before and after injection of CNT-7 and CNT-T. Each bar represents the mean of 3 replicates $\pm$ SEM. The results were statistically analyzed using ANOVA test followed by Dunnett's test. *** = $p < 0.001$ indicates a statistically significant difference with controls.

Macrophages replenishing the peritoneal cavity after non-mesotheliomagenic CNT-T are self-renewing

In comparison to monocyte-derived MHCII^{high} SPM macrophages, RNA-seq data revealed that several genes related to cell cycle and mitosis were specifically expressed by MHCII^{low} LPM macrophages after CNT-T (Figure 5 A). To confirm their proliferative ability, we next studied BrdU incorporation and ki-67 expression. Based on BrdU data, MHCII^{low} macrophages from CNT-T-treated rats proliferated more than macrophages from untreated and CNT-7-treated rats (Flow cytometry dot plot and bar graph, Figure 5 B). Similar data were obtained by analysing the expression of ki-67. MHCII^{low} CNT-T macrophages expressed more ki-67 transcripts (qRT-PCR, Figure 5 D) and protein (Flow cytometry dot plot and bar graph, Figure 5 C) than macrophages from saline- or CNT-7-treated rats. Altogether, these results indicate that self-proliferating MHCII^{low} LPM become the prevalent macrophage subset in response to CNT-T in contrast to CNT-7.

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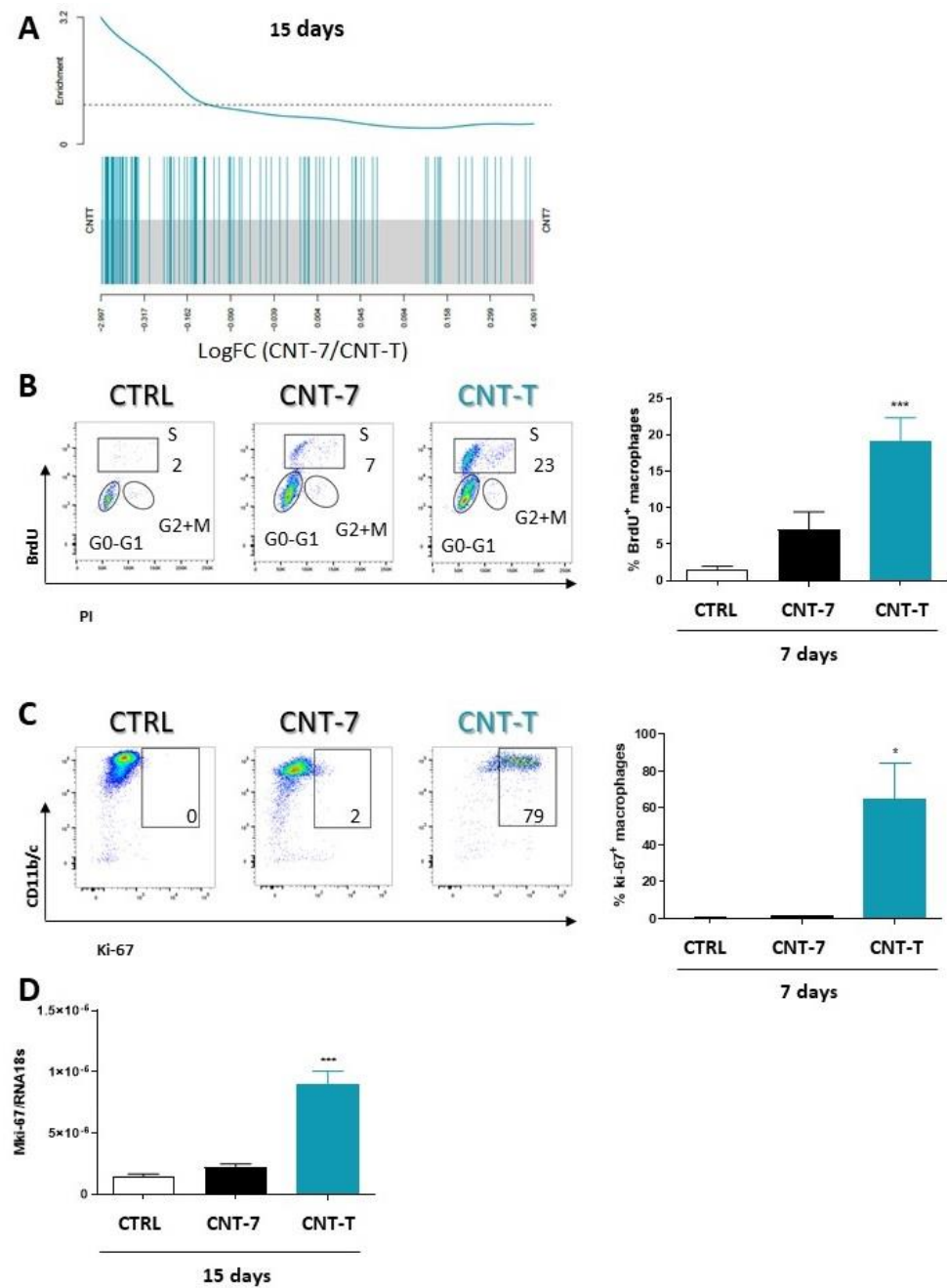


Figure 5: Macrophages repopulating the peritoneal cavity after CNT-T treatment are self-renewing. (A) Barcode plot showing enrichment of cell cycle related signature in the CNT-7 vs CNT-T macrophage comparison. Macrophages were purified from the peritoneal cavity of rats treated with CNT-7 or CNT-T (2 mg I.P.) and sacrificed after 15 days. The worms show relative enrichment. (B) Flow cytometry dot plots show the proportions of peritoneal macrophages in S phase (BrdU-positive) of untreated Wistar rats or rats injected (2 mg I.P.) with mesotheliomagenic CNT-7 or non-mesotheliomagenic CNT-T and sacrificed at 7 days. Graph of BrdU-positive macrophages: each bar represents mean $\pm$ SEM of 6 observations. (C) Flow cytometry dot plots show the proportions of peritoneal macrophages ki-67-positive from untreated or CNT-7/CNT-T treated rats sacrificed at 7 days. The graph represents the ki-67-positive macrophages from untreated rats and CNT-7 or CNT-T-treated rats. Each bar represents mean $\pm$ SEM of 6 observations. (D) mki-67 from macrophages purified from untreated and CNT-treated rats (at 15 days) was quantified by qRT-PCR. Values are means $\pm$ SEM of 4 animals. The results were statistically analyzed using ANOVA test followed by Dunnett's test. * = $p < 0.05$, *** = $p < 0.001$ indicates a statistically significant difference with controls.

2.5. Discussion

CNT are attractive materials with unique physical and chemical properties, including tensile strength, stiffness and good electrical conductivity which are currently used in engineering and material sciences for rechargeable batteries, automotive parts, sporting goods and water filters (De Volder et al. 2013). Experimental studies in rodents demonstrated that the long and straight CNT-Mitsui-7 can induce mesothelioma like asbestos fibers, and experts classified CNT-7 in the IARC Group 2b, i.e. as possibly carcinogenic for humans (Orsi et al. 2020). The numerous applications of other CNT justify the need of carefully characterizing the mesotheliomagenic potential (Aoki and Saito 2020; Heller et al. 2020).

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Like for asbestos, the pathogenicity of CNT is linked to their shape and geometry (Dong and Ma 2015). In rats, more sensitive than mice to mesothelioma development, a single intraperitoneal injection of CNT with a straight and acicular shape (CNT-7) induced peritoneal mesothelioma with a 100 % of incidence after 52 weeks. On the other side, tangled CNT (CNT-T) did not induce peritoneal mesothelioma in long-term studies (3 years) (Muller et al. 2009; Nagai et al. 2013; Rittinghausen et al. 2014; Sakamoto et al. 2018).

In homeostatic conditions the peritoneal cavity is populated by resident macrophages, free-floating GATA6⁺ cells able to proliferate locally without the contribution of bone marrow-derived monocytes (Okabe and Medzhitov 2014). Inflammatory stimuli trigger a phenomenon called Macrophage Disappearance Reaction (MDR), during which resident macrophages become irretrievable from the lavage of the serous cavity. This phenomenon was already observed after different inflammatory insults (Accarias et al. 2016; Cassado Ados et al. 2015; Meza-Perez and Randall 2017; Okabe and Medzhitov 2014), but is still incompletely understood. MDR can be associated to cell death, adhesion to neighbouring tissues or migration to the draining lymph nodes or the omentum (Accarias et al. 2016; Cassado Ados et al. 2015). MDR is a strategy to face and annihilate the infection by which macrophages, under the control of GATA6, move from the peritoneum to the closest tissues in order to alert the immune system. At the same time, they express factor V of the coagulation cascade to promote clot formation in order to trap free-floating bacteria or foreign particles and facilitate their phagocytosis (Zhang et al. 2019b). Here, we report a clear disappearance reaction in the rat peritoneal cavity only 3 hours after the injection of both kinds of CNTs (CNT-T and CNT-7). Interestingly, MDR was not linked to a migration to neighbouring tissue but was caused by the cytotoxic activity of CNT to peritoneal resident macrophages.

In steady state conditions, even if the peritoneal cavity is easily accessible from blood vessels, blood monocytes do not contribute to the existing pool of resident macrophages (Guilliams and Scott 2017). However, when resident macrophages are depleted during inflammatory stimuli, macrophages from circulating monocytes or self-proliferating resident macrophages represent the two competing mechanisms to achieve the repopulation of empty cavities. This phenomenon is called 'niche model' and occurs when the compartment is accessible (no physical barriers), available (not occupied by other macrophages) and without other competitive progenitors (Guilliams and Scott 2017).

The regeneration of macrophages by monocytes is orchestrated by dying macrophages releasing master inflammatory mediators (mainly members of the IL-1 and TNF families). These inflammatory molecules temporarily trigger macrophage niche (epithelial, endothelial and stromal cells) to release monocyte-recruiting (CCL2 and adhesion molecules) and differentiating (CSF family) factors that result in monocyte engraftment and differentiation (Bonnardel et al. 2019). In contrast, during mild or minor inflammation, there are very few monocytes engrafting in the empty macrophage niche, which is mainly restored by remaining macrophages or newly differentiated macrophages which proliferate (Guilliams et al. 2020). The exact growth factors orchestrating monocyte differentiation or macrophage proliferation are still debated but the most accepted hypothesis is that the expression of soluble or membrane forms of M-CSF, IL-34 or GM-CSF and the complex association of their corresponding receptors are crucial to fill the empty niches and finalize the macrophage repopulation (Guilliams et al. 2020).

Our data support the new concept that the regeneration of macrophages during mesotheliomagenic responses to CNT-7 is exclusively tributary of the monocyte

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pool. The initial depletion depends on the cytotoxic activity of the particles to macrophages, and the intensity and duration of the inflammation, which is related to the type of macrophage death. Indeed, the rigid, needle-like shape of CNT-7 is responsible for a pronounced inflammatory reaction initiated by pyroptosis/inflammasome mediated-cell death (IL-1 β activation and release) and release of inflammatory cytokines (IL-6, IL-17A, CCL3) involved in neutrophil recruitment. The cytotoxic and inflammatory activity of CNT-7 in turn triggers the recolonization of the niche by inflammatory monocytes which differentiate into pathogenic macrophages.

The dynamic of macrophage replenishment after mesotheliomagenic CNT-7-induced depletion could rely on the CCL2 and M-CSF axis (Figure 4 B). CCL2 (MCP-1) is a chemokine regulating monocyte infiltration in tissues. The major source and the main cell target of CCL2 is the monocyte/macrophage lineage. CCR2^{high} monocyte-derived macrophages typically infiltrate cancer promoting tumor growth, immunosuppression, angiogenesis, and cancer cell dissemination leading to a bad prognosis (Lim et al. 2016). Anti-CCL2 neutralizing antibodies reduce mesothelioma growth and tumor volume, switching the polarization of tumor infiltrating macrophages to a more anti-tumor phenotype (Fridlender et al. 2011). M-CSF controls the production, differentiation, survival and functions of macrophages, monocytes and bone marrow progenitors. Like CCL-2, M-CSF is involved in monocyte chemoattraction and promotes monocyte survival (Jeannin et al. 2018). M-CSFR (also known as CSF1-R) inhibitors induce the depletion of tumor infiltrating macrophages or their polarization towards a pro-inflammatory phenotype, with beneficial effects for mesothelioma regression (Dammeijer et al. 2017; Hoves et al. 2018).

After the macrophage disappearance reaction, monocytes from CNT-7-treated rats engrafted the empty peritoneal cavity and differentiated into resident cells, possessing a unique MHCII^{high} SPM phenotype. Serous cavities physiologically comprise distinct macrophages subsets. Monocyte-derived MHCII^{high} macrophages are characterized by highly phagocytic activity and constitutively producing RELM- α , a cytokine signature of alternatively activated macrophages (M2) that controls the CD4⁺ T cell response during inflammatory events and promotes tissue repair process (Nair et al. 2009). It was recently discovered that MHCII^{high} macrophages present the antigens to naïve CD4⁺ T cells, thanks to DNAM-1, an immunoreceptor, co-immunostimulatory signal which binds its counterpart (CD155 ligand) on CD4⁺ T cells (Takenaka et al. 2018). These data are in accordance with our previous results showing that only mesotheliomagenic particle injection induced an accumulation of monocytes and macrophages possessing the ability to limit T cell functions (Huaux et al. 2016). This alternative polarization of monocyte-derived macrophages after CNT-7, is possibly kept until the installation of cancer, contributing to prevention of T cell killer activity, as widely described in the literature (Chu et al. 2019).

We showed that non-mesotheliomagenic CNT-T macrophages express high levels of CD163 and low levels of MHC II. MHCII^{low} macrophages are maintained through local self-renewal in naïve conditions (Wang and Kubes 2016). They express GATA6, important for the proliferation, survival and physiologic metabolic activity of macrophages. Moreover, these cells represent an important reservoir of mature macrophages, which can be mobilized to repair injured tissues and orchestrate return to homeostasis (Wang and Kubes 2016). We demonstrated that MHCII^{low} macrophages presenting the characteristics of proliferating and self-renewing cells also accumulate after CNT-T. At the same time, these proliferating macrophages

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partially depend on hematopoietic progenitors since they are not completely replenished after monocyte depletion (Figure 4 C). These findings agree with those published by Cain and co-authors (Cain et al. 2013). They showed that after resident cell depletion via chemical or radioactive treatment, circulating monocytes differentiate first into F4/80^{low} MHCII^{high} macrophages and later into F4/80^{high} MHCII^{low} proliferating macrophages. This represents the physiological process allowing to restore the reservoir of resident cells with bone marrow derived circulating monocytes after depletion. We thus propose that MHCII^{low} macrophages derive at very early time point from MHCII^{high} macrophages. Then, MHCII^{low} macrophages proliferate and reconstitute the peritoneal macrophage stock by proliferation.

Altogether, our findings attest that macrophage replenishment is a key event during responses to particles. Pathogenic needle-like CNT are highly cytotoxic for resident macrophages in the peritoneal cavity. This cytotoxicity induces the release of pyroptotic-related inflammatory cytokines and chemokines, which, in turn, together with the need to repopulate the empty niche, orchestrate early immune responses characterized by the recruitment of monocytes. They differentiate into MHCII^{high} macrophages that remain blocked into an immature state, inapt to differentiate into resident MHCII^{low} macrophages to restore the steady state conditions. In contrast, the macrophage repopulation after non-mesotheliomagenic, tangled-like CNT is caused by the proliferation of newly differentiated MHCII^{low} macrophages from very early recruited monocytes. These macrophages re-colonize the peritoneum and can restore the normal equilibrium disrupted after particle-induced depletion. These findings may open a horizon for a new therapeutic strategy for mesothelioma based on reprogramming of MHCII^{high} macrophages towards a MHCII^{low} profile and the reactivation of their cell cycle to revert their functions into non-pathogenic cells. Moreover, the regeneration and

niche model proposed in this study validates the interests for *in vivo* bioassays assessing MDR and macrophage repopulation for anticipating the hazardous potential of a new particle or fiber sample.

Acknowledgements

Not applicable.

3. PAPER III

The early macrophage response to mesotheliomagenic carbon nanotubes precedes tumour development and immunosuppressive tumour-associated macrophages

Additional experiments required before publication

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3.1. Abstract

Compelling evidence suggests that macrophages exposed to mesotheliomagenic particles polarize towards M1-like inflammatory cells that promote mesothelial cell transformation and proliferation. Conversely, once mesothelioma is established, M2-polarized immunosuppressive macrophages (or tumor-associated macrophages, TAM) invade tumors and prevent anti-tumor T lymphocytes.

In a model of mesothelioma induced by carbon nanotube (CNT-7), we previously identified a new population of monocyte-derived macrophages (Small Peritoneal Macrophages or SPM) that replenish the empty macrophage niche caused by the acute cytotoxic activity of CNT. Here, we determine whether this specific early population contribute to inflammation extension or create an early immunosuppressive microenvironment.

We observed that early SPM macrophages collected from CNT-7-treated rats display an *in vitro* immunosuppressive activity towards activated T cells and express most of the immunoregulatory signatures of TAM. In contrast, Large Peritoneal Macrophages (LPM)-associated with responses to non-mesotheliomagenic CNT-T were not immunosuppressive. SPM corresponded to a subpopulation of immunosuppressive syndecan-1 (SDC-1)-positive macrophages and release the SDC-1 soluble form which possesses intrinsic immunoregulating functions. RNA-seq analyses indicate that SPM arise from the transition of inflammatory M1/immunosuppressive M2 macrophages specifically induced by the mesotheliomagenic CNT-7.

Altogether, we identified a intermediate population of immunosuppressive SDC-1⁺ macrophages, which links together acute and chronic phases of CNT-induced carcinogenesis. This subset contributes to establishing an early immunosuppressive

environment predisposing to tumor cell expansion and mesothelioma. The proximity in phenotype and function between early SPM and late TAM represents a new opportunity to enrich existing bioassays currently available to predict the carcinogenic potential of particles.

3.2. Background

Most solid tumors are infiltrated by immunosuppressive leukocytes which negatively regulate natural T cell anticancer activity. Beside regulatory T cells (T reg) and Myeloid Derived Suppressor Cells (MDSC) (Lei et al. 2020), Tumor-Associated Macrophages (TAM) are abundantly recruited by tumor cells and polarized into a pro-tumoral immunosuppressive M2-like phenotype (Biswas and Mantovani 2010; Mantovani et al. 2002; Roca et al. 2009; Solinas et al. 2010).

TAM employ diverse mechanisms and mediators to prevent anticancer T cell activity. TAM cell surface is rich in inhibitory checkpoint proteins (e.g. PDL-1 and B7-1) responsible for the hampering of the priming of T cells (Hashimoto et al. 2018). TAM express amino acid metabolizing enzymes including arginase (Arg1) and indoleamine-2,3-dioxygenase (IDO) in order to deprive the tumor microenvironment from arginine and tryptophan, that are pivotal in ensuring T cell fitness (Groth et al. 2019). In addition, TAM produce a plethora of immunoregulatory molecules such as nitrogen oxygen species (NOS), which trap and deactivate T cell in the tumor stroma (Joyce and Fearon 2015), and immunosuppressive TGF- β 1, that inhibit T cell activation (Cheng et al. 2006; Wu et al. 2015). TAM-derived IL-10 inhibits APC functions (Mannino et al. 2015),

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suppresses dendritic cell maturation in the tumor (Vicari et al. 2002) and, moreover, arrests T cell proliferation (Wu et al. 2015). Finally, PGE₂, once released by TAM, keeps the M2 immunosuppressive polarization state of macrophages (Wang et al. 2015), prevents the NK cell cytotoxicity towards tumor cells and stimulates the T reg immunosuppressive functions (Nakanishi and Rosenberg 2013). Furthermore, PGE₂ decreases the production of immunostimulatory IFN- γ and IL-12 (Wu et al. 2015). TAM not only exert a direct immunosuppressive activity on T cells, but they also orchestrate and coordinate the establishment of a favorable tumor microenvironment by fostering cancer cell glycolysis (Vitale et al. 2019), tumor hypoxia (Jeong et al. 2019) and neoangiogenesis (Jackaman et al. 2017). Such an environment is ideal for cancer cell survival and proliferation (Vitale et al. 2019). Also, TAM produce proteolytic enzymes and matrix-associated proteins, which interact with the extracellular matrix surrounding the tumor to allow malignant cell invasion (Afik et al. 2016).

Malignant mesothelioma affecting pleura, peritoneum or pericardium is one of the most aggressive solid tumors (Kao et al. 2010) with poor prognosis (Berzenji and Van Schil 2018) and, currently, lacking efficient therapies. The role of immunosuppressive instead of inflammatory macrophages defined as M2-like immunosuppressive TAM has become a major area of interest in mesothelioma research (Cornelissen et al. 2015; Dammeijer et al. 2017). Indeed, recent data outline that high CD163⁺ M2-like TAM and low CD8⁺ lymphocytes infiltrating peritoneal and pleural mesothelioma are associated with pathological features of aggressiveness and correlated with a decreased overall survival (Burt et al. 2011; Chene et al. 2016; Cornelissen et al. 2014; Ujiie et al. 2015). The exact mechanisms or mediators by which immunosuppressive TAM can induce T cell anergy in malignant mesothelioma have been recently investigated. Evidence suggested that TAM-released immunosuppressive mediators like TGF- β 1, IL-10 and prostanoic

PGE₂ could explain the T cell deactivation in malignant mesothelioma (Donnenberg et al. 2019; Li et al. 2016; Lievense et al. 2016). Investigators, indeed, noticed that high levels of TGF- β 1, IL-10 and PGE₂ in pleural effusions of malignant mesothelioma patients correlate with negative prognosis. Moreover, it was demonstrated that TGF- β 1 produced by mesotheliomagenic TAM induces T cells to decrease the production of anti-tumoral molecules such as IFN- γ and granzyme B (Li et al. 2016). Similarly, the autocrine production of PGE₂ polarizes macrophages towards an immunosuppressive phenotype able to arrest the T lymphocyte proliferation in mesothelioma (Lievense et al. 2016).

These recent observations on immunosuppressive/M2 TAM in mesothelioma have strongly challenged the dogmatic view that particle-activated inflammatory/M1 macrophages represent the exclusive macrophage population implicated in malignant mesothelioma (Toyokuni 2019; Urso et al. 2020). Indeed, compelling experimental elements on macrophages demonstrated that needle-like shaped and high length/diameter ratio particles cause an uninterrupted frustrated phagocytosis and pyroptotic-related cell death (Jiang et al. 2008). During this process, macrophages release genotoxic reactive oxygen species (ROS) (Ito et al. 2020) and cytokines playing a role in inflammation and remodeling, such as alarmins (IL-1 α and HMGB1), neutrophil (IL-1 β , IL-8 and TNF- α)- and monocyte (CCL2)-chemotactic cytokines and fibroblast growth factors (TGF- β 1 and PDGF) (Liu and Tang 2020; Nakayama 2018). This persistent inflammatory reaction results in genomic instability, transformed mesothelial cell survival and proliferation, matrix protein deposition and mesothelioma establishment (Chew and Toyokuni 2015; Toyokuni 2019).

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The mechanisms responsible of the macrophage transition between the two distinct macrophage subpopulations infiltrating mesothelioma (M2-like immunosuppressive- and M1-like inflammatory-macrophages) and polarized respectively by tumor cells and particles remain undetermined and need to be clarified.

The peritoneal cavity hosts two distinct resident macrophage subsets: bone-marrow-derived Small Peritoneal Macrophages (SPM) and embryonic, self-proliferating Large Peritoneal Macrophages (LPM). LPM and SPM differ in size, in the expression of certain surface markers including Gr-1 and MHCII and in the transcription factors GATA6 and IRF4 (Ghosn et al. 2010; Kim et al. 2016). LPM maintain the peritoneal cavity's physiological conditions by clearing apoptotic and senescent cells and regulating homeostasis and the adequate amount of surfactant in the serous cavity. More controversial are the functions of SPM. Indeed, SPM are supposed to be inflammatory cells in charge of phagocytosing bacteria, releasing inflammatory mediators such as TNF- α , MIP-1 α , and RANTES and presenting antigen to T cells in response to inflammatory stimuli (Bain and Jenkins 2018; Cassado Ados et al. 2015). Nevertheless, Han and coworkers discovered that an SPM-like macrophage subpopulation expressing syndecan-1 glycoprotein (SDC-1) has an immunoregulatory phenotype involved in the resolution of inflammation in infections and autoimmune diseases (Han et al. 2017).

By using a rat model of carbon nanotube (CNT)-induced peritoneal mesothelioma, we recently demonstrated that mesotheliomagenic CNT-7 perturb the peritoneal cavity homeostasis, induce resident macrophage depletion and promote a specific influx of a SPM-like subset (SPM). In contrast, peritoneal responses to non-mesotheliomagenic CNT-T are characterized by the recruitment of a LPM-like homeostatic subpopulation (Paper II). In this study, we clarify whether early SPM

specifically recruited by mesotheliomagenic CNT-7 contribute to the inflammatory or immunosuppressive microenvironment characterizing mesothelioma development. To address this question, we compare SPM with LPM (recruited by non-mesotheliomagenic CNT) and TAM (accumulated in CNT-7-induced peritoneal mesothelioma) for their ability to block T cell proliferation and express immunosuppressive and inflammatory genes.

3.3. Results

Small Peritoneal Macrophages (SPM) recruited after mesotheliomagenic particles injection inhibit T cell proliferation and are immunosuppressive

We first determined whether CNT-7-related SPM and CNT-T-related LPM differently affect T lymphocyte activity in co-culture. Indeed, modulation of splenic T cell proliferation discriminates inflammatory and immunosuppressive peritoneal leukocytes (Huaux et al. 2016). Peritoneal macrophages were identified by flow cytometry as CD11b/c^{high} HIS48^{int} population (Supplemental Figure 1 A–B). SPM and LPM were respectively collected from peritoneal cavity of mesotheliomagenic CNT-7 and non-mesotheliomagenic CNT-T-treated rats at 15 days when macrophage replenishment is accomplished (Paper II). Moreover, SPM- or LPM-like phenotypes can also be mimicked and expanded *in vitro* by using bone-marrow (BM) precursors differentiated with recombinant GM-CSF and M-CSF, respectively (Lukic et al. 2017; Na et al. 2016; Ohradanova-Repic et al. 2018; Wang et al. 2017). Figure 1 shows that SPM obtained from mesotheliomagenic CNT-7-treated rats (Figure 1 A) or GM-CSF-polarized macrophages (Figure 1 C) inhibit T cell proliferation, activated by

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anti-CD3/CD28 antibodies, in a number-dependent way. In contrast, LPM collected from non-mesotheliomagenic CNT-T-treated rats (Figure 1 B) or M-CSF-differentiated macrophages (Figure 1 D) do not impact T cell proliferation. Furthermore, we performed a co-culture system with peritoneal macrophages obtained from rats exposed to additional mesotheliomagenic (asbestos) and non-mesotheliomagenic (crystalline SiO₂) particles. We confirmed that peritoneal macrophages from mesotheliomagenic asbestos-treated rats exert an immunosuppressive activity on activated T cells (Figure 1 E), unlike macrophages from non-mesotheliomagenic SiO₂-treated rats (Figure 1 F). These results outline that early SPM-like macrophages accumulated in responses to mesotheliomagenic particles are specifically immunosuppressive towards T cells.

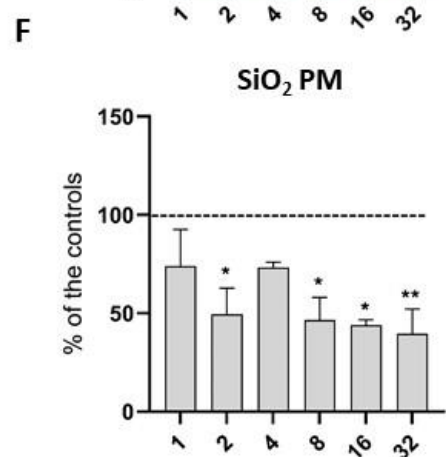
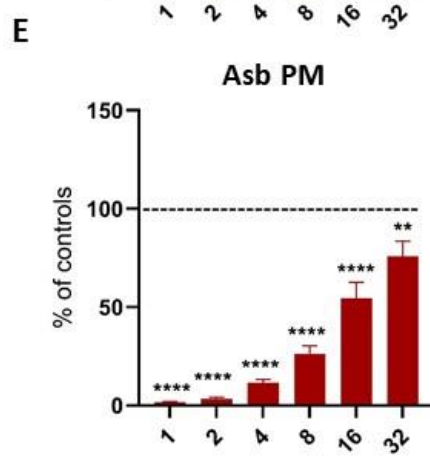
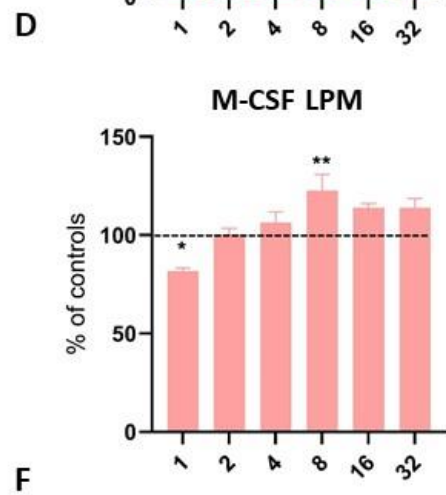
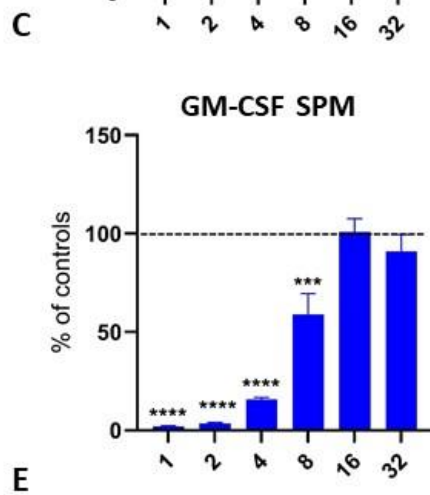
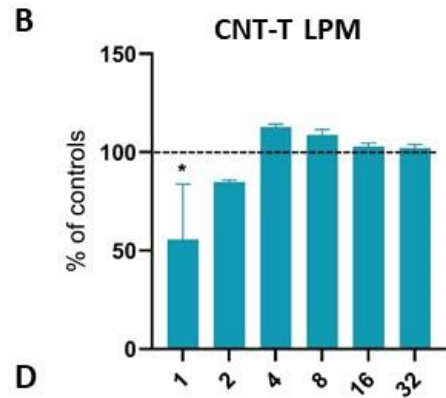
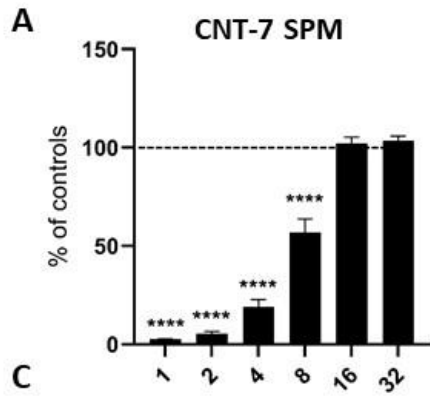


Figure 1: Inhibition of T lymphocyte proliferation by CNT-7-recruited Small Peritoneal Macrophages. *In vitro* antiproliferative activity of peritoneal macrophages purified 15 days after CNT-7 (A), CNT-T (B), asbestos (E), and SiO₂ (F)-treated rats (2 mg) on spleen T lymphocytes activated with anti-CD3/CD28 antibodies. *In vitro* antiproliferative activity of macrophages differentiated *in vitro* with GM-CSF (C) and M-CSF (D) on spleen T lymphocytes activated with anti-CD3/CD28 antibodies. Graphs represent the average level of ³H-thymidine incorporation expressed as percent of controls (activated T lymphocytes alone) for each splenocytes/macrophages ratio. Each bar represents mean ± SEM. These results were statistically analyzed using Anova test followed by Dunnett's Multiple Comparison test. * = p < 0.05, ** = p < 0.01, *** = p < 0.001, **** = p < 0.0001 indicate a statistically significant difference with activated splenocytes cultured alone.

Early SPM macrophages are reminiscent of Tumor-Associated Macrophages (TAM)

We then compared the characteristics of SPM to TAM obtained from rat bearing peritoneal mesothelioma 6 months after CNT-7 injection (Huaux et al. 2016).

First, we confirmed that mesothelioma TAM blocked T cell proliferation in a number-dependent manner, unlike mesothelioma neutrophils (Figure 2). These data indicate that SPM and TAM from CNT-7-treated animals possess comparable immunosuppressive activity toward T lymphocytes.

In order to arrest T cell expansion and prevent T cell anticancer activity, TAM overexpress a specific panel of immunoregulating genes and pathways favoring mesothelioma expansion (Vitale et al. 2019). Therefore, we examined the phenotypic proximity between SPM (recruited by mesotheliomagenic particles) and TAM (infiltrating tumoral microenvironment). By RNA-sequencing, we compared the expression of TAM-related genes and pathways of immunosuppressive SPM and homeostatic LPM.

The barcode plot is a visual representation of RNA-sequencing data. All the genes (vertical bars) of a specific pathway are ranked from left to right by increasing log-fold change (ratio of the gene-expression level between SPM and LPM) and the curve above the barcode plot is the relative local enrichment of the bars in the plot. We observed that most of the genes belonging to the M2 profile signature (blue, bottom panel) are expressed by SPM, supporting their proximity with TAM (Figure 3 A). However, some genes of M1 profile (red, top panel) are overexpressed in SPM-like subset. Indeed, SPM-like macrophages overexpress the IL-1 (Figure 3 B) and the toll-like receptor signaling pathways (Figure 3 C), both belonging to the M1 inflammatory phenotype (Mantovani et al. 2002). In addition, we report that SPM-like macrophages have a glycolytic metabolism (Figure 3 D–E), like M1 inflammatory macrophages, while M2 TAM rely on oxidative phosphorylation (Viola et al. 2019). These data showed that SPM present an intermediate profile between M2-like and M1-like polarization.

We then investigated the immunosuppressive genes and pathways expressed by TAM to control T lymphocyte responses. Like TAM (Groth et al. 2019), SPM highly metabolize tryptophan and arginine (Figure 3 F–G) and overexpress the arachidonic acid metabolic pathway (Figure 3 H) whose metabolites comprise immunosuppressive compounds (Mantovani et al. 2017). Moreover, like TAM (Hashimoto et al. 2018), they also express the inhibitory immune checkpoint genes (Figure 3 I) more than their LPM counterparts. Also, the pathways promoting a favorable microenvironment for cancer cell seeding and proliferation (Chanmee et al. 2014; Vitale et al. 2019), including angiogenesis (Figure 3 L), extracellular matrix interaction (Figure 3 M) and HIF-1 α /hypoxic (Figure 3 N) pathways are overexpressed in SPM compared to LPM. To conclude, these findings suggest that

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SPM share with TAM most of the immunosuppressive and pathological pathways determining their specific phenotypic features.

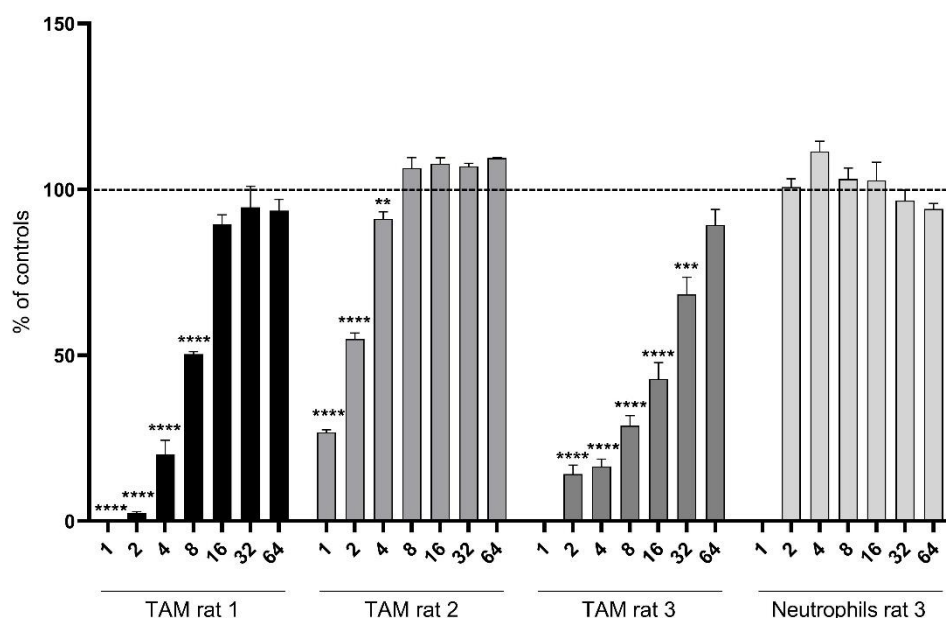
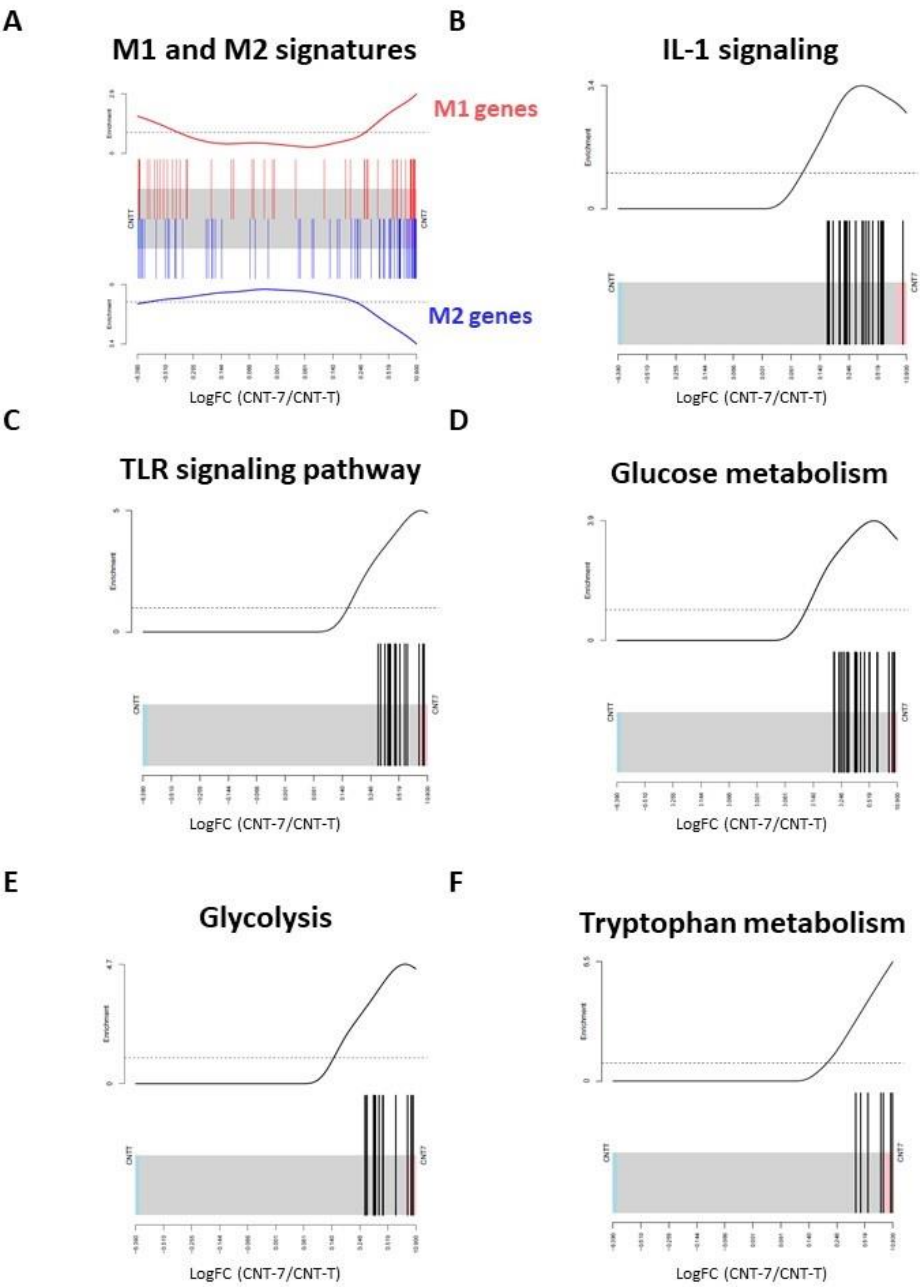
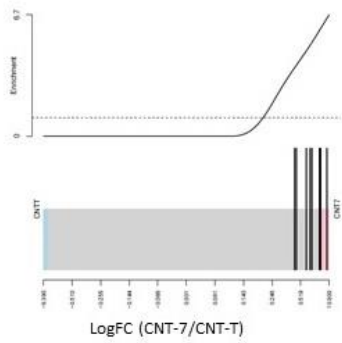


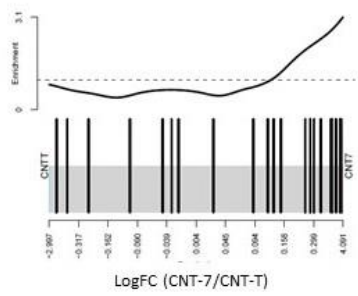
Figure 2: Inhibition of T lymphocyte proliferation by TAM from tumor bearing rats. *In vitro* antiproliferative activity of peritoneal Tumor-Associated Macrophages and neutrophils purified from 3 rats bearing mesothelioma on spleen T lymphocytes activated with anti-CD3/CD28 antibodies. Graphs represent the average level of ^3H -thymidine incorporation expressed as percent of controls (activated T lymphocytes alone) for each splenocytes/macrophages ratio. Each bar represents mean $\pm$ SEM. These results were statistically analyzed using Anova test followed by Dunnett's Multiple Comparison test. ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$ indicate a statistically significant difference with activated splenocytes cultured alone.



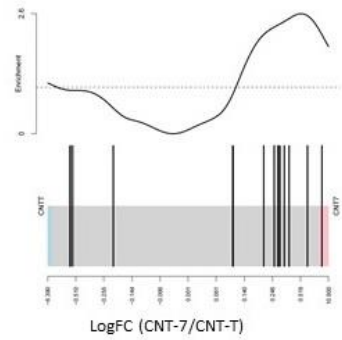
G
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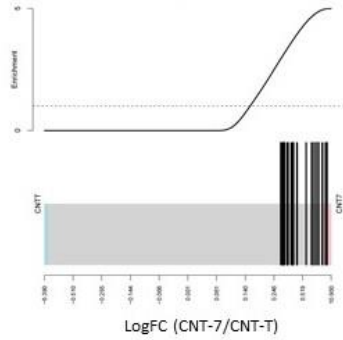
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Arachidonic acid metabolism



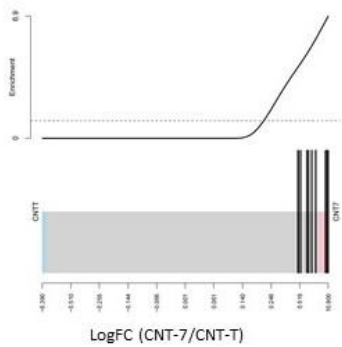
I
Immunocheckpoint proteins



L
Angiogenesis



M
ECM-receptor interaction



N
HIF-1 signaling pathway

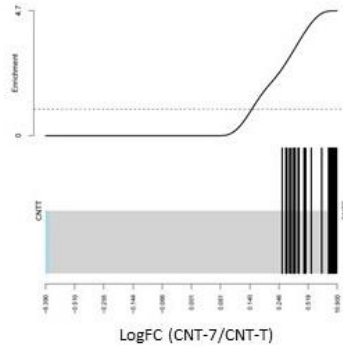


Figure 3: Similarities in immunosuppressive signatures between TAM and SPM accumulated after CNT-7 exposure. Barcode plots showing enrichment of M1 (top panel) and M2 (bottom panel) signatures in the CNT-7 vs CNT-T macrophage comparison. Red bars show up M1 signature genes, blue bars show up M2 signature genes. The worms show relative enrichment (A). Barcode plots showing enrichment of IL-1 signaling (B), Toll-like receptor signaling pathway (C), glucose metabolism signature (D), glycolysis (E), tryptophan metabolism signature (F), arginine and proline metabolism signature (G), arachidonic acid metabolism signature (H), immune checkpoint protein signature (I), angiogenesis signature (L), ECM–receptor interaction (M), HIF–1 signaling pathway (N) in the CNT-7 vs CNT-T macrophage comparison. The worms show relative enrichment.

TGF- β 1, IL-10 and PGE₂ do not explain the immunosuppressive activity of SPM

To determine which mediator explains the immunosuppressive activity of SPM, we then explored the potential role of TGF- β 1, IL-10 and PGE₂. Indeed, previous studies using a comparable co-culture system demonstrated that these molecules exert a strong immunosuppressive activity on T cells when released by TAM from patients developing malignant peritoneal mesothelioma (Blondy et al. 2020; Li et al. 2016; Lievense et al. 2016).

We first evaluated the amount of TGF- β 1 (active and inactive forms) present during acute inflammation and macrophage depletion (from 3 hours until 7 days after particle injection, Paper II) and when immunosuppressive CNT-7 SPM and homeostatic CNT-T LPM replenishment is accomplished (at day 15 and 30). We noticed that, in comparison with untreated (CTRL) and CNT-T-treated rats, peritoneal levels of total TGF- β 1 were higher after CNT-7 at each studied time-point (Figure 4 A). However, released TGF- β 1 was in inactive form when SPM are differentiated (Figure 4 B–C, 15 days). Additionally, we did not observe any difference in the surface expression of LAP between CTRL macrophages, LPM and

SPM, ruling out a role of activated TGF- β in the immunosuppressive activity of SPM (Figure 4 D). Results also suggested that IL-10 was not specifically related to mesotheliomagenic CNT-7-related immunosuppressive responses, since IL-10 levels were significantly higher in CNT-7-treated rats than in untreated (CTRL) or CNT-T conditions only at 3 hours (Figure 4 E). Finally, we quantified PGE₂ after CNT injection. We noticed that the amount of PGE₂ was similar for untreated and CNT-treated rats (Figure 4 F, 15 days). Therefore, we concluded that the immunosuppressive activity of SPM is not directly related to TGF- β 1, IL-10 or PGE₂.

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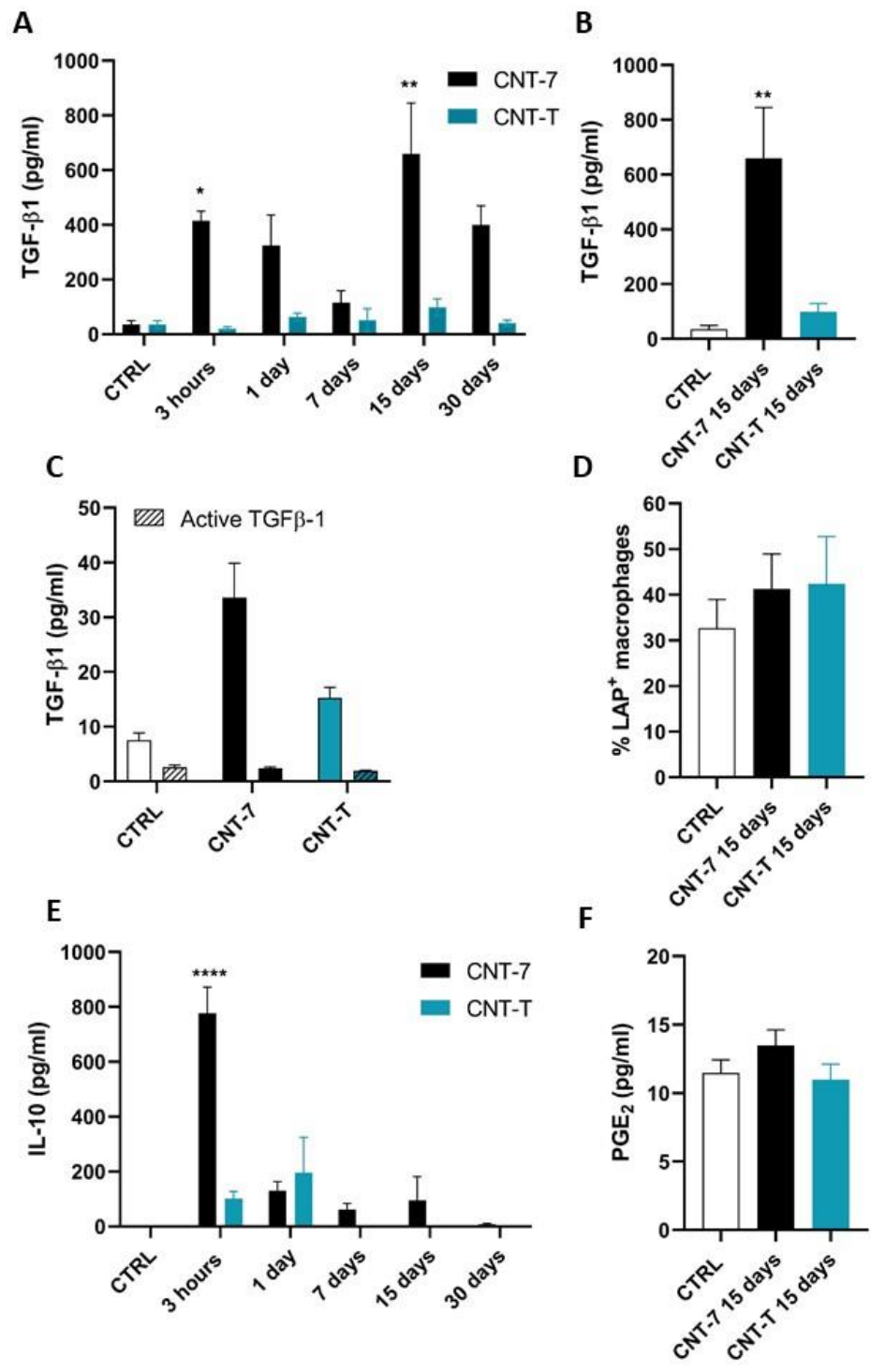


Figure 4: TGF- β 1 IL-10 and PGE₂ are not persistently released in the peritoneal cavity after CNT-7 exposure. TGF- β 1 released in the peritoneal fluid of CNT-7 and CNT-T-treated rats (at 0, 3 hours, 1, 7, 15 and 30 days after the injection) were tested using Bio-Plex TGF- β assay. Each bar represents the mean of 4 replicates $\pm$ SEM (A). Amount of TGF- β 1 released in the peritoneal fluid of CNT-7 and CNT-T-treated rats at 15 days. Each bar represents the mean of 4 replicates $\pm$ SEM (B). The release of active TGF- β 1 (dashed boxes) by macrophages from CNT-T-treated and untreated rats is under the detection limits (C). Percentage of LAP-positive macrophages from untreated or CNT-7/CNT-T-treated rats sacrificed 15 days after. Each bar represents the mean of 6 replicates $\pm$ SEM (D). IL-10 released in peritoneal fluid of CNT-7 and CNT-T-treated rats (at 0, 3 hours, 1, 7, 15 and 30 days after the injection) was tested using Bio-Plex multiplex assay. Each bar represents the mean of 4 replicates $\pm$ SEM (E). PGE₂ released in peritoneal fluid of CNT-7 and CNT-T-treated rats at 15 days were tested using ELISA test. Each bar represents the mean of 4 replicates $\pm$ SEM (F). These results were statistically analyzed using Anova test followed by Dunnett's Multiple Comparison test. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$ indicate a statistically significant difference from control (untreated rats).

SPM specifically express syndecan-1 (SDC-1)

To find possible immunosuppressive mediators which could explain the antiproliferative activity of SPM on T cells and early immunosuppression, we used a semi-blind approach - in contrast to the knowledge-driven approach – which led us to investigate TGF- β 1, IL-10 and PGE₂. To do this, we compared the transcriptome of SPM and LPM-like populations purified at day 15, via RNA-sequencing analysis, and selected the genes significantly upregulated in the SPM subset (Table 1). The proteoglycan syndecan-1 (SDC-1) was selected as a good candidate, because it was recently demonstrated that the expression of SDC-1 confers to SPM subset (CD138⁺ / SDC⁺ SPM-like macrophages) an anti-inflammatory

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and immunosuppressive phenotype, which is essential during the regulatory phase of immune responses in mice (Guillonnet al. 2017; Han et al. 2017).

We first confirmed by qRT-PCR that SDC-1 gene was overexpressed in purified CNT-7 SPM macrophages (Figure 5 A, day 15). We then measured the amount of the SDC-1 shed form in the cell culture supernatant, coming from purified peritoneal macrophages of untreated or CNT-treated rats. An increased release of soluble SDC-1 was noted in SPM cultures in comparison to naïve and LPM macrophage conditions (Figure 5 B). The effect of mesotheliomagenic CNT-7 was additionally noted in the peritoneal fluids from treated rats when SPM are accumulated (Figure 5 C). To confirm that mesotheliomagenic fibers induce the release of soluble SDC-1, we assessed SDC-1 after injection of asbestos and noted significantly higher levels in these treated rats (Figure 5 D). We finally evaluated the release of SDC-1 in the peritoneal cavity during the inflammatory phase when resident macrophages are depleted (from 3 hours up to 7 days) (Figure 5 E). We noticed that SDC-1 is additionally and strongly released after CNT-7 treatment comparatively to CNT-T and saline. Overall, these data suggest that SDC-1-positive SPM and a soluble SDC-1 release are intimately associated to mesotheliomagenic CNT responses.

Table 1: List of genes differentially expressed 4-fold or more in CNT-7 peritoneal macrophages. RNA-seq.

ENTREZID	SYMBOL	FULL NAME	adj.p	logfc
474169	Rnase2	ribonuclease A family member 2	8.62E-08	8.72117082
364303	Rnase111	ribonuclease, RNase A family, 1-like 1	1.14E-06	8.20604967
103690354	LOC103690354	ribonuclease pancreatic beta-type	1.18E-06	7.81642282
24362	Fbp1	fructose-bisphosphatase 1	1.18E-06	7.53764405
24443	Hdc	histidine decarboxylase	6.10E-06	7.52432535
78967	Mcam	melanoma cell adhesion molecule	6.10E-06	6.17739428
296257	Cst7	cystatin F	3.17E-05	6.16985154
301265	Pla2g7	phospholipase A2 group VII	1.61E-05	5.96999823
364304	Rnase1	ribonuclease A family member 1	1.73E-06	5.72135852
499879	Duoxa2	dual oxidase maturation factor 2	0.00010471	5.51807186
117522	Xpnpep2	X-prolyl aminopeptidase 2	6.09E-06	5.24094068
25216	Sdc1	syndecan 1	2.84E-06	5.14490464
60325	Serpinb2	serpin family B member 2	7.27E-06	5.0570129
292794	Ffar2	free fatty acid receptor 2	0.00010471	4.96940436
117551	Ccl22	C-C motif chemokine ligand 22	0.0018772	4.80034661
79107	Duox2	dual oxidase 2	0.00062316	4.77793652
25426	Cyp1b1	cytochrome P450, family 1	9.42E-05	4.51013939
24482	Igf1	insulin-like growth factor 1	9.84E-05	4.4329005
140915	Slco3a1	solute carrier organic anion transporter	0.0086267	4.27995762
58812	Apln	apelin	6.44E-05	4.22624639
690906	Lilrc2	leukocyte immunoglobulin-like receptor	0.00010952	4.21374785
29704	Pacsin1	protein kinase C and casein kinase sub.	0.00057035	4.18940655
310392	Slc7a11	solute carrier family 7 member 11	0.00102509	4.14731199
25335	Mmp7	matrix metalloproteinase 7	0.00144334	4.13676281
360504	Hba2	hemoglobin alpha, adult chain 2	0.00035071	4.0777356
56083	Tgm2	transglutaminase 2	0.0001681	4.06909647

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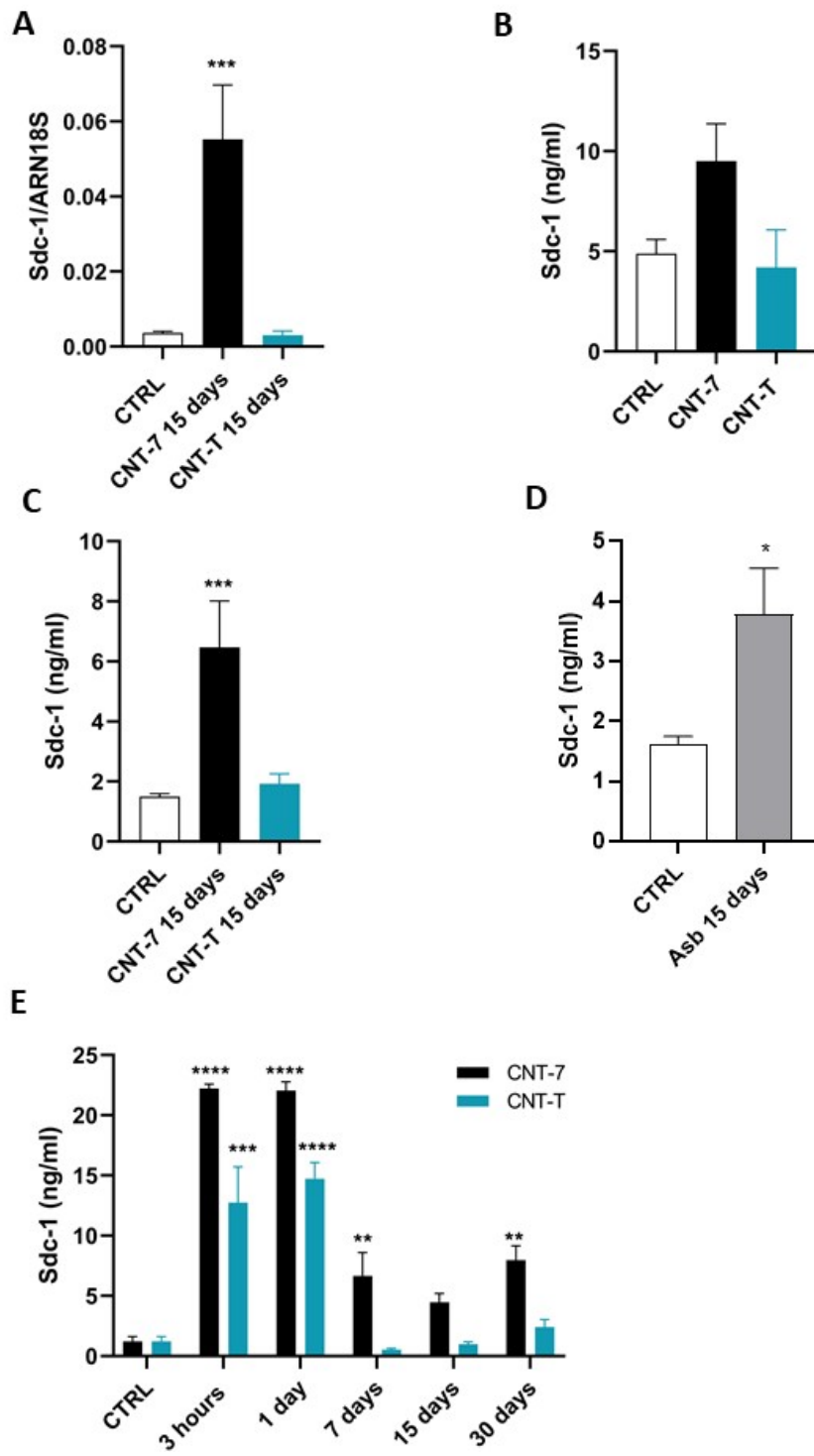


Figure 5: Syndecan-1 is overexpressed by SPM from mesotheliomagenic CNT-treated rats.

Syndecan-1 was quantified by qRT-PCR. The samples are the same used for RNA-sequencing (macrophages purified from CNT-7/CNT-T-treated rats) and macrophages from untreated rats. Values are means $\pm$ SEM of 4 animals (A). Shed syndecan-1 released in 24 hours of culture by peritoneal macrophages from CNT-7 or CNT-T-treated rats at 15 days were tested using ELISA assay. Values are means $\pm$ SEM of 3 animals (B). Shed syndecan-1 released in the peritoneal cavity fluid of CNT-treated rats at 15 days were tested using ELISA assay. Values are means $\pm$ SEM of 10 animals (C). Syndecan-1 released in the peritoneal fluid of asbestos-treated rats at 15 days were tested using ELISA assay. Values are means $\pm$ SEM of 6 animals (D). Shed syndecan-1 released in the peritoneal fluid of CNT-7 and CNT-T-treated rats (at 0, 3 hours, 1, 7, 15 and 30 days after the injection) were tested using ELISA assay. Values are means $\pm$ SEM of 4 animals (E). These results were statistically analyzed using Anova test followed by Dunnett's Multiple Comparison test; ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$ indicate a statistically significant difference from control (untreated rats).

3.4. Discussion

Inflammation and immunosuppression are two distinct processes both contributing to the initiation and maintenance of malignant mesothelioma after the exposure to CNT. The protracted and unresolved inflammation governed by macrophages engenders genetic instability and uncontrolled mesothelial cell proliferation (Murphy et al. 2012; Yang et al. 2010). In parallel, an early immunoregulatory microenvironment induced by mesotheliomagenic particles is also rapidly installed by immunosuppressive mediator release (TGF, IL-10 and PGE₂) and monocytic-MDSC recruitment (Huaux et al. 2016). Immunosuppression culminates with a massive infiltration of immunosuppressive M2/TAM in the tumor

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microenvironment, once mesothelioma is established. TAM inhibit the natural anticancer T cell activity and organize a favorable microenvironment for tumor seeding and proliferation (Cornelissen et al. 2015; Miselis et al. 2008).

Our data support this concept and have demonstrated that macrophages replenishing the cavity after macrophage depletion specifically participate in the establishment of an early immunosuppressive microenvironment induced by mesotheliomagenic particles. We observed that early CNT-7 SPM and TAM are very similar. Early recruited SPM are immunosuppressive towards T cells (Figures 1 and 2) as are mesothelioma TAM. In a similar way, SPM that have been purified after asbestos injection or generated *in vitro* possess the same anti-proliferative capacity, suggesting that mesotheliomagenic particles recruit a specific immunosuppressive SPM-like macrophage subset. Like TAM, expanded SPM highly metabolize and deplete arginine and tryptophan, two crucial components for T cell survival (Lemos et al. 2019). Interestingly, both TAM and SPM overexpress inhibitory checkpoint proteins like PDL-1, PDL-2, B7-1 and B7-2, which are responsible for dampening the T cell priming (Haanen and Robert 2015). Also, these two populations upregulate the HIF-1 α transcription factor which in turn induces the release of VEGF and the promotion of angiogenesis (Chanmee et al. 2014) and express a panel of metalloproteinase involved in extracellular-matrix interaction favoring cancer cell dissemination (Chanmee et al. 2014).

We demonstrated that the SPM subpopulation presents characteristics of syndecan-1 (SDC-1)-positive immunosuppressive SPM (Han et al. 2017). SDC-1 is a transmembrane proteoglycan characterized by heparansulfate and chondroitinsulfate side chains at its extracellular domain. This glycoprotein is the coreceptor of different growth factors like VEGF, EGF, HGF, Wnt factors or TGFs (Guillonnet et al. 2017). It can be cleaved by proteolytic enzymes at its

juxtamembrane site (shedding of syndecan-1) and be released as a soluble form (Kind et al. 2019a; Kind et al. 2019b). SDC-1 is a valid immunosuppressive candidate which could explain the immunosuppressive functions of the SPM-like subset. Indeed, investigators recently noticed that SDC-1 has immunoregulatory properties in humans (Saleh et al. 2019). In breast cancer SDC-1 inhibits the polarization of Th cells towards T helper 1, resulting in an increase of cancer aggressiveness (Saleh et al. 2019). Interestingly, recent discoveries highlighted that IL-34 – which are produced specifically by CD8⁺ and CD4⁺ Foxp3⁺ Tregs (Bezie et al. 2015) and already known as the second ligand of MCSF-R after CSF-1 – can also bind SDC-1 (Guillonnet al. 2017). The functions of SDC-1/IL-34 signaling are not completely elucidated. However, it is known that IL-34 can differentiate macrophages towards an M2 polarization state, able to prevent T cells and NK and expand immunosuppressive T regs (Foucher et al. 2013; Ge et al. 2019). Furthermore, SDC-1 can modulate the CSF1R/IL-34 signaling, limiting or increasing the proximity between ligand and receptor (Guillonnet al. 2017). The signaling CSF1R/IL-34 mediates TAM survival (Cannarile et al. 2017) and neoangiogenesis (Ge et al. 2019; Segaliny et al. 2015).

The overexpression of SDC-1 was observed in many cancers (Kind et al. 2019a). Concerning malignant mesothelioma, the transmembrane form of SDC-1 is a positive prognostic factor (Kumar-Singh et al. 1998) and its overexpression inhibits mesothelial cell proliferation (Zong et al. 2010). On the other side, the soluble form of syndecan-1 in mesothelioma patients' pleura effusions and serum correlates with a bad prognosis (Mundt et al. 2014). SDC-1 has direct pro-tumoral functions which were elucidated in myeloma. First the heparanase trims the sulfate chains on the extracellular domain of SDC-1, then it allows matrix metalloproteinase 9 in

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charge of shedding the glycoprotein at the juxtamembrane site to intervene. The shed SDC-1 favors the coupling of VEGFR2 to the VLA-4 clusters, which activates a downstream signal for invasion and angiogenesis (Jung et al. 2016). We showed that the shed form of the glycoprotein SDC-1 is more released in mesotheliomagenic CNT-7-treated rats and that its production is at least in part directly attributed to SPM macrophages. Probably other leukocyte populations like B cells or epithelial cells (Regos et al. 2020) participate in the acute release of the protein, since a high level of SCD-1 was measured in the absence of macrophages, i.e. during the macrophage disappearance reaction window (3 hours and 1 day, Figure 5).

Finally, our RNAseq data indicate that SPM possess an intermediate phenotype in between M1 and M2 polarization states. Indeed, despite the M2 polarization signature in common with TAM, SPM macrophages express also specific M1 markers such as MHCII proteins and toll-like receptors. Another element which correlates with the M1 polarization of SPM concerns metabolism. TAM indeed adopt an oxidative phosphorylation-based metabolism to guarantee glucose availability for cancer cell proliferation (Vitale et al. 2019), whereas SPM show here a high glycolytic metabolism. Indeed, it is known that M1 prefer a glycolytic metabolism, because it provides a rapid amount of ATP, ROS and inflammatory cytokines which are important to neutralize pathogens (Viola et al. 2019). These data show that there is a potential link between the early inflammatory and the immunosuppressive macrophages recruited by mesotheliomagenic particles. It remains, however, to be determined which signals trigger this macrophage transition if existing.

3.5. Conclusions

Overall, we discovered that early SPM are immunosuppressive towards T cells like TAM. They also share with them most immunoregulatory features. As a possible mediator of their immunosuppressive activity, we identified the immunoregulatory syndecan 1 glycoprotein, abundantly expressed and produced by SPM-like macrophages. Our findings are in line with previous studies describing a strong immunoregulatory role of syndecan 1 in malignant mesothelioma. We also observed that SPM-like macrophages present an intermediate profile between M1 and M2 macrophages in the early phases of mesothelioma development. SPM-like macrophages may represent the link between inflammatory M1 macrophages and immunosuppressive M2 TAM in mesothelioma well described in the literature on mesothelioma.

3.6. Materials and Methods

Carbon nanotubes

CNT-7 are multi-walled carbon nanotubes MWCNT-XNRI-7 from Mitsui & Co. (Ltd., Tokyo, Japan, Lot # 05072001 K28), sub-sampled at Norwegian Research Centre for the Working Environment (NRCWE) with the code NRCWE-006. CNT-T (tangled, kindly provided by Prof. S. Toyokuni, Nagoya University, Japan) and their physico-chemical characteristics have been previously documented in detail (Muller et al. 2009). All particles were first treated at 200 °C for 2 h in order to remove any possible trace of endotoxin, and suspensions were prepared immediately before

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administration by sonication and manual vortexing in sterile phosphate-buffered saline (PBS) containing 1.4 mg bovine serum albumin/ml.

Animals

Specific pathogen-free (SPF) female Wistar rats (8 weeks old, 200 g) (RGD ID: 13792727) were purchased from Janvier SAS (St.-Berthevin, France). The animals were housed at the local animal facility under SPF conditions, with a 12-h light–dark cycle and controlled temperature and humidity. All animal experiments were approved by the local committee for animal research at the Université Catholique de Louvain, Comité d'Éthique pour l'Expérimentation Animale, Secteur des Sciences de la Santé, Brussels, Belgium (No LA1230312). International recommendations for doses administered, controls and group size were followed (Cullen et al. 2002). The animals were injected intraperitoneally with the different particles suspended in a volume of 1 ml of PBS-BSA. They were given tap water and sterilized pellets *ad libitum*. Groups of 4 to 5 Wistar rats were injected with 2 mg of test material and sacrificed after 3 hours and 1, 3, 7 and 15 days. The peritoneal cavity was opened, washed with 15 ml of saline and the cells in the peritoneal fluid were collected.

Cell analysis and purification

Leukocyte counts were determined on a Burkér cell chamber. Cytocentrifuge preparations of peritoneal cells were stained with Diff-Quick (Baxter, Lessines, Belgium). Rat peritoneal cell suspensions were also stained using antibodies specific for CD11b/c-BV421 (Cat. # 743977, RRID: AB_2741898) from BD Biosciences, His48-PE (Cat. # sc-19613PE, RRID: AB_627686) from SantaCruz Biotechnology and LAP-APC (Cat. # 141405, RRID: AB_10896418). Samples were acquired on a FACSCantoII (BD Biosciences) and analyzed using FlowJo software (RRID:SCR_008520). At least 10,000 events were recorded. Rat peritoneal leukocytes were separated and isolated using flow cytometry cell sorter (FACS Aria III, BD Biosciences). The purity

of the obtained leukocyte cell preparations was routinely > 93 % as assessed by Diff-Quick staining.

In vitro macrophage polarization

Bone marrow progenitors from tibia and femur of untreated Wistar rats were collected and put in culture with complete medium DMEM. M-CSF at 20 ng/ml (Cat. # NBP2-76302) by Novus Biologicals or GM-CSF at 40 ng/ml (Cat. # 415-ML-010/CF) by R&D Systems were then added a first time 24 hours after, and a second time 4 days after culturing. At day 7 differentiated macrophages were collected and counted with Trypan blue.

Immunosuppressive in vitro test

Similarly to Blondy and coworkers who used a system of co-culture to demonstrate that mesotheliomagenic TAM dampen T cell activation (Blondy et al. 2020), we used here co-cultures combining purified peritoneal macrophages from treated rats and CD3/CD28-activated splenic T cells.

The suppressive activity on T lymphocytes of peritoneal leukocyte subpopulations was assessed in a co-culture assay in which splenocytes from untreated rats were seeded in triplicates in 96-well round bottom plates (50×10³/well, Cellstar®, 96 well, U-Botto, Greiner Bio-One, Wemmel, Belgium). Splenocytes were cultured in the presence of increasing ratios of rat purified peritoneal cells or GM-CSF or M-CSF polarized cells and stimulated with anti-CD3 (Cat. # 554829) and anti-CD28 (Cat. # 554993) antibodies by BD Biosciences. On day 3 of the co-culture, cells were pulsed with 3[H]-thymidine (0.05 mCi per well) (Cat. # NET027A001MC) by Perkin Elmer and 18 h later 3[H]-thymidine incorporation was assessed.

RNA sequencing

Peritoneal macrophages from CNT-7 or CNT-T-treated rats, sacrificed after 15 days, were separated and isolated using flow cytometry cell sorting (FACS Aria III, BD Biosciences). RNA was isolated using RNeasy mini kit (Cat. # 74104) from Qiagen. RNA quantity was measured with Qubit™ RNA HS Assay Kit (Cat. # Q32852) from Thermofisher. RNA integrity and quality were assessed using an Agilent Bioanalyzer using the Agilent RNA 6000 Nano kit (Cat. # 5067-1511). All RNA used had an RNA Integrity Number between 9 and 10. Libraries were prepared starting from 100 ng of total RNA using the Illumina® TruSeq® Stranded mRNA Sample Preparation Kits, following the standard protocol. Libraries were sequenced using an Illumina HiSeq1500 platform. Approximately 50 million paired-end reads (2x125bp) per sample were obtained. All sequencing data were analyzed using the Automated Reproducible MODular workflow for preprocessing and differential analysis of RNA-seq data (ARMOR) pipeline (Orjuela et al. 2019). In this pipeline, reads underwent a quality check using FastQC (RRID:SCR_014583) (Andrews 2010). Quantification and quality control results were summarized in a MultiQC report (RRID:SCR_014982) (Ewels et al. 2016) before being mapped using Salmon (RRID:SCR_017036) (Patro et al. 2017) to the transcriptome index, which was built using all Ensembl cDNA sequences obtained in the *Rattus_norvegicus*.Rnor_6.0.cdna.all.fa file (Cunningham et al. 2019). Then, estimated transcript abundances from Salmon were imported into R using the tximeta package (Love et al. 2019) and analyzed for differential gene expression with edgeR (RRID:SCR_012802) (Robinson et al. 2010). Multiple testing correction of the p-value was performed within edgeR by the Benjamini-Hochberg procedure in order to control the false discovery rate (FDR) at a level of 0.10. Accordingly, genes associated with an adjusted p-values < 0.10 were considered significant and were

therefore expected to include 10 % of false positive result. A downstream analysis was performed in order to interpret the differential expression results by considering a global biological context. In that context, pathway enrichment analysis was performed by using the WebGestaltR bioconductor package and the KEGG database (RRID:SCR_012773). Barcode gene enrichment plots were generated with limma bioconductor package (RRID:SCR_010943).

Both raw and processed RNA-seq data were deposited on the Gene Expression Omnibus (GEO) and made publicly available (GSE157487).

ELISA cytokine analysis

Levels of TGF- β 1 in peritoneal fluid supernatants and in macrophage cell cultures were analyzed using the Bio-Plex Pro™ TGF- β 3-plex Assay (Cat. # 171W4001M) from Biolegend.

Levels of active or total TGF- β 1 in peritoneal fluid supernatants were analyzed using the Mouse TGF- β 1 DuoSet (Cat. # DY1679) from R&D systems.

Levels of PGE₂ in peritoneal fluid supernatants were analyzed using Prostaglandin E Metabolite ELISA Kit (Cat. # 514531-96) from Cayman Chemical.

Levels of syndecan-1 in peritoneal fluid supernatants were analyzed using Rat Syndecan-1/CD138(SDC1) ELISA Kit (Cat. # CSB-E17115r) from Gentaur.

BioPlex cytokine analysis

Levels of soluble IL-10 in peritoneal fluid supernatants were analyzed using the Bio-Plex Pro™ Rat Cytokine Assay (Cat. # 171K1001M, RRID: AB_2861403) from Bio-Rad.

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qRT-PCR

Total RNA extraction was performed using TriPure Isolation Reagent (Cat. # 11667165001) from Roche Diagnostics. Reverse transcription was carried out from 1 µg of extracted RNA resuspended in Hyclone water, using the M-MLV Reverse Transcriptase (Cat. # 28025013) from Thermofisher and random hexamers from Eurogentec. The resulting cDNA was used as template in subsequent polymerase chain reactions (PCR). Sequences of interest were amplified by PCR using the following forward primers from Invitrogen: CCC TCC CGC AAA TTG TCA (Syndecan-1) CGGCTACCACATCCAAGGAA (h18sRNA) and reverse primers: AAA GTC ATA TCT GGC AAA GCA CCT (Syndecan-1), ATACGCTATTGGAGCTGGAATTACC (h18sRNA). PCR was performed with AmpliTaq Gold polymerase (Invitrogen) according to the manufacturer's instructions with the following temperature program: 10 min 95 °C, (15 s 95 °C, 1 min 60 °C) ×40 cycles. Amplified DNA fragments were purified from a 2 % agarose gel using PCR clean-up Gel extraction kit(s) (Macherey-Nagel, Düren, Germany) and then serially diluted to serve as standards in real-time PCR. Reverse transcribed mRNAs were finally quantified by real-time PCR using SYBR Green technology on an ABI Prism 7000 Sequence Detection System (Applied Biosystems) according to the following program: 2 min 50 °C, 10 min 95 °C, (15 s 95 °C, 1 min 60 °C) ×40 cycles. In order to verify specific amplification, a dissociation curve was obtained by increasing temperatures up to 95 °C over a 20-minute period. Results were calculated as a ratio of gene expression to the expression of the reference gene, RNA 18s.

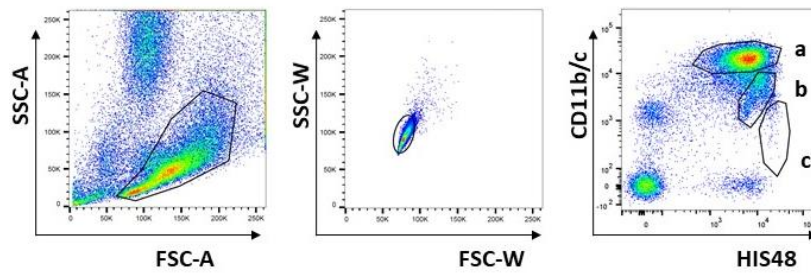
Statistics

Data were evaluated by one-way analysis of variance (ANOVA) using the Dunnett's Multiple Comparison Test when appropriate. Statistical significance was considered

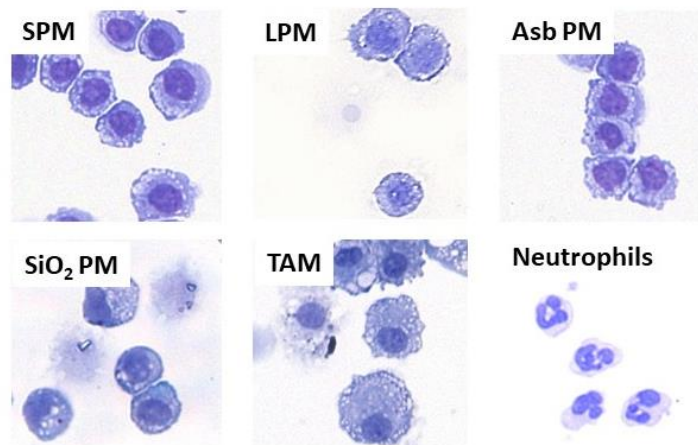
at $p < 0.05$. Data were evaluated by an unpaired t test, when appropriate. Statistical significance was considered at $p < 0.05$.

3.7. Supplemental Figures

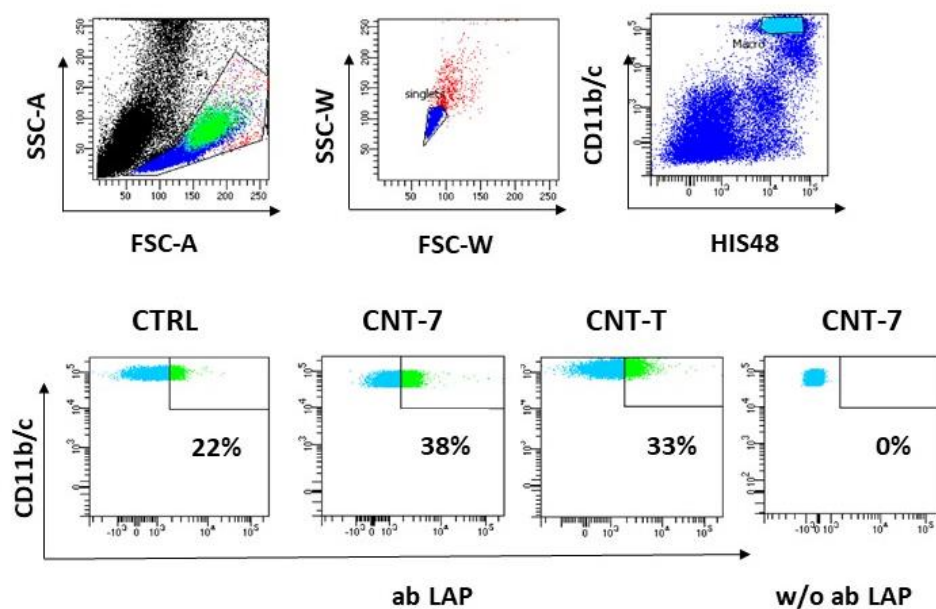
A



B



Supplemental Figure 1: Gating strategy in flow cytometry for peritoneal macrophage purification. Flow cytometry gating strategy for peritoneal leukocyte identification. Dot plots (CD11b/c, His48) of peritoneal cells (a = macrophages, b = neutrophils, c = monocytes) of untreated or CNT-treated Wistar rats (15 days, 2 mg) (A). Microscopic views (Diff-Quick staining, 400x) of FACS-sorted CD11b/c^{high} and His48^{int} macrophages from CNT-7 (SPM); CNT-T (LPM), asbestos, SiO₂-treated rats at 15 days and Tumor-Associated Macrophages at 6 months and neutrophils (B).



Supplemental Figure 2: FACS strategy for TGF/LAP complex assessment. Flow cytometry gating strategy for LAP expression assessment on peritoneal macrophages. Dot plots of CD11b/c and His48 peritoneal macrophages (15 days, 2 mg) and CD11b/c – LAP.

4. General Discussion

Overall, this work has led to identify a macrophage subset involved in the establishment of an early immunosuppressive environment which results from the exposure to mesotheliomagenic CNT.

The peritoneal cavity of the Wistar rat was chosen as a mesothelioma model to define macrophage origin and turnover and characterize gene expression profile after injection of mesotheliomagenic (CNT-7) or non-mesotheliomagenic (CNT-T) carbon nanotubes. The mesothelial cell composition of the peritoneal and the pleural cavities being similar, the former is generally accepted as a good model for the study of pleural mesothelioma (Fukushima et al. 2018).

We showed that both the intraperitoneal injection of CNT-7 and CNT-T results in a very rapid depletion of resident peritoneal macrophages. This mechanism is already observed after exposure to several other inflammatory stimuli and described as “macrophage disappearance reaction” (MDR) (Accarias et al. 2016; Cassado Ados et al. 2015; Meza-Perez and Randall 2017; Okabe and Medzhitov 2014). In our studies, MDR is explained by a macrophage cell death caused by the cytotoxicity of the particles instead of a migration to the omentum. Importantly, this depletion phase was followed by a macrophage replenishment of the peritoneal cavity with new macrophage subsets intimately linked to the carcinogenicity of CNT. The different cytotoxic properties of CNT-7 (highly cytotoxic, paper II) and CNT-T (moderately cytotoxic) may entail two distinct dynamics of replenishment which may be explained with the “niche model” described by Guilliams for lung alveoli and liver (Guilliams et al. 2020). The whole elimination of resident cells by highly toxic CNT-7 triggers a massive recruitment of bone-marrow derived monocytes

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which replenish the peritoneal cavity differentiating into MHCII⁺ SPM-like macrophages. On the other hand, because of their lower toxicity, CNT-T particles potentially induce an incomplete macrophage depletion followed by the proliferation of residual resident cells together with a moderate recruitment of monocytes which differentiate first into MHCII⁺SPM and then into self-proliferating MHCII⁺CD163⁺LPM. This last mechanism agrees with Cain (Cain et al. 2013) and Van de Laar (van de Laar et al. 2016) showing that when monocytes arrive in the tissue of residence, they assume the appearance, the gene profile and the proliferative ability of their embryonic and homeostatic counterparts.

Interestingly, SPM-like macrophages strongly resemble to the immunosuppressive M2-polarized Tumor-Associated Macrophages (TAM) in terms of immunoregulatory signatures and impact on T cell proliferation. In mesothelioma microenvironment TAM infiltrate tumors, prevent anticancer T cell activity and determine bad prognosis (Burt et al. 2011; Chene et al. 2016; Cornelissen et al. 2014; Ujiie et al. 2015). Early recruited SPM and their monocytic precursors (M-MDSC) are also immunosuppressive towards T cells. In addition, SPM share many similarities with TAM in gene expression. We noticed that most of the immunosuppressive pathways characterizing TAMs are similarly expressed by SPM. We newly propose that the early responses to mesotheliomagenic particles comprise an unsuspected and early immunosuppressive environment orchestrated by macrophages and favouring a tumoral cell evasion from T cell surveillance before mesothelioma development.

Despite the similarities between SPM and TAM, some differences exist in their polarization. While TAM generally display a M2 polarization state, an intermediate M1-M2 phenotype was intriguingly observed in SPM-like macrophages. Indeed, despite the fact that SPM show most of the typical characteristics of a M2 profile,

they also express clear inflammatory signatures, such as the toll-like receptor and the inflammatory cytokine signaling pathways, which are specific markers of M1 phenotype (Mantovani et al. 2002). Furthermore, SPM also overexpress the proteins belonging to MHCII machinery, traditionally associated to the M1 skewing (Viola et al. 2019). Finally, while TAM exhibit an oxidative phosphorylation-based metabolism (Vitale et al. 2019), we noticed that SPM-like macrophages show a glycolytic metabolism representative of M1 inflammatory macrophages (Viola et al. 2019). The combined M1 and M2 profile observed in our study supports the hypothesis that M1/M2 SPM may represent a possible missing link between early inflammatory/M1 macrophages (activated by particles) and late immunosuppressive/M2 TAM in mesothelioma development (activated by tumor cells).

It is still unclear how inflammatory SPM switch to immunosuppressive SPM during the early responses to mesotheliomagenic particles. Efferocytosis, i.e. the phagocytosis of death cell, is carried out by macrophages (Boada-Romero et al. 2020). Effective efferocytosis results in a reprogramming of macrophage activation profile with the release of anti-inflammatory and immunosuppressive cytokines, such as IL-10 and TGF- β (Boada-Romero et al. 2020). Thus, in addition to allow elimination of pyroptotic macrophages, efferocytosis also result in cytokine secretion which leads to an M2-related cellular environment supporting the accumulation of immunosuppressive SPM. The apparent opposite accumulation of inflammatory and immunosuppressive SPM is not exclusive and may be, in contrast, concomitant. The view that immunoregulatory and immunostimulatory responses and SPM co-exist is supported by the co-presence of inflammatory and immunosuppressive cells such as inflammatory neutrophils and MDSC during carcinogenesis induced by CNT in rats (Huaux et al. 2016).

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Experimental evidence suggested that pro-inflammatory and immunosuppressive cytokines released by particle-activated macrophages directly alter mesothelial cells function *in vitro* and *in vivo* (Wang et al. 2004). Cytokines (in particular IL-1 β and TNF- α) resulting from inflammatory macrophages induce proliferation and differentiation of mesothelial cells (Xu et al. 2012). The alarmin HMGB1 triggers the NF- κ B signaling pathway in activated mesothelial cells, inducing the expression of several genes allowing cell survival (Affar and Carbone 2018; Carbone and Yang 2017). TGF and other immunosuppressive are known to activate tumor cell proliferation and dissemination. Because of this potential role played by macrophages in triggering and maintaining the stemness of tumor cells, transformed mesothelial cells should be named MAT (Macrophage-Associated Tumor) in mesothelioma.

Conversely to what is described in literature on malignant mesothelioma (Lievence et al. 2016), IL-10 cannot explain the immunosuppressive response exerted by SPM on T cells in our model. However, during the macrophage disappearance reaction (at 3 hours), the amount of IL-10 after CNT-7 exposure is exceptionally high. The most valid explanation is that IL-10, together with TNF- α , IL-1 β and IL-6, is probably released by resident macrophages after the encounter with the fibers and the activation of the inflammasome, as shown in literature (He et al. 2011).

The immunosuppression exerted by early SPM toward T cells is possibly mediated by syndecan 1 glycoprotein, which is upregulated in SPM-like macrophages in comparison to LPM and released at a higher extent in CNT-7 than CNT-T-treated rats. Syndecan 1, a transmembrane glycoprotein belonging to the syndecan family, is overexpressed in many types of cancer (Kind et al. 2019a). In mesothelioma, in particular, the membrane-bound form of syndecan 1 in mesothelioma cells correlates with a good prognosis (Zong et al. 2010), while the soluble form is an

indicator of poor prognosis (Mundt et al. 2014). Moreover, it was demonstrated that syndecan 1 characterizes an immunosuppressive SPM-like macrophage subpopulation involved in the dampening of chronic inflammation (Han et al. 2017). The present findings may open a horizon for new tests in toxicology to predict the mesotheliomagenic potential of particles and fibers. A simple *in vitro* cytotoxicity test exposing macrophages and mesothelial cells, as performed by Donaldson and coworkers (Murphy et al. 2012), is useful to predict and anticipate the hazardous and carcinogenic potential of a CNT sample. However, it is not exhaustive in explaining the impact of particles on the dynamic change of macrophage subsets in their natural niches. In contrast, an *in vivo* model, like the one presented by Poland (Donaldson et al. 2010; Poland et al. 2008), allows to understand the entity of inflammation triggered by a particle (e.g. number of neutrophils and levels of inflammatory cytokines released) in the early time points (from 3 hours until 7 days) after the exposure. Furthermore, when the macrophage replenishment is accomplished, our *in vivo* model allows to finely predict the organization of an early immunosuppressive microenvironment through the characterization of the recruited macrophage subpopulations (SPM vs LPM-like) and their related immunosuppressive mediators (as syndecan-1). The combination of these *in vitro* and *in vivo* assays should help toxicologists to determine the danger and risk for human health of particles or fibers newly produced in industry.

Limitations

Different models of malignant mesothelioma already exist, such as wild-type rats injected with asbestos or mesotheliomagenic CNT, genetically engineered mice exposed or non-exposed to asbestos or CNT, and orthotopic or heterotopic mesothelioma xenografts of human mesothelioma cell lines in immunodeficient mice (Jean and Jaurand 2018; Wu et al. 2017). Different advantages are associated with the rat model. In particular, rats are sensitive to mesothelioma development without the need of previous genetic mutations in proto-oncogenes or tumor suppressor genes. This allows investigating the successive events leading to mesothelioma formation (short- and long-term responses to particles). This model is also simple, reproducible and less time consuming than other models; Wistar rats develop mesothelioma only 6 months after a single CNT injection (Huaux et al. 2016). Unfortunately, very few immunological tools specific for rats are currently available on the market. This aspect limited the possibility to characterize in a more detailed and specific way macrophage subsets present in the peritoneal cavity. In murine models, peritoneal LPM and SPM subsets can be easily identified via flow cytometry using a large set of markers, including F4/80, CD11b, CD11c, MHC-II, CD163, GR1, Ly6C, CD62L, Dectin-1, DC-Sign, TLR4, CD80, CD86, CD40, 12/15-LOX, TIM4 (Cassado Ados et al. 2015). In our experimental set-up, it was only possible to evaluate two specific macrophage markers: MHCII and CD163.

The easy-to-perform intraperitoneal injection is the generally accepted way of exposure to induce mesothelioma (Rittinghausen et al. 2014). However, it clearly does not represent the real exposure route to mesotheliomagenic fibers, which accumulate after inhalation. Furthermore, it was recently demonstrated that CNT-T, which are not carcinogenic in the peritoneum, induce adenocarcinoma in the lungs when administered via the airway (Saleh et al. 2020). This finding opens an

interesting question regarding the role of the anatomical niche (peritoneal/ pleural cavities or lung) in modulating macrophage and tumor onset (see below). In this context, we are developing in the laboratory a new model based on the use of A/J mice, known to be more susceptible to carcinogen-induced tumors (Falcone et al. 2017; Zeidler-Erdely et al. 2013). In this model, CNT-T and CNT-7 exposure would be performed via whole-body inhalation or pharyngeal instillation, therefore allowing the possibility to investigate the macrophage phenotype and turnover not only in pleural cavity, but also in lungs.

The dose is also a matter of debate. We used 2 mg of CNT per rat, because this is the mass of CNT-7, that corresponds to 0.67×10^9 WHO fibers, able to induce mesothelioma in rat (Cullen et al. 2002; Huaux et al. 2016). However, because of the different physico-chemical properties of CNT-7 and CNT-T, it would be interesting to administer the same number of fibers instead of the same mass. Another option is to use lower and repetitive doses of CNT, closer to human exposure conditions. Smaller doses could emphasize slighter differences in the biological effects caused by CNT-7 and CNT-T during MDR, macrophage repopulation and immunosuppression.

The recruitment of an immunosuppressive SPM-like macrophage subset after mesotheliomagenic particle exposure is probably a tissue-specific phenomenon. First, only mesothelial cavities (pleura and peritoneal) host SPM and LPM subpopulations. Furthermore, it was recently demonstrated that CNT have different carcinogenic effects depending on the exposed anatomical site. Indeed, only needle-like CNT-7 are mesotheliomagenic for the peritoneal cavity after intraperitoneal injection, however both kinds of CNT are carcinogenic for lung and pleura when administered into the lungs of rats (Saleh et al. 2020). In addition, as

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described by Guilliams, macrophages are intimately connected with their anatomical niches which provide them the nutrients and confer them their tissue-specific identity (Guilliams et al. 2020). Altogether this evidence supports the idea that the macrophage disappearance reaction and the following replenishment by SPM-like subset is limited to the peritoneal and pleural cavities.

An additional limitation of our study is the co-culture system used to assess the immunosuppressive activity of peritoneal macrophages on CD3/CD28 activated splenocytes. The experiment is long (several days of culture) and based on a solely readout i.e. T cell proliferation assessed via the incorporation of ^3H -thymidine. In the future, we should consider additional markers of inhibitory effects as the immune checkpoint protein expression or the release of inflammatory cytokines. Similarly to the test proposed by Li and co-workers (Li et al. 2016), we should assess the proportion of PD-1⁺, Tim3⁺ and CTLA4⁺ T cells (CD4⁺ and CD8⁺ populations) or measure the amount of INF- γ and granzyme B in co-culture (Li et al. 2016).

Perspectives

During this work, we only defined the identity of peritoneal macrophages at 15 days when macrophage repopulate the peritoneal cavity. We could broaden our study and define macrophage phenotype at later time points and up to mesothelioma establishment (6 months). This study would allow us to clarify the dynamic of macrophage turnover. Indeed, the macrophage disappearance reaction may be not limited at early time points (3 hours) and may be a recurring phenomenon leading to successive and alternate replenishment phases. A finely detailed kinetic will allow us to determine whether the TAM-related profile of SPM is maintained all along the mesothelioma onset or if it is only restricted to the early responses to the mesotheliomagenic particles. Indeed, we cannot exclude that SPM-like phenotype progressively differentiates into LPM or vice versa. In parallel, it would be also interesting to measure the level of syndecan-1 at later time points to confirm that it is persistently and specifically released by CNT-7-macrophages until mesothelioma establishment.

Many strategies can be used to thwart the immunosuppressive microenvironment established early after mesotheliomagenic fiber exposure and target SPM turnover or the syndecan-1.

As observed, the replenishment of the cavity by SPM probably relies on monocyte recruitment via CCL2/CCR2 and CSF-1/MCSF-R signaling. The blockage of these pathways would limit the SPM replenishment and their subsequent immunosuppressive activity. Indeed, it was observed that the suppression of both CCR2 and CCL2 using inhibitors or neutralizing antibodies in murine models of pancreatic (Li et al. 2017; Sanford et al. 2013; Teng et al. 2017) and lung cancers

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(Fridlender et al. 2011; Wang et al. 2018b), reduced tumor growth and metastasis. These effects were accompanied by a decrease in M-MDSC infiltration and a reduction in M2-polarization of TAM. In a similar way, the inhibition of MCSF-R reduced the monocyte recruitment in the tumor microenvironment and prevented the M2-polarization of TAM in murine models of mammary carcinoma, cervical carcinogenesis, lung and breast carcinoma (Strachan et al. 2013; Van Overmeire et al. 2016).

The same approach should be evaluated in rats by different strategies, i.e. by using the available CCR2 neutralizing antibodies, (RS504393) (Liu et al. 2017; Wang et al. 2018a), or inhibitory CCR2 antagonists (RS504393 or CCX872) (Flores-Toro et al. 2020; Xu et al. 2017).

M2 polarization phenotype of TAM is known to be responsible for the establishment of an immunosuppressive microenvironment (Chanmee et al. 2014). The reprogramming of M1/M2 phenotype of SPM into a fully pro-inflammatory M1 polarization state could boost the antitumor immunity. As demonstrated by Lyamina and coworkers (Lyamina et al. 2012) peritoneal macrophages can be repolarized *in vitro* towards an M1 state by adding INF- γ and LPS. Furthermore, investigators recently discovered that Super paramagnetic iron-oxide nanoparticle (SPIONs), Glycocalyx-mimicking nanoparticles (glycol-NPs) and polystyrene nanoparticles can revert the polarization state from M2 to M1 *in vitro* and *in vivo* (Miao et al. 2017). An interesting alternative is the use of nanocarriers targeting the SPM and containing the mRNA of IRF5, a M1 polarization transcription factor. This method, developed by Zhang & co. (Zhang et al. 2019a), was successfully studied in models of ovarian cancer, metastatic melanoma, and glioblastoma. A significant reduction in tumor progression, accompanied by a decrease in M2 macrophage number and an increase in M1 polarized TAM subset, was observed.

Another strategy is the depletion of SPM-like macrophages producing syndecan 1 (or CD138). The antibody-drug conjugated indatuximab and ravtansine targets and eliminates CD138-expressing cells. For this reason, it is currently under study in the development of a combination therapy in breast cancer (Schonfeld et al. 2018) and in multiple myeloma in preclinical (Ikeda et al. 2009) and clinical studies (Jagannath et al. 2019). In a similar way, the depletion of CD138⁺ SPM-like macrophages can be achieved using the CAR T cells transduced with a CD138-specific chimeric antigen receptor. The CAR T cells for CD138⁺ SPM depletion are currently tested in clinical trials (Lin et al. 2019).

Recently, the silencing of syndecan 1 transcript in SPM-like macrophages by molecular biology techniques was investigated and successfully performed in breast cancer human cell line to assess the role of syndecan 1 in promoting metastasis in the brain (Sayyad et al. 2019). miR-494 is specifically able to down regulate the expression of syndecan 1 (Yang et al. 2018), thus suppressing the immunosuppression exerted by syndecan 1 on T cells.

The shed form of syndecan 1 was recently associated to bad prognosis in malignant mesothelioma patients and we have observed that it is highly released in CNT-7-treated rats.

The syndecan 1 shedding can be orchestrated by numerous extracellular and membrane-bound proteases belonging to the metzincin family (zinc endopeptidases): metalloproteinases (MMP), membrane-tethered MMPs (MT-MMPs), and A-disintegrin and-matrix-metalloproteinases (ADAMs) (Stepp et al. 2015). Among them ADAM17, MMP7, MMP2, MMP9, MT1-MMP and MT3-MMP are the most well-known and they could be the perfect therapeutical targets (Manon-Jensen et al. 2013).

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In particular, MMP9 correlates often with bad prognosis in different cancer pathologies like breast cancer (Manon-Jensen et al. 2013). The employment of inhibitory antibody towards MMP9 inhibits tumor cell invasion and promotes the CD8+ T cell activation in a breast cancer mouse model (Owyong et al. 2019).

Other sheddase inhibitors, which are now under investigation in models of ischemic acute kidney injury (Lu et al. 2018), could be used to modulate the release of syndecan 1 and prevent its immunosuppressive activity.

During this work, we limited our research to the syndecan 1, while other differentially expressed genes (see the table 1, Paper III) represent additional candidates able to explain the immunosuppression exerted by CNT-7 macrophages towards activated T cells. Among them, we identified CCL22 as a promising immunosuppressive mediator. CCL22 is a chemokine released by TAM and cancer cells that binds its receptor CCR4 on T reg thus inducing their recruitment to the tumor microenvironment (Martinenaite et al. 2016; Wang et al. 2019). High levels of CCL22 were measured in malignant pleural effusion patients with lung cancer and correlates with poor survival (Wang et al. 2019). Moreover, it was demonstrated that PGE₂ contributes to CCL22 secretion (Rohrle et al. 2020). The CCL22-CCR4 axis is thus an interesting therapeutic target for cancer immunotherapy and the cell-depleting monoclonal CCR4-antibody mogamulizumab (KW-0761) is currently in use for the treatment of cutaneous T cell lymphoma and CCR4-positive peripheral T cell lymphoma (Rohrle et al. 2020).

Other interesting candidates are transglutaminase 2 and phospholipase A2 (Pla2g7). Tg2 is a transpeptidase and a calcium-dependent protein-cross linking enzyme that interacts with fibronectin and collagen to form a ternary complex important for the cell-ECM and integrin interaction. This interaction is pivotal for macrophage adhesion and migration (Huang et al. 2015). The members of

phospholipase A2 family hydrolyse membrane phospholipids. The cleavage of arachidonic acid begins the biosynthesis of the immunosuppressive PGE₂. Interestingly both transglutaminase 2 and phospholipase A2 are markers of IL-4 differentiated M2 macrophages in human and mouse (Chrobok et al. 2017; Yamaguchi et al. 2016). Moreover, it was recently demonstrated that the function and the expression of TG2 are regulated by phospholipase A2 via the PGE₂ synthesis (Yamaguchi et al. 2016). Both Pla2 and TG2 can be therapeutic targets. Regarding transglutaminase 2, it is largely expressed in human mesothelioma tumor and in mesothelioma cancer cell lines and it is important for cancer cell survival in hypoxic condition (Zonca et al. 2017). Tg2 can be inhibited either with NC9, a Tg2 inhibitor or via knock down (Adhikary et al. 2018) or via 1–155 selective inhibitor (Zonca et al. 2017).

Concerning Pla2-specific shRNA or inhibitors like AACOCF3 and PLA-695 are currently under study in ovarian cancer cells and NSCLC. The inhibitors can reduce the angiogenesis and cause cancer cells apoptosis (Chen et al. 2017).

5. Conclusions

In a rat model of peritoneal mesothelioma, we showed that exposure to mesotheliomagenic particles changes very rapidly the general profile of macrophage subsets, leading to an early immunosuppressive microenvironment. The depletion of resident macrophages, resulting from the cytotoxic activity of particles, is rapidly followed by a replenishment by bone marrow-derived Small Peritoneal Macrophages (SPM), which differ from self-proliferating and homeostatic Large Peritoneal Macrophages (LPM), that renew the cavity after exposure to non mesotheliomagenic particles.

SPM show similar immunosuppressive profile and the same ability to block T cell expansion of Tumor Associated Macrophages (TAM), known to infiltrate the mesothelioma microenvironment. We also demonstrated that SPM release the shed form of the syndecan-1 glycoprotein (SDC-1), an immunoregulatory molecule already detected in malignant mesothelioma. SDC-1 could play a crucial role in the early immunosuppressive activity of SPM.

Overall, our current observations can pave the way for the development of short-term in vivo bioassays (SPM accumulation) to evaluate the mesotheliomagenic potential of new particles and fibers. Furthermore, our results open a new horizon for the treatment of malignant mesothelioma, by either silencing syndecan-1 or depleting the immunosuppressive SPM.

6. Annexes

6.1. Mesothelioma Response to Carbon Nanotubes Is Associated with an Early and Selective Accumulation of Immunosuppressive Monocytic Cells

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Mesothelioma response to carbon nanotubes is associated with an early and selective accumulation of immunosuppressive monocytic cells

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Abstract

Background: The asbestos-like toxicity of some engineered carbon nanotubes (CNT), notably their capacity to induce mesothelioma, is a serious cause of concern for public health. Here we show that carcinogenic CNT induce an early and sustained immunosuppressive response characterized by the accumulation of monocytic Myeloid Derived Suppressor Cells (M-MDSC) that counteract effective immune surveillance of tumor cells.

Methods: Wistar rats and C57BL/6 mice were intraperitoneally injected with carcinogenic multi-walled Mitsui-7 CNT (CNT-7) or crocidolite asbestos. Peritoneal mesothelioma development and immune cell accumulation were assessed until 12 months. Leukocyte sub-populations were identified by recording expression of CD11b/c and His48 by flow cytometry. The immunosuppressive activity on T lymphocytes of purified peritoneal leukocytes was assessed in a co-culture assay with activated spleen cells.

Results: We demonstrate that long and short mesotheliomagenic CNT-7 injected in the peritoneal cavity of rats induced, like asbestos, an early and selective accumulation of monocytic cells (CD11b/c^{int} and His48^{hi}) which possess the ability to suppress polyclonal activation of T lymphocytes and correspond to M-MDSC. Peritoneal M-MDSC persisted during the development of peritoneal mesothelioma in CNT-7-treated rats but were only transiently recruited after non-carcinogenic CNT (CNT-M, CNT-T) injection. Peritoneal M-MDSC did not accumulate in mice which are resistant to mesothelioma development.

Conclusions: Our data provide new insights into the initial pathogenic events induced by CNT, adding a new component to the adverse outcome pathway leading to mesothelioma development. The specificity of the M-MDSC response after carcinogenic CNT exposure highlights the interest of this response for detecting the ability of new nanomaterials to cause cancer.

Keywords: Immunosuppression, Inflammation, Mesothelioma, Myeloid cells, Carbon nanotubes, Asbestos, Crocidolite, Fibers, Rats and mice

Abbreviations: CNT, Carbon nanotubes; CNT-7, Multi-walled Mitsui-7 CNT; CNT-M, Muller carbon nanotubes; CNT-T, Tangled carbon nanotubes; Asb, Crocidolite asbestos; SiO₂, DQ12 silica; MDSC, Myeloid-derived suppressor cells; M-MDSC, Monocytic myeloid-derived suppressor cells; PMN-MDSC, Polymorphonuclear myeloid-derived suppressor cells

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Background

Excessive inhalation of long and biopersistent fibrous materials such as asbestos has been associated with the development of multiple respiratory manifestations, including lung cancer and mesothelioma [1]. Concerns about similar health risks have been expressed for carbon nanotubes (CNT). The International Agency for Cancer Research (IARC) has recently recognized a specific type of CNT, i.e. Mitsui-7 CNT (CNT-7), as carcinogenic in experimental animals [2]. It is, however, not possible to generalize this carcinogenic property to other types of CNT due to the lack of coherent experimental data. There is, therefore, a need to develop bioassays and biomarkers to predict the carcinogenic activity of existing, new or emerging CNT.

The currently prevailing paradigm accounting for the pathogenic activity of carcinogenic fibers such as asbestos or CNT is relatively simple: a chronic inflammatory status, in which immune cells are activated and release toxic mediators, damages the pulmonary architecture, modulates the accumulation of mesenchymal cells and their connective tissue products, and transforms lung epithelial or mesothelial cells through direct and indirect genotoxic and epigenetic pathways [3]. Experimental monitoring of this inflammatory response can be used to predict the carcinogenic activity of CNT in short-term bioassays. In their seminal work, Poland and colleagues revealed the potential of long straight CNT to induce mesothelioma by documenting their capacity to elicit an inflammatory response, similar to asbestos fibres, in the mouse peritoneal cavity [4].

There is now growing evidence that, besides inflammation, immunosuppressive responses also contribute to the pathological processes involved in carcinogenesis and cancer progression [5, 6]. Tumors harbor immunosuppressive cells (i.e. regulatory macrophages, monocytes, DC, neutrophils and lymphocytes) that inhibit both innate and adaptive immunity, subverting immune surveillance and preventing efficient natural or therapeutic anti-tumor immune responses [7]. The current understanding of immunosuppression in malignant mesothelioma has advanced significantly within the past decade [8]. Tumor-associated immunosuppressive cells include T regulatory lymphocytes (Tregs) and tumor-associated macrophages (M2 macrophages). Indeed, the stromal space in primary human mesothelioma tissue harbors significant proportions of immunosuppressive Tregs and M2 macrophages that suppress adaptive anti-tumor immunity in this disease [9, 10].

Myeloid-derived suppressor cells (MDSC) include a small group of discrete myeloid progenitors and immature cells morphologically and phenotypically similar to monocytes (M-MDSC) or polymorphonuclear cells (PMN-MDSC) [11]. MDSC are currently the focus of intense research

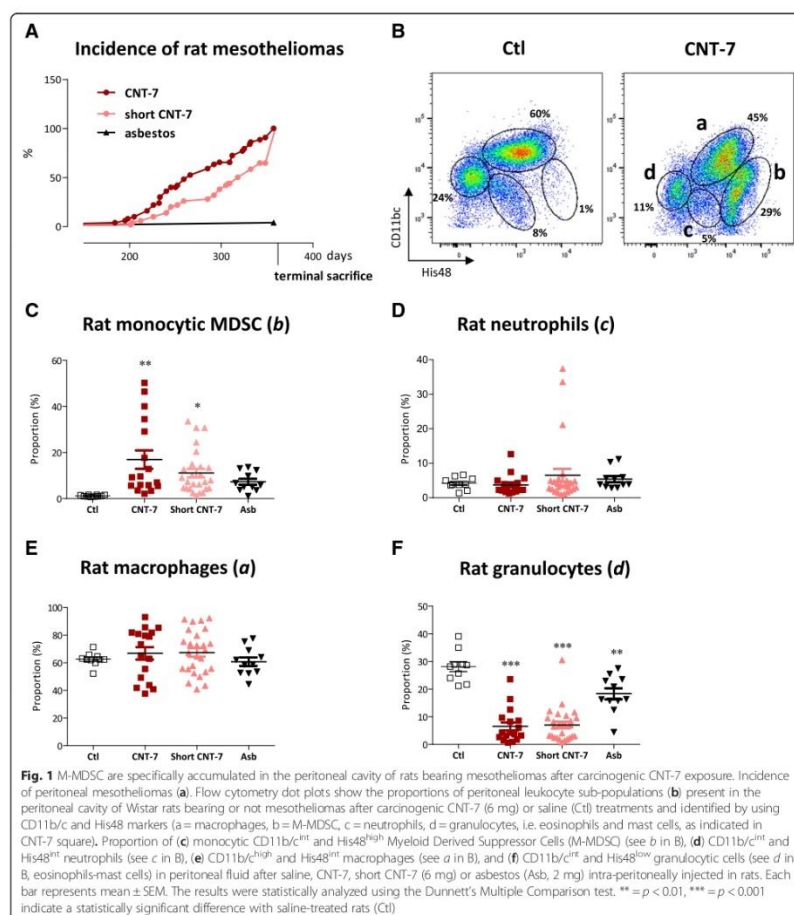
efforts as their accumulation in neoplastic tissues represents a prognostic marker of tumor progression and poor therapeutic efficiency [12]. MDSC are generally associated with established neoplastic tissue, their recruitment being induced by tumor-derived factors [11, 12]. Treatment of mesothelioma-bearing mice with the COX-2 inhibitor celecoxib prevented the local and systemic expansion of MDSC, suggesting that prostaglandins such as PGE2 play an important role in the recruitment of MDSC in established mesothelioma [13]. Immunotherapeutic approaches targeting MDSC or their immunosuppressive function have recently been suggested in patients with mesothelioma [8].

The direct exposure of mesothelial cells to particles in the rat peritoneum bioassay is very useful to identify the capacity of fibrous materials to induce mesotheliomas [4, 14–16]. While this model does not cover all the aspects of what occurs in humans occupationally or environmentally exposed, it allows easily sampling the mesothelial cavity for studying immune cells associated with mesotheliomagenesis [4]. We used this model and a panel of CNT previously characterized for their capacity to induce or not experimental mesothelioma [17–19] to define the inflammatory and immunosuppressive functions of accumulated leucocytes after CNT exposure. We newly show here that M-MDSC are selectively recruited during the early peritoneal response to carcinogenic CNT, well before the establishment of mesothelioma.

Results

Monocytic MDSC accompany CNT-induced mesotheliomas in rats

We first traced MDSC in the peritoneal cavity of rats treated with carcinogenic multi-walled Mitsui-7 CNT (CNT-7) known to induce mesotheliomas in Wistar rats, a strain susceptible to carcinogenic fibers [17–19]. Rats were treated with a single intra-peritoneal injection of CNT-7 (6 mg, median length, 7.1 μ m; 78 % >5 μ m), short CNT-7 (6 mg, median length, 2.8 μ m; 14 % >5 μ m) or crocidolite asbestos [17]. As expected, the first mesotheliomas occurred 6 months after CNT-7, the majority of animals developing tumors within 12 months (Fig. 1a). Short CNT-7 also induced mesotheliomas although to a lesser extent than the long ones (Fig. 1a). Mesothelioma diagnosis was confirmed by histological analyses (Additional file 1: Figure S1 A-F). CNT-7 and short CNT-7 appeared extremely active in comparison to asbestos fibers over the 12-month duration period (Fig. 1a). One rat injected with asbestos developed a mesothelioma. This low response to asbestos after 12 months was consistent with our previous work in the same rat strain in which the same asbestos sample did not induce mesothelioma until 14 months after injection [19]. None of the controls developed mesothelioma. Leucocytes were detected in the peritoneal



lavage performed when rats were euthanized in the course of the experiment because of clinical evidence of mesothelioma (mainly presenting with a dilated abdomen and ascites) or at terminal sacrifice for rats injected with CNT-7 or asbestos. Leukocytes were identified by recording expression of CD11b/c and His48 by flow cytometry [20, 21]. Monocytic cells and macrophages were the

two main populations present in the peritoneal cavity of rats bearing mesothelioma after CNT treatment (Additional file 1: Figure S1 G and I). Compared to control rats, increased proportions of monocytic CD11b/c^{int}His48^{high} cells were noted in animals injected with CNT-7 (Fig. 1b-c), short CNT-7 or asbestos (Fig. 1 c and Additional file 1: Figure S1 H), and the extent of their

accumulation was associated with the ability of the fibers to induce mesotheliomas over the 12 months observation period (Fig. 1a). In contrast, mesotheliomas were not associated with an increased proportion of neutrophils (CD11b/c^{int} and His48^{int}) (Fig. 1d) and macrophages (CD11b/c^{high} and His48^{int}) (Fig. 1e). The proportion of granulocytic cells (eosinophils-mast cells, CD11b/c^{int} and His48^{low}) was significantly decreased upon mesothelioma-genic particle exposure (Fig. 1f). We thus concluded that M-MDSC specifically accumulated in the peritoneal cavity of rats bearing mesotheliomas induced by CNT.

Early accumulation of M-MDSC in response to carcinogenic CNT

We next determined the early response to CNT by examining the time course of peritoneal leukocyte influx from day 1 to 30. CNT and asbestos (2 mg) induced an acute accumulation of peritoneal leukocytes after injection (Additional file 1: Figure S2 A). We found that monocytic CD11b/c^{int} His48^{high} cells appeared in the peritoneal cavity as early as 24 h after injection and persisted for at least 30 days (Fig. 2a-b). Monocytic cells purified from 1 to 30 days after CNT displayed a strong immunosuppressive activity on spleen T lymphocytes activated with anti-CD3/CD28 antibodies (Fig. 2d), confirming that this cell population comprised M-MDSC. In contrast, monocytes purified from blood or peritoneal cavity of naive rats did not significantly affect activated T cell proliferation in co-cultures (Additional file 1: Figure S4 A). A neutrophilic CD11b/c^{int} His48^{int} cell population was also recruited at the early stages after CNT administration (Fig. 2a and c) but these cells did not display immunosuppressive activity (Fig. 2e), indicating that they consisted of inflammatory neutrophils. Like CNT, asbestos strikingly induced M-MDSC and inflammatory neutrophil accumulation at all-time points examined (Fig. 2b-c and f-g). Finally, the proportions of regulatory macrophages and other inflammatory granulocytes (eosinophils and mast cells) were decreased by carcinogenic fiber treatment (Additional file 1: Figure S3).

We next demonstrated that infiltrated M-MDSC proliferate as appreciated by BrdU incorporation (Additional file 1: Figure S4 B) and weakly expressed the monocyte/macrophage lineage markers CD68 and CD163 [22] (Additional file 1: Figure S4 C-D). These observations supported the notion that monocytic cells recruited after CNT-7 exposure were undifferentiated and proliferating cells and corresponded to bona fide MDSC. Finally, we confirmed the inflammatory profile of neutrophilic cells in CNT-7-treated rats by revealing that these cells weakly expressed Rp-1 (Additional file 1: Figure S4 E), a marker of PMN-MDSC in rat mammary carcinoma [21]. Altogether, these results indicated that, besides the inflammatory reaction (inflammatory

neutrophils), an early accumulation M-MDSC was selectively associated with the peritoneal response to carcinogenic CNT.

Early peritoneal M-MDSC response appears specific for mesotheliomagenic CNT

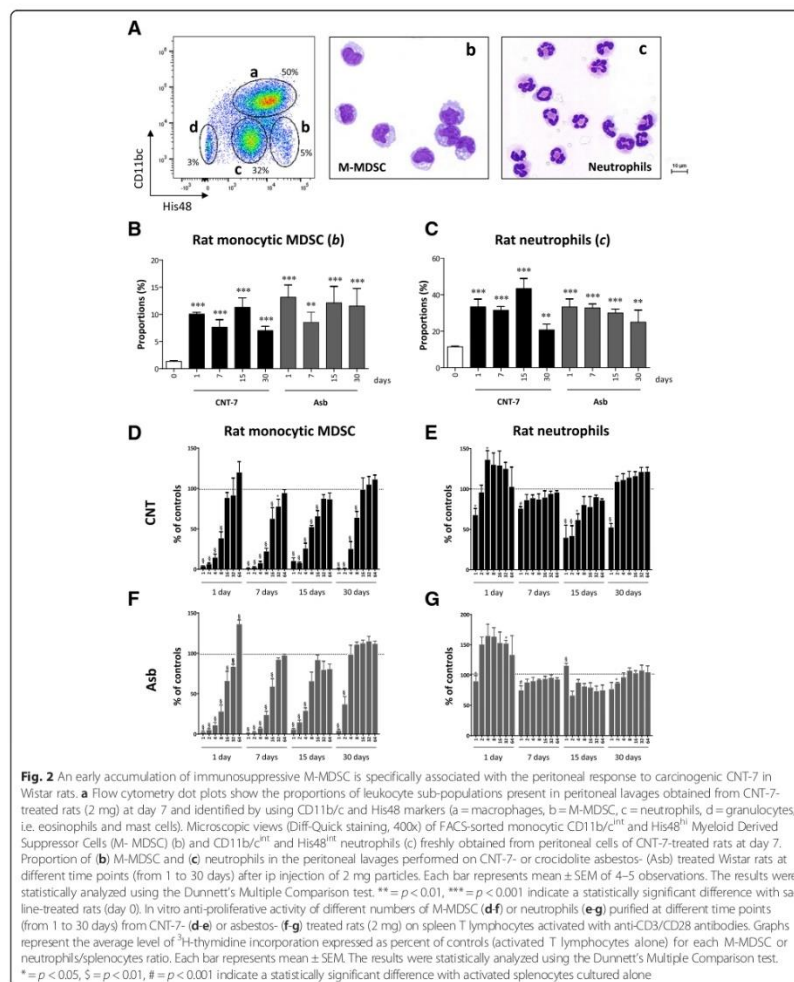
We next determined whether M-MDSC were recruited in mice, which do not develop mesothelioma upon intra-peritoneal injection of CNT or asbestos fibers [23–25]. In our hands, conventional C57BL/6 mice did not develop mesothelioma even after multiple injections of CNT-7 (0.2 mg/mouse/week, not shown). The kinetics of total leukocyte recruitment in the peritoneal cavity of CNT- and asbestos-treated mice was similar to that recorded in rats (Additional file 1: Figure S2 B). The leukocyte subpopulations were identified in mice by using CCR2 and Ly6G markers [11]. We found that while the proportions of monocytic and neutrophilic cells were increased after CNT-7 and asbestos injections (Additional file 1: Figure S5 A-C), these cells did not possess a significant and persistent immunosuppressive activity (Additional file 1: Figure S5 D-G). Macrophages and granulocytes were not accumulated after particle treatment and did not affect T cell proliferation in vitro (Additional file 1: Figure S6). These data indicated that only inflammatory leukocytes are recruited in CNT-treated mice. Combining observations in rats and mice supported, therefore, the idea that the development of peritoneal mesotheliomas after CNT exposure is related to an early recruitment of both inflammatory neutrophils and M-MDSC.

We further evaluated the specificity of the early M-MDSC accumulation by comparing their influx at early time points (days 1 and 7) after a single injection of different agents inducing mesotheliomas (CNT-7, asbestos, 2 mg) or not (silica 2 mg, LPS dose 5 µg) in Wistar rats. We recorded an inflammatory reaction in all groups as illustrated by the accumulation of inflammatory neutrophils. However, an accumulation of M-MDSC was only evident in animals treated with CNT-7 or asbestos (Fig. 3a-c and Additional file 1: Figure S7).

We also compared the capacity of CNT-7 and non-carcinogenic CNT (CNT-M [19] and CNT-T [17]) at a dose of 2 mg to acutely recruit M-MDSC in the peritoneal cavity of Wistar rats. Only CNT-7 induced an early and persistent accumulation of M-MDSC while this influx was weak and transient after CNT-M and CNT-T (Fig. 3d and Additional file 1: Figure S7). Altogether, an early and sustained peritoneal M-MDSC response appeared specific for a mesotheliomagenic CNT.

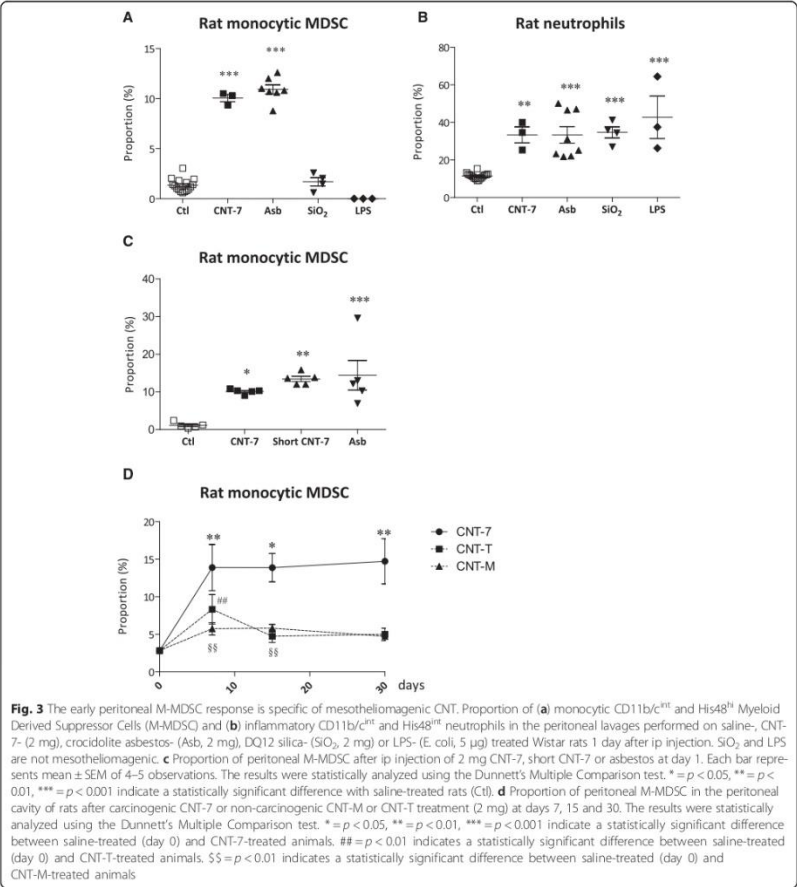
Discussion

While the mechanistic evidence for a mesothelioma-genic activity of CNT is still limited, recent experimental studies have shown that CNT-7 (like asbestos)



induce peritoneal mesothelioma in rats [17, 18, 26]. These data are challenging because, in humans, malignant mesothelioma remains highly refractory to existing

therapies [27]. There is, therefore, an urgent need for additional investigations to identify pathological pathways implicated in CNT-induced mesotheliomas and new



biomarkers predicting the carcinogenic activity of newly developed CNT.

Major advances in understanding the mechanisms leading to tumor immunity have recently been made [28–30]. The development of cancers is now viewed as related to the ability of tumors to develop a tolerant micro-environment by activating diverse immunosuppressive mechanisms counteracting antitumor host immunity [31].

Previous studies have indicated that immunosuppressive responses in mesothelioma consist of tumor-induced accumulation of regulatory T lymphocytes (Tregs) and immature myeloid cells [32]. By using orthotopic implantation of established mesothelioma cell lines in syngenic mice, it has been reported that tumor-associated Tregs contribute to mesothelioma development by limiting the accumulation of IFN-gamma producing effector T

lymphocytes [9, 33]. COX-2-dependent MDSC have also been reported in established mesothelioma [13, 34]. In a mouse model of lung carcinoma, CNT increased the recruitment and accumulation of tumor-associated MDSC that produced TGF β and increased lung tumor burden [35, 36]. Our data demonstrate for the first time that carcinogenic CNT possess the intrinsic capacity to induce a selective, rapid and sustained accumulation of monocytic MDSC before mesothelioma is established. This unsuspected observation indicates that a suitable environment for tumoral cell evasion from T cell surveillance is rapidly installed (within the first days) after carcinogenic CNT exposure. M-MDSC can be generated in the bone marrow in response to factors such as colony stimulating factors (CSFs), inflammatory cytokines and vascular endothelial growth factors and are recruited through a gradient of chemokines such as CCL2. Resident peritoneal macrophages and mesothelial cells treated *in vitro* with asbestos or CNT release these MDSC-related factors [37, 38]. Our data also revealed that beside monocytic cells, peritoneal macrophages also possess immunosuppressive functions upon CNT treatment (Additional file 1: Figure S3). The immunosuppressive M-MDSC and macrophages may thus represent a new crucial pathological pathway in CNT-induced mesothelioma. Our observation also showed that M-MDSC weakly expressed the markers of the classical monocyte/macrophage lineage CD68 and CD163. Previous study also demonstrated that tumor-associated macrophages are not related to MDSC in experimental mesothelioma [39]. Altogether, these data suggest that monocytic cells weakly differentiate in macrophages and that other mechanisms explain macrophage accumulation during peritoneal responses to CNT. Interestingly, it has been demonstrated that macrophages self-maintain independently of monocytes in response to IL-1 α released after silica instillation [40] and hypothetically after intra-peritoneal administration of CNT. Regulatory neutrophilic MDSC (PMN-MDSC) that highly express Rp-1, a marker of undifferentiated neutrophils, have been detected in rat bearing carcinoma [21]. In contrast, neutrophils accumulated locally after short-term exposure to mesotheliomagenic CNT are ineffective for inhibiting T cell proliferation and weakly express Rp-1. It remains, however, to determine whether regulatory Rp-1⁺ PMN-MDSC infiltrate mesotheliomas in long-term studies with CNT-treated rats as proposed in other models [41].

In recent years, experimental observations have supported a scenario whereby a series of chronic and diverse cell injuries and immune stimuli explain the long latency period of mesothelioma development. It thus is probable that the persistent accumulation of M-MDSC represents one of the immune components contributing to mesothelioma development. Indeed, considerable evidence

also suggests that inflammatory neutrophils play a critical role in cancer progression. Early in the neoplastic process, neutrophils trigger genomic instability, produce a favorable environment for tumor growth, and promote angiogenesis [42]. Neutrophils accumulated after asbestos generate substantial amounts of DNA damaging free-radicals [43]. The neutrophil influx in the peritoneal cavity of CNT-treated animals therefore contributes to explain the carcinogenicity of CNT and has been proposed as an early biomarker for predicting the carcinogenic activity of CNT [3, 4]. We confirmed here that inflammatory neutrophils are massively and early recruited after CNT or asbestos injection. The joint presence of immunosuppressive M-MDSC and inflammatory neutrophils supports a scenario in which synchronized immunosuppressive and inflammatory mechanisms contribute to the emergence of neoplastic cells, induce immunological tolerance allowing tumor cell evasion, and finally promote tumor progression. In contrast to M-MDSC, we noted that the accumulation of inflammatory neutrophils was not specific for mesotheliomagenic agents because silica or LPS similarly induced an acute accumulation of neutrophils. Thus, the early and sustained accumulation of M-MDSC after carcinogenic CNT treatment may represent a more robust indicator of a potential to induce mesothelioma than solely the inflammatory neutrophil response. The capacity of a given CNT to maintain a sustained M-MDSC response probably integrates its biopersistence, which is an important parameter determining the carcinogenic potential of CNT and fibers [3].

Conclusion

Our data provide new insight on how CNT may establish a carcinogenic response by mounting an early and sustained immunosuppressive microenvironment facilitating, in conjunction with inflammation, mesothelioma development. M-MDSC represent a new component of the adverse outcome pathway leading to the development of mesothelioma. The specificity of the M-MDSC accumulation after exposure to carcinogenic CNT highlights the potential interest of this response for detecting in short-term bioassays the ability of new or emerging CNT to cause mesothelioma.

Methods

Carbon nanotubes and asbestos fibers

CNT-7 were multi-walled carbon nanotubes MWCNT-XNRI-7 from Mitsui & co (Ltd., Tokyo, Japan, Lot# 05072001 K28) sub-sampled at Norwegian Research Centre for the Working Environment (NRCWE) with the code NRCWE-006. Short CNT-7 were obtained by grinding CNT-7 in an oscillatory agate ball mill (Pulverisette 0, Fritsch), with a vertical vibration of 1 mm applied during 6 h. CNT-7 and short CNT-7 were annealed at

1500 °C under argon during 60 min. Multi-walled CNT-M (Muller) and CNT-T (tangled, kindly provided by prof S. Toyokuni, Nagoya university, Japan) and their physico-chemical characteristics have been documented in details previously [17, 19, 44]. The main characteristics of CNT-7, short CNT-7 and CNT-M are summarized in the Additional file 2: Table S1. Respirable crocidolite fibers (positive control) were obtained from the Union internationale contre le Cancer (UICC, Geneva, Switzerland) [45]. All particles were first treated at 200 °C during 2 h to remove any possible trace of endotoxin and suspensions were prepared immediately before administration by sonication and manual vortexing in sterile phosphate-buffered saline (PBS) containing 1.4 mg bovine serum albumin/ml.

Animals

Specific pathogen free (SPF) male Wistar rats (10 weeks old, 200 g) were obtained from Charles River (Brussels, Belgium). C57BL/6 mice (10 weeks old, 20 g) were purchased from Janvier SAS (St Berthevin, France). International recommendations for doses administered, controls and group size were followed [46]. After a week of acclimatization, the animals were injected intraperitoneally with the different particles suspended in a volume of 2 ml (rats) or 0.5 ml (mice) of PBS-BSA. For long-term experiments (12 months), groups of 50 rats were injected with 6 mg of CNT-7 (2×10^9 WHO fibres), short CNT-7 (0.36×10^9 WHO fibres). Positive controls were injected with 2 mg (6×10^9 WHO fibres) of crocidolite asbestos ($n = 26$). Vehicle controls ($n = 26$) were injected with an equal volume of PBS-BSA. The animals were housed at the local animal facility under SPF conditions, with a 12-h light–dark cycle and controlled temperature and humidity. They were given tap water and sterilized pellets ad libitum. The animals were observed daily for mortality/morbidity and debilitated animals were euthanized with an intra-muscular injection of pentobarbital. Full gross necropsy was performed on all animals found dead or euthanized during the experiment, and grossly visible lesions or tumors were preserved in 10 % formalin. The experiment was terminated after 12 months when the majority of the animals injected with CNT-7 or short CNT-7 had developed tumors. At this point in time, positive control animals were not justified anymore and we sacrificed the rats treated with crocidolite before they had developed tumors, except one. All animals were sacrificed and systematically autopsied. External lesions and tumors were recorded and samples were preserved for subsequent histopathological analysis. The peritoneal cavity was opened and the cells present in the peritoneal fluid were collected and analyzed (see below). Then, the peritoneal cavity was examined macroscopically for the presence of anomalies and tissues were preserved in formalin for histopathological examination.

In short-term experiments to analyze cellular responses, groups of 4 to 5 Wistar rats or C57BL/6 mice were injected with 2 mg (rats) or 0.2 mg (mice) of test material and sacrificed after 1, 7, 15 and 30 days. In these conditions, injection in rats of 2 mg CNT-7 corresponds to 0.67×10^9 WHO fibres, 2 mg short CNT-7 to 0.12×10^9 WHO fibres and 2 mg crocidolite asbestos to 6×10^9 WHO fibres. In mice, 0.67×10^8 WHO fibres of CNT-7 (0.2 mg) were injected per animal.

Histopathological analysis

The different tissues were processed for light microscopic examination by serial desiccation in ethanol and paraffin embedded specimens were cut in 5- μ m slices and stained with hematoxylin-eosin (HE). The preparations were examined by an experienced pathologist.

Cell analysis and purification

The peritoneal cavity was lavaged with 15 ml (rats) or 8 ml (mice) of saline, and leukocyte counts were determined on a Burkert cell chamber. Cyto centrifuge preparations of peritoneal cells were stained with Diff-Quick (Baxter, Lessines, Belgium). Rat peritoneal cell suspensions were also stained using antibodies specific for CD11b/c-APC (clone OX-42) obtained from BioLegend (San Diego, USA), His48-FITC (clone anti-granulocytes) from eBioscience (Hatfield, UK), CCR2-PE (clone 890231) from R&D system (Abingdon, UK), CD68 and CD163 (AbD Serotec, Oxford, UK), Rp-1 (BD Pharmingen, Erembodegem, Belgium) and isotype controls (clone MOPC-21, MCA1209F, 11E10 and MG2a) from BioLegend, Gentaur (Kampenhout, Belgium), eBioscience and Invitrogen (Frederick, USA). Mouse peritoneal cells were stained by the following antibodies: CD45-PerCP Cy5.5 (clone 30-F11), CD11b-BV 421 (clone M1/70), Ly6G-FITC (clone 1A8) from BD Biosciences (Erembodegem, Belgium), CCR2-PE (clone 475301) from R&D Systems and isotype controls (clone R35-95, A95-1) from BD Biosciences. Samples were fixed in a 1.25 % paraformaldehyde in PBS, acquired on a FACSCalibur (BD Biosciences) and analyzed using the FlowJo software. Rat and mouse peritoneal leukocytes were separated and isolated using flow cytometry cell sorting (FACS Aria III, BD Biosciences). APC (CD11b/c), FITC (His48) and PE (CCR2) fluorescence measured by FACS was used to select rat peritoneal cell subpopulations and PerCP-Cy5.5 (CD45), BV 421 (CD11b), FITC (Ly6G), and PE (CCR2) fluorescence to select mouse peritoneal cell subpopulations. The purity of the obtained leukocyte cell preparations was routinely >93 % as assessed by Diff-Quick staining.

BrdU incorporation

15 mg of BrdU (BD Biosciences) was injected intraperitoneally in rats 24 h before the sacrifice (7 days after saline

or CNT-7 treatment, 2 mg). Cell suspensions from the peritoneal cavity were stained for surface markers followed by intracellular BrdU staining using the BrdU Flow kit according to the manufacturer's instructions (BD Biosciences). Stained cells were acquired on a FACSCalibur (BD Biosciences) and analyzed using the CellQuest software (BD Biosciences).

Immunosuppressive *in vitro* test

The suppressive activity on T lymphocytes of peritoneal leukocyte subpopulations was assessed in a co-culture assay in which splenocytes from 3 naive rats or mice were pooled and seeded in triplicates in 96-well round bottom plates (50x 10³/well, Cellstar[®], 96 well, U-Botto, Greiner Bio-One, Wemmel, Belgium). Splenocytes were cultured in the presence of increasing ratios of rat or mouse purified peritoneal cells and stimulated with anti-CD3 (anti-rat clone G4.18, anti-mouse clone 145-2C11, 5 µg/ml, BD Biosciences) and anti-CD28 (anti-rat clone J319, anti-mouse clone 37.51, 2 µg/ml, BD Biosciences) antibodies. On day 3 of the co-culture, cells were pulsed with ³[H]-thymidine (0.05 mCi per well, Perkin Elmer, Waltham, USA) and 18 h later ³[H]-thymidine incorporation was assessed.

Statistics

Data were evaluated by one-way analysis of variance (ANOVA) using the Dunnett's Multiple Comparison Test when appropriate. Statistical significance was considered at $p < 0.05$.

Additional files

Additional file 1: Figure S1. Mesotheliomas and leukocytes in peritoneal cavity of Wistar rats after mesotheliogenic CNT-7 exposure. **Figure S2.** Peritoneal cell numbers after CNT and asbestos treatment in rats and mice. **Figure S3.** Proportions and *in vitro* activity of macrophages and granulocytes during the early peritoneal response to carcinogenic CNT-7 in Wistar rats. **Figure S4.** Characterization of peritoneal M-MDSC and inflammatory neutrophils accumulated after CNT-7 injection in Wistar rats. **Figure S5.** Accumulation of inflammatory monocytes and neutrophils during the peritoneal response to carcinogenic CNT-7 in C57BL/6 mice. **Figure S6.** Proportions and *in vitro* activity of macrophages and granulocytes during the early peritoneal response to carcinogenic CNT-7 in C57BL/6 mice. **Figure S7.** Early peritoneal M-MDSC response is specific of mesotheliogenic CNT in Wistar rats. (PPTX 5835 kb)

Additional file 2: Table S1. Characteristics of the carbon nanotubes. (DOCX 200 kb)

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Availability of data and materials

The datasets supporting the conclusions of this article are included within the article and its additional files. There are 7 Figure and 1 supplemental table.

Authors' contributions

FH, VD, MP, MO, PV, CB, RM, EM and DL were involved in the conception and design of the experiments, analyzed the data and wrote the manuscript. FU, RD, SL, YY, NP, and MPP performed the experiments. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Experimental protocols with animals were approved by the local ethical committee for the use and care of animals in biomedical research (2014/UCL/MD/018) and conformed to the Belgian and European Community regulations (LA1230312 and CEE n° 86/609).

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6.2. A New Paradigm Explaining Mesothelioma Development: The Collective Contribution of Inflammatory and Immunosuppressive Particle- and Tumor-Associated Macrophages.

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6.2.1. Abstract

The different stages of mesothelioma development have been exclusively explained by the accumulation of particle-activated inflammatory macrophages responsible of mesothelial cell transformation and tumor expansion. Emerging data highlights, however, a supplemental pathway in which immunosuppressive macrophages deriving from both particle- and tumoral cell-shaped microenvironments enable transformed cells to escape tumor immune surveillance. In this review, we recapitulate clinical and animal findings supporting the new paradigm of a polarized-macrophage continuum shaped by particles and tumor cells and driving mesothelioma progression.

In this merged model, first responses to mesotheliomagenic particles are initiated by resident macrophages (RM) which sense particles and fibers with their array of innate receptors and programmed cell death-related mechanisms. The recognition of reactive particles and dead cell debris polarizes macrophages in inflammatory cells (inflammatory particle-associated macrophages or inflammatory PAM) which orchestrate neutrophilic inflammation by releasing inflammatory mediators (IL-1 and TNF family, alarmins and oxidants). These molecular and cellular elements trigger genetic instability, transform mesothelial cells and promote their growth.

In order to regulate their inflammatory activity and limit persistent inflammation, PAM acquire a regulatory phenotype (immunosuppressive PAM) and release powerful immunosuppressive molecules (TGF- β , IL-10 and PGE₂) establishing an additional environment for tumor development by acting as stromal-stimulating and immune-suppressing mediators. In these conditions, transformed mesothelial cells develop into a tumor consolidated with matrix proteins in an immune silent microenvironment. Finally, when mesothelioma is established, immunosuppressive

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macrophages (tumor-associated macrophages, TAM) are recruited by tumors and sustain tumor cell escape from T cells by developing supplementary inhibitory mechanisms (immune check-points, amino acid consumption and ROS).

The conjunctional requirement of inflammatory and immunosuppressive macrophages explains the long latency period of mesothelioma and the limited effectiveness of current targeted therapies.

Keywords:

Macrophages, inflammation, immunosuppression, mesothelioma, asbestos, carbon nanotubes, fibrosis, T lymphocytes.

Abbreviation:

TAM: tumor-associated macrophages

PAM: particle-associated macrophages

MDSC: myeloid derived suppressor cells

6.2.2. Particles and Malignant Mesothelioma: A General Overview.

Malignant mesothelioma is a cancer of mesothelial cells belonging to peritoneum, pleura, pericardium and tunica vaginalis (Moore et al. 2008). The most common form of disease is malignant pleura mesothelioma (MPM). In 1960, Wagner and coworkers established for the first time a cause-effect relationship between a massive exposure to the asbestos fibers and the onset of malignant mesothelioma (MM) (Wagner et al. 1960). Asbestos is a mineral fibrous silicate, which was largely used around 1970s for thermal insulation, electrical wiring, fireplace cement, pipes, friction material, gaskets and compounds (Carbone et al. 2012; Prazakova et al. 2014); before been completely banned in 55 countries and in the whole European Union in 2005. Other countries still use this material without restrictions such as China, Brazil and India (Carbone et al. 2012). Not only asbestos but also other materials such as some carbon nanotubes (CNT) showed mesotheliomagenic effects in rodent models (Donaldson et al. 2010; Dong and Ma 2019; Murphy et al. 2013; Sakamoto et al. 2018; Toyokuni 2013) .

According to the latest studies, asbestos cause 255,000 death annually, of which 90 % are work-related (Furuya et al. 2018). Asbestos-induced MM is characterized by a long latency period ranging from 15 to 60 years after the exposure (Moore et al. 2008; Prazakova et al. 2014) : this is the reason why a peak in mesothelioma cases in Europe is expected for 2020 (Peto et al. 1999). There is not yet an efficient treatment for MM. Many clinical trials are exploring the combination of different therapies, because surgery, chemotherapy or radiotherapy alone are not effective as single-modality treatment. On the other side, immunotherapy seems to be a promising possibility (Berzenji and Van Schil 2018).

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The need to find a new efficient therapy pressed the investigators to explore the cellular mechanisms behind mesothelioma progression. In this review, we focused the attention on the role of macrophages during mesothelioma development, because macrophages are the first cells involved in the recognition and phagocytosis of foreign fibers and they are the major immune cells infiltrating the mesothelioma microenvironment.

Because of their peculiar physico-chemical properties, asbestos and carbon nanotubes can be inhaled and reach the distal alveolar space. Once there, they are sensed and recognized by macrophages. Depending on their physico-chemical properties, particles are phagocytosed and dissolved by lung macrophages or, due to biopersistence, retained in tissue and cause persistent inflammation by prolonged recruitment and activation of particle-associated macrophages, whose specific role is to sense, alert, remove and destroy the foreign agents, triggering an inflammatory reaction. In the case of biopersistent fibers, such as asbestos and CNT, the prolonged inflammation result in the development of lung diseases including fibrosis, cancer and mesothelioma.

Among the several recognized pathogenic mechanisms of cancer development, the continuous and prolonged inflammatory reaction subsequent to the inhalation of asbestos fibers, is so far the best known. However, it was recently shown that the huge infiltration of immunosuppressive M2 like-polarized macrophages in the mesothelioma microenvironment inhibits the natural T cell anti-cancer activity. In this sense, macrophages act as immunoregulatory cells, dampening the local inflammation.

Here, we point out that, in the early and late mesothelioma microenvironment, macrophages can modulate their phenotype switching from a pro-inflammatory

state (M1), necessary to eliminate particles, to an anti-inflammatory state (M2) damping inflammation, but strategically exploited by cancer cells to survive and proliferate. Understanding the early mechanisms by which macrophages skew their phenotype from physiological to pathological state can represent a milestone in deciphering disease pathogenesis.

Early Recognition of Mesotheliomagenic Particles by Resident Macrophages Initiates Macrophage Polarization and Inflammatory Premises

Resident macrophages possess a large set of surface receptors (named pattern-recognition receptors or PRR) that mediate recognition, binding and internalization of pathogens, particles (Figure 1) and damaged cell residues (Bournazos and Ravetch 2017; Ishikawa et al. 2017; PrabhuDas et al. 2017; Taylor et al. 2005; Underhill and Goodridge 2012). Several studies have been carried out to determine by which PRR receptors macrophages specifically recognize inorganic mesotheliomagenic particles, including asbestos and CNT. It was demonstrated that macrophages recognize particles by scavenger receptors (SR), mannose receptors (MR), and Toll-like receptors (TLR) (Aderem and Underhill 1999). During the '90s, it was first evidenced that recombinant SR-A I and II were able to bind asbestos, and the use of SR-A ligands, such as polyinosinic and polyguanylic acids, suppressed their particle binding ability (Resnick et al. 1993). More recently, it was observed the occurrence of less pulmonary responses in SR-A II- or MARCO-deficient mice upon exposure to asbestos, confirming the role of scavenger receptors in macrophage activation after fiber sensing (Murthy et al. 2015). Involvement of SRA-II receptor was also described in the *in vitro* recognition and

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phagocytosis of CNT (Hirano et al. 2012). Mukherjee & coworkers (Mukherjee et al. 2018) also found that the exposure of macrophages to CNT induced chemokine secretion via TLR2 and 4 associated with the MyD88/NFkB-related inflammatory pathway. These data indicate that particle-related recognition activity occurs via diverse PRR (Gay et al. 2014). The exact physico-chemical interactions and biochemical mechanisms that drive asbestos or CNT sensing by macrophages remain unclear. A scenario implicating negative surface charges of particles is often referenced to explain innate receptor activation (Tsugita et al. 2017). An alternative mechanism was recently supported by Ng & coworkers whereby macrophage stimulation is also triggered by membrane lipid alterations without the requirement of specific PRR (Ng et al. 2008).

After sensing, the engulfment and accumulation of internalized fibers and particles in phagocytic resident macrophages (Figure 1) result in inflammasome activation (Donaldson et al. 2010; Roggli 1990; Sanchez et al. 2009). This multiprotein complex is composed by the NOD-like receptor family pyrin domain containing 3 sensor (NLRP3), adaptor-apoptosis-associated speck-like protein containing a caspase-recruitment domain (ASC) and caspase-1 effector protein (Dostert et al. 2008; Hillegass et al. 2013; Sayan and Mossman 2016). Inflammasome activation results in the proteolytic maturation of caspase-1, which in turn triggers the release of proinflammatory mature cytokines (IL-1 β , IL-8) by activated resident macrophages. These effects were evidenced after exposure of macrophage but also mesothelial cells (Dostert et al. 2008; Hillegass et al. 2013). As for asbestos, CNT can be internalized by animal macrophages (Ryman-Rasmussen et al. 2009) and induce inflammasome activation, ROS production and pro-inflammatory cytokine secretion (Hamilton et al. 2012; Hindman and Ma 2019; Sager et al. 2014; Sun et al. 2015). Palomaki & coworkers, in particular, observed comparable ROS-dependent inflammasome activation in human primary macrophages after treatment with

long needle-like CNT and asbestos fibers (Palomaki et al. 2011). Interestingly, defects in IL-1 β secretion and early macrophage responses in NLRP3 knock-out mice evidenced the critical role played by inflammasome during particle recognition and macrophage activation (Chow et al. 2012). Furthermore, the inflammasome sensing can trigger pyroptosis, a caspase-1 dependent macrophage-death pathway (Bergsbaken et al. 2009; Malik and Kanneganti 2017; Man et al. 2017). Besides mature IL-1 production, DNA fragmentation and plasma membrane rupture, this process is also characterized by the release of inflammatory mediators previously stocked in resident macrophages, including the alarmins IL-1 α and HMBG1 (Figure 2) (Bergsbaken et al. 2009; Stegmann et al. 2015). It is now well accepted that macrophage pyroptosis and its associated mediators represent the first events which play a central major role in the initiation of inflammation caused by mesotheliomagenic particles (Dinarello 2018).

The Inflammatory Cascade Orchestrated by Particle-Associated Macrophages (PAM) Results in Transformed Mesothelial Cells and Mesothelioma Progression

The ability of inorganic fibers to persist over time in the body is known as biopersistence or biodurability and defined by particle retention half-time. A long retention-half time allows particle to reach the mesothelioma site, i.e. pleura or peritoneal cavity (Laux et al. 2017). This process strongly impacts tissue responses (Andersen et al. 2012; Sanchez et al. 2009) and is largely determined by particle characteristics, including size and shape (Donaldson et al. 2010; Padmore et al. 2017). Lung accumulation of biopersistent particles that cannot be degraded or dissolved by resident macrophages cause so-called macrophage frustrated phagocytosis resulting in an unsolved deleterious vicious cycle of macrophage

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pyroptosis, inflammation and lung clearance failure (Frank et al. 2015; Pan et al. 2011; Poland et al. 2008). Release of the pyroptotic-related mediators by frustrated macrophages is triggered by asbestos fibers or CNT (Dostert et al. 2008; Murphy et al. 2012). This uncontrolled and persistent response is commonly characterized by accumulation and activation of inflammatory leukocytes, in particular pro-inflammatory macrophages with a M1 phenotype, here referred as inflammatory particle-associated macrophages or inflammatory PAM (Figure 1). Inflammatory PAM amplify the inflammatory microenvironment through the additional release of an array of soluble interleukins, chemokines, alarmins, growth factors and oxidants (including IL-1-related cytokines IL-6 and IL-8, CCL-2, HMGB1, GM/M-CSF, and ROS/RNS) (Laskin et al. 2011) (Figure 1). These data were confirmed by studying iNOS and TNF expression, well-known markers of M1-polarized macrophages, after exposure to CNT (Xue et al. 2018). The occurrence of an inflammatory/M1 PAM phenotype was further supported by the increased levels of two specific additional biomarkers of inflammatory signaling pathways, STAT1 and IRF5 (Dong and Ma 2018).

The impact of cytokines and chemokines on mesothelial cells during frustrated responses to mesotheliomagenic particles was extensively investigated. Experimental evidence suggested that pro-inflammatory cytokines released by activated inflammatory PAM directly alter mesothelial cells function *in vitro* and *in vivo* (Wang et al. 2004). Cytokines (in particular IL-1 β and TNF- α) resulting from inflammatory PAM induce proliferation and differentiation of mesothelial cells (Xu et al. 2012). The alarmin HMGB1 trigger the NF- κ B signaling pathway in activated mesothelial cells, inducing the expression of several genes allowing cell survival (Affar and Carbone 2018; Carbone and Yang 2017). In addition, these mediators activate inflammatory phenotype in mesothelial cells, promoting the release of

additional inflammatory, growth and differentiation factors for stem and endothelial cells (M-CSF, GM-CSF and VEGF, respectively) (Nowak et al. 2020). It is well accepted that asbestos and CNT are directly genotoxic for exposed mesothelial cells (Mossman et al. 2013). Main genotoxic responses triggered by fibers include DNA strand breakage, DNA oxidation, micronuclei induction, chromosomal aberrations and cell-cycle related mutations (van Berlo et al. 2012). For instance, the direct sensing between fibers and mesothelial cells can lead to mitogen activated protein kinases (RTKs) phosphorylation and stimulation of growth-promoting or anti-apoptotic pathways, which may also be triggered by cytokines, including TNF- α released by inflammatory PAM (Goldberg et al. 1997). Genotoxicity can also occur via oxidative stress (ROS, RNS) resulting from activated inflammatory PAM. This process leads to aberrant mesothelial cell growth and malignant transformation, resulting in carcinogenesis (Figure 2) (Benedetti et al. 2015; Jaurand 1997; Jaurand et al. 2009; Ma 2010; Urso et al. 2020). DNA damage upon formation of 8-nitroguanine, a mutagenic DNA lesion, was induced by ROS/RNS released from activated inflammatory PAM in asbestos-exposed mice (Hiraku et al. 2010). This observation, also evidenced in human lung tissues (Hiraku et al. 2014), supports the role played by radical species generated by activated macrophages during inflammatory response. It was recently suggested that DNA methylation upon treatment with CNT, represents a pathogenic mechanism explaining mesothelial cell transformation, tumor cell proliferation and mesothelioma development. Altogether, this evidence demonstrates that the asbestos- and CNT-induced inflammatory and genotoxic processes attributed to macrophages promote mutated mesothelial cell proliferation and malignant mesothelioma progression (Yang et al. 2006) (Figure 2).

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The PAM-driven inflammatory response triggers the recruitment of other inflammatory cells, including neutrophils. Influx of neutrophils was observed in mice after treatment with CNT and asbestos only sixteen hours after exposure (Rydman et al. 2015). They are recruited around the particle deposition site via pro-inflammatory signals released by inflammatory PAM (Grommes and Soehnlein 2011; Powell and Huttenlocher 2016). They crucially contribute to particle-induced lung injury and damage (Bass et al. 1986; Chollet-Martin et al. 1992; Reddy et al. 2011). It is known that neutrophils release ROS after CNT-exposure, resulting in additional genotoxic effects towards mesothelial cells (Figure 1) (Knaapen et al. 2006). In addition, neutrophils recruit additional neutrophils and macrophages, amplifying the immune response to particle and tissue damage (Kumar and Sharma 2010). Overall, these macrophage- and neutrophil-related processes avoid mesothelial cells to undergo apoptosis, induce migration of aberrant mesothelial cells through tissues and favor tumor development (Figure 1)

Fibroblasts belong to the family of connective-tissue cells that, in physiological conditions, participate to wound healing and are in charge to produce extra cellular matrix components such as type I, type III collagen and elastin (Kalluri and Zeisberg 2006; Parsonage et al. 2005; Tomasek et al. 2002; Watt and Fujiwara 2011). Extensive inflammatory damage to surrounding cells and tissue induced by inflammatory PAM stimulate a subsequent repair phase comprising fibroblast activation and granuloma development (Figure 1) (Donaldson et al. 2010). This concept was supported by Boyles & colleagues, who evidenced that frustrated phagocytosis by inflammatory PAM of long CNT and asbestos fibers (> 15 μm) triggers pro-fibrotic and pro-angiogenic activities (Boyles et al. 2015). Cytokines and ROS released by inflammatory PAM activate NF- κB (nuclear factor- κB) signaling pathway, increasing synthesis and release of growth factors for fibroblasts (TGF- β1 and platelet-derived growth factor (PDGF)-A) that promote matrix protein

deposition and fibrosis (He et al. 2012; He et al. 2011; Vietti et al. 2016b). Additional studies consolidated the interplay between inflammatory PAM and fibrotic response in the uncontrolled tissue remodeling existing at early and late stages of particle-induced lung responses (Brown et al. 2016; Cesta et al. 2010; Cole et al. 2019; Dong et al. 2015). It is now assumed that continuous remodeling and fibrosis in affected tissue facilitate support networks resulting to tumor expensive spread (Figure 2).

Early Macrophage Polarization Shift: An Unexpected Immunosuppressive Environment Mediated by PAM in Response to Mesotheliomagenic Particles

Intriguingly, it was observed that the development of malignant mesothelioma in NLRP3-deficient or wild-type mice treated with asbestos was comparable (Chow et al. 2012). This suggests that inflammation and inflammatory PAM may not be critical in mesothelioma development as expected, and, furthermore, that additional pathways besides inflammation are operative to explain particle-induced mesothelioma.

While inflammatory/M1 macrophages are involved in the orchestration of inflammatory responses, anti-inflammatory/M2 macrophages orchestrate the resolution phase of inflammation through immunoregulatory responses. The two phenotypic states of pro- and anti-inflammatory macrophages are not fixed or immutable but dynamically modulated by specific stimuli (Atri et al. 2018; Tarique et al. 2015; Xu et al. 2013). M2 phenotype is triggered by the Th2-like cytokines, such as IL-4, IL-13, IL-10 and TGF- β and characterized by high levels of IL-10 and arginase-1 (Klopfleisch 2016), all of which are considerate as powerful

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immunoregulating mediators. In a normal tissue, the pro-inflammatory/anti-inflammatory ratio is highly regulated but unbalanced during inflammatory or immunosuppressive processes (Fujisaka et al. 2009; Hu et al. 2018).

TGF- β family is composed by a set of multifunctional polypeptides that play a major immunoregulatory role (Stockhammer et al. 2020). Immunosuppressive activity of TGF- β was firstly evaluated in TGF- β 1 deficient mice, which died after uncontrolled systemic inflammation and accumulation of autoreactive T lymphocytes (Culliton 1992). Strong immunosuppressive functions are also attributed to IL-10 (Armstrong et al. 1996; Bogdan et al. 1991; de Waal Malefyt et al. 1991; Fiorentino et al. 2016), much as downregulation of proinflammatory mediators released by M1-macrophages (Standiford et al. 1995). Interestingly, macrophages expressing IL-10 increase mesothelial cell proliferation and induce resistance to mesothelioma treatment (Chene et al. 2016; Maeda et al. 2008). Also, IL-10 regulates the expression of PGE₂, a key-mediator in the control of lung fibrosis and regulation of immune responses (Kalinski 2012; Moore et al. 2000). Indeed, PGE₂ possesses immunosuppressive activity by downregulating the production of Th1 and M1 cytokines and upregulating Th2 and M2 cytokine release (Li et al. 2013).

In contrast to the dogmatic inflammatory concept, several groups noted that these potent immunosuppressive mediators (TGF- β , IL-10 and PGE₂) were produced by PAM after exposure to asbestos and CNT *in vivo*. Indeed, anti-inflammatory M2-like macrophages and tissue IL-10 and TGF- β expression were observed 7-days after CNT exposure in mice (Dong and Ma 2018; Park et al. 2011a). Other studies evidenced that CNT were able to induce strong release of immunosuppressive TGF- β 1 by activated PAM (Shvedova et al. 2005; Wang et al. 2013). Early and long-lasting release of TGF- β by PAM in particle deposition site was also reported after treatment with asbestos (Nishimura et al. 2007; Perdue and Brody 1994). Interestingly, Sullivan et al. (Sullivan et al. 2008) showed that TNF- α enhances the

production of latent TGF- β in alveolar surfaces of asbestos-exposed animals. Its activation was triggered by asbestos-derived ROS. TGF- β expression was suppressed in lungs of TNF- α knock-out mice, confirming that the pro-inflammatory TNF- α is necessary for immunosuppressive growth factor release (Liu and Brody 2001). In addition, osteopontin (OPN, another pro-inflammatory cytokine) acts upstream of TGF- β 1 release since TGF- β 1 is absent in the lungs of OPN knock-out mice treated with CNT (Khaliullin et al. 2017). Slowdown of tumor growth was observed in TGF- β deficient-mice (Shvedova et al. 2015), demonstrating that TGF- β and its immunosuppressive activities are essential in CNT-induced mesothelioma. Besides TGF- β , early increase of IL-10 expression by macrophages was observed in rats after exposure to CNT. The release of IL-10 induces a clear deficiency of IL-2 resulting in limited T cell proliferation and anti-tumoral-activity (Kido et al. 2014). Interestingly, Mitchell & coworkers observed that the activation of cyclooxygenase enzymes (COX) is a critical step in the suppression of systemic humoral immunity resulting from CNT exposure. TGF- β is necessary for PGE₂ and IL-10 expression. After exposure to CNT, PGE₂ production was further observed in murine alveolar macrophages via COX-2 expression and ERK-1,2 dependent mechanisms (Lee et al. 2012). In response to CNT and asbestos, the role of COX-2 in immunosuppressive PGE₂ production was furthermore demonstrated (Bissonnette et al. 1990; Sayers et al. 2013). PGE₂ is also able to recruit Myeloid derived Suppressive Cells (MDSC) in mesothelioma (Brown et al. 2009) and potentiate their immunosuppressive phenotype and activity (Tomic et al. 2019). It also acts as promoter of immunosuppressive M2 polarization of macrophages (Digiacoimo et al. 2015). The early recruitment of M-MDSCs accompanied by the release of TGF- β were also evidenced after the injection of mesotheliomagenic CNT *in vivo* (Huaux et al. 2016; Shvedova et al. 2008; Shvedova et al. 2013).

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The presence of immunoregulatory PAM suggests that an early resolution phase is triggered and implemented after mesotheliomagenic particles. This phase plays a crucial role in the onset, stabilization and resolution of inflammation and comprise, at least in part, a gradual switch between inflammatory M1-like PAM to an anti-inflammatory M2-like PAM that takes place upon the exposure to mesotheliomagenic fibers (Figure 2). Additionally, anti-inflammatory M2-like PAM possess immunosuppressive activity because their mediators (IL-10, TGF- β , and prostaglandins) strongly affect the function of T cells highlighting a continuum between inflammatory PAM and immunosuppressive PAM (Mitchell et al. 2007; Mitchell et al. 2009). Altogether, these findings indicate that an early and potent immunosuppressive environment orchestrated by immunosuppressive PAM (Figure 1) is generated by mesotheliomagenic particles to regulate inflammation. As for the frustrated phagocytosis concept, PAM-related immunosuppressive responses can be persistent as mesotheliomagenic particle are not eliminated, but are biodurable. This repetitive phase provides a silent immune microenvironment favorable for transformed mesothelial cell survival, immune evasion and mesothelioma progression (Figure 2).

A cross talk between alternatively activated M2/immunosuppressive macrophages and fibroblasts exists and is largely described in responses to microorganisms (Wynn and Vannella 2016) and particles (Ma 2020). Indeed, M2-polarized macrophages secrete various pro-fibrotic mediators comprising VEGF α , IFG-1, ROS and TGF- β which attract, stimulate and polarize fibroblasts towards a smooth muscle-like or “tissue repair” phenotype, characterized by high contractility, proliferation, and secretion (Bernardo and Fibbe 2013; Murray and Wynn 2011). The alternatively activated fibroblasts deposit the ECM proteins, MMPs and TIMPs (tissue inhibition of metalloproteinase) (Tampe and Zeisberg 2014) and release TGF- β , VEGF, CXCL10, CXCL12, IL-6, and EGF in order to promote remodelling

(Ireland and Mielgo 2018). The clear relationship between immunosuppression, immunosuppressive PAM and fibrosis development has been recently detailed in response to silica (Huaux 2018a). It is possible that this remodeling environment contribute transformed mesothelial cells to develop into a tumor consolidated with matrix proteins and activated fibroblasts (Figure 1).

It is still unclear how inflammatory PAM switch in immunosuppressive PAM during the early responses to mesotheliomagenic particles. Efferocytosis, i.e. the phagocytosis of death cell, is carried out by macrophages (Boada-Romero et al. 2020). Effective efferocytosis results in a reprogramming of macrophage activation profile with the release of anti-inflammatory and immunosuppressive cytokines, such as IL-10 and TGF- β (Boada-Romero et al. 2020). Thus, in addition to allow elimination of pyroptotic macrophages, efferocytosis also result in cytokine secretion which leads to an M2-related cellular environment supporting the accumulation of immunosuppressive PAM (Figure 2). The apparent opposite accumulation of inflammatory and immunosuppressive PAM is not exclusive and may be, in contrast, concomitant. The view that immunoregulatory and immunostimulatory responses and PAM co-exist is supported by the co-presence of inflammatory and immunosuppressive cells such as inflammatory neutrophils and MDSC during carcinogenesis induced by CNT in rats (Huaux et al. 2016).

Infiltration of Immunosuppressive Tumor-Associated Macrophages (TAM) Ensures Mesothelioma Survival and Progression

Infiltration of TAM macrophages in mesothelioma tumors is associated with poor prognosis and correlates with chemotherapy resistance (Chen et al. 2019). Indubitably, TAM possess pro-tumoral activities and promote malignant progression by suppressing anti-tumor immunity, stimulating angiogenesis and increasing tumor cell growth (Cassetta and Pollard 2018)(Figure 1).

TAM inhibit T or NK activity against cancer cells, via the stimulation of immune checkpoints (Figure 2) (Burugu et al. 2018). Among these immune checkpoints or signals, the PD-1/PDL-1 axis is the most studied for its relevance as therapeutic target in cancer (Sun et al. 2018). The binding between the ligands expressed on TAM surface and the corresponding receptors on T cells and NK leads to the inactivation of their killer activities. In mesothelioma, between 20 % and 70 % of patients are positive for PD-L1 (Combaz-Lair et al. 2016). Inhibitory antibodies targeting PD-1 (pembrolizumab, durvalumab, nivolumab) administrated in combination with cisplatin/pemetrexed as first line therapy are currently under evaluation for the treatment of unresectable malignant mesothelioma (Hotta and Fujimoto 2020). Another immune checkpoint is CTLA-4 which is expressed on T cell surface. Together with CD28 co-stimulatory molecule, CTLA-4 binds CD80 (B7-1) and CD86 (B7-2) molecules on macrophage cell surface. CTLA-4/CD28 activation inhibits T cell survival and proliferation. In malignant mesothelioma tremelimumab, an anti-CTLA-4 is investigated as salvage setting therapy (Hotta and Fujimoto 2020).

IL-10 is a strong immunosuppressive cytokine largely expressed by TAM (Oft 2014). IL-10 causes a down regulation of MHCII (Steinbrink et al. 1999) and MHCI (Adris et al. 1999) expression, inhibiting antigen presentation by APC and tumor cells

respectively (Mannino et al. 2015). Pleural effusions (Chene et al. 2016) and blood (Matsuzaki et al. 2017) from malignant mesothelioma patients are especially rich in IL-10, which is mainly released by macrophages from person exposed to asbestos (Murthy et al. 2015). TGF- β 1 is another immunosuppressive cytokine with pro-oncogenic properties released by TAM. TGF- β 1 exerts its immunosuppressive role by inhibiting T cell effector and activating immunosuppressive regulatory T cells (or Treg) (Massague 2008). High level of TGF- β 1 has been detected in pleural effusion of malignant mesothelioma patients (Maeda et al. 1994). Stevenson et al. evaluated the efficacy of GC1008, a TGF- β neutralizing antibody on pleural malignant mesothelioma patients: 3 out of 13 patients showed an increased immune response which correlated with an increase of the overall survival (Stevenson et al. 2013). Finally, the compound SM-16 which inhibits the kinase activity of the T β RI activating receptor-like kinase (ALK5) in the TGF- β signaling was tested on murine malignant mesothelioma model. It was demonstrated to reduce the tumor growth and TAM accumulation (Suzuki et al. 2007).

PGE₂ is a metabolite of arachidonic acid, produced by COX-2 enzyme. The expression of COX-2 by TAM and the consequent production of PGE₂ correlates with bad prognosis in mesothelioma (Edwards et al. 2002). PGE₂ binds G-coupled receptors on T effector cells, activating protein kinase A (PKA) which in turn prevents T cell signaling, proliferation and cytotoxic activity (Newick et al. 2016). Lievense and co-workers observed that PGE₂, highly contained in pleural effusions of mesothelioma patients, polarizes macrophages into an M2 phenotype responsible to inhibit T cell proliferation *in vitro* (Lievense et al. 2016). Celecoxib, COX-2 inhibitor, was tested in a murine model of mesothelioma and it is effective in decreasing PGE₂ levels (Veltman et al. 2010a).

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ROS (reactive oxygen species) and RNS (reactive nitrogen species) produced by TAM and MDSC participate in the anergy of natural killer cells (NK), CD8+ and CD4+ and in the expansion of Treg population (Lu and Gabrilovich 2012). The enzymes involved into ROS/NOS production are arginase 1, Nox1 and Nos2. Arginase 1 catalyses the conversion of arginine to urea and L-ornithine, participating also in $O_2^{\cdot-}$ generation, while Nos2 transforms arginine to citrulline and NO. NADPH oxidase (Nox1) reduces the O_2 to superoxide anion $O_2^{\cdot-}$, which then reacts with nitric oxide (NO) generated by Nos2 to produce peroxynitrite ($ONOO^-$). Peroxynitrite catalyses the nitration of four amino acids: tyrosine, cysteine, methionine and tryptophan (Ekmekcioglu et al. 2000; Haqqani et al. 2002). In tumor microenvironment, peroxynitrite induces the nitration of the T cell receptor (TCR), CD8 and CD4 on T cells, inhibiting their binding to antigen-MHC complex (Nagaraj et al. 2007). Regarding mesothelioma an overexpression of iNOS has been measured in 74 % to 100 % of mesothelioma biopsies (Marrogi et al. 2000). Moreover, high peroxynitrite level correlates with tumor progression in mesothelioma patients (Nagaraj and Gabrilovich 2008).

When naïve T cells are activated to T effector cells, there is an up regulation of aerobic glycolysis to provide biochemical intermediates such as amino acids, fatty acids and nucleotides. In parallel, there is also an increase of mitochondrial biogenesis, oxidative (OXPHOS) metabolism, glutaminolysis and amino acid uptake (Bantug et al. 2018). L-arginine and tryptophan are pivotal for T cell survival and fitness (Sikalidis 2015). Arginase 1 (Arg1) is employed by myeloid suppressor cells and TAMs to restrain the T cell activity. Arg1 metabolizes arginine to ornithine and urea (Arlauckas et al. 2018) and the L-arginine depletion leads to a downregulation of the expression of the ζ -chain of CD3 resulting in T cell anergy (Bronte and Zanovello 2005) and limited cell-cycle progression (Rodriguez et al. 2007). L-

arginine supply increases the T cell survival and antitumor functionality (Geiger et al. 2016). Regarding mesothelioma Rehrauer and co-workers observed an upregulation of Arg1 gene in inflamed tissues and tumor from mice intraperitoneally injected with asbestos (Rehrauer et al. 2018).

Another fundamental amino acid for T cell fitness is tryptophan: its depletion causes the T cell-cycle arrest and the accumulation of its catabolites such as 3-hydroxyanthranilic and quinolinic acids induce T cell apoptosis (van Baren and Van den Eynde 2015). Indoleamine 2,3-dioxygenase 1 (IDO1), tryptophan-2,3-dioxygenase (TDO) and the most recently discovered indoleamine 2,3-dioxygenase 2 (IDO2) belong to the rate-limiting step in the kynurenine pathway, involved in the metabolism of tryptophan to nicotinamide adenine dinucleotide (NAD⁺) (Ball et al. 2007; Selvan et al. 2016). These three enzymes are largely expressed by TAM and are responsible of T cell anergy. In mesothelioma, IDO is constitutively expressed (Uyttenhove et al. 2003): this is the reason why increasing efforts are invested in the research of IDO inhibitors for mesothelioma therapy.

Furthermore, TAMs contribute to maintain low pH, hypoglycaemia and hypoxia in tumor microenvironment. The interaction between PD-1 (expressed on T effector cells) and PDL-1 (expressed by tumor cells and TAMs) controls the metabolism of cells in TME. The signal transmitted by this network promotes a glycolytic metabolism in cancer cells (Warburg effect) and in effector cells and an oxidative phosphorylation-based metabolism in TAMs, which avoid in this way to be in competition with cancer cells for glucose (Vitale et al. 2019). The low pH and the high level of lactate resulting from the glycolytic metabolism of cancer cells increases the immunosuppressive functions of TAMs and MDSC and induces T cell anergy, overexpression of PD-1 inhibitory checkpoint, polarization of T cell towards

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Treg and impairment of T cell motility (Davoodzadeh Gholami et al. 2017). The hypoxia, induced by a rapid proliferation of cancer cells, activates in TAMs the HIF-1 α transcription factor, which in turn upregulates the CXCR4 chemokine receptor and increases the CXCL12 release. The CXCR4/CXCL12 binding stimulates TAM recruitment in hypoxic area and their switch towards M2 alternative activated phenotype (Chanmee et al. 2014). A recent study in mesothelioma patients confirmed the presence of hypoxic area in most of tumors (Francis et al. 2015). Moreover, an *in vitro* study of Kim and co-workers showed that hypoxia significantly increases the aggressiveness of transformed mesothelial cells (Kim et al. 2018).

When the cancer is established, the collaboration between TAM and cancer-associated fibroblasts (CAFs) is maintained (Figure 2) (Ireland and Mielgo 2018). In cancer, persistent signals keep fibroblasts in a chronically activated state which leads to continuous tissue remodelling and the formation of a dense granuloma surrounding the cancer. CAFs infiltrate the stroma of the tumor microenvironment and experience many activating cytokines and chemokines such as TGF- β (Massague 2012; Pickup et al. 2013), FGF, PDGF and MCP-1 (Marsh et al. 2013). CAFs are source of factors for TAM recruitment, regulation and differentiation such as CCL2, CCL3, CCL5, IL-6, CSF1, CSF2, VEGF and CXCL8 (Chomarat et al. 2000; Kalluri 2016; Torres et al. 2013; Wu et al. 2011). In particular, the high level of IL-6 production seems to have a direct effect on STAT3 phosphorylation on macrophages sticking them in an immature, immunosuppressive state (Chomarat et al. 2000). High levels of IL-6 were observed in the biopsies of MM patients (Hegmans et al. 2006) and could explain why the mesothelioma cells are not eliminated despite the presence of T cells. After stimulation, CAFs release immunosuppressive factors, such as PGE₂, CTGF and CXCL7 (Hanahan and Coussens 2012; Ireland and Mielgo 2018). Moreover, fibroblast growth factor (FGF) and its

receptor (FGFR) are over expressed in MPM and correlate with poor prognosis (Marek et al. 2014; Schelch et al. 2018b; Strizzi et al. 2001). The inhibition of the FGF/FGFR axis could be a novel therapeutical strategy in mesothelioma treatment (Blackwell et al. 2016; Pattarozzi et al. 2017; Schelch et al. 2014; Schelch et al. 2018a).

During epithelial-mesenchymal transition, cells lose their polarity, acquiring stemness-related features which contribute to cancer proliferation and migration. In some cases (pancreatic cancer cells) M2 TAM contribute to EMT by activating the IL10/TLR4 signaling pathways (Liu et al. 2013). TAM that produced-TGF- β 1, together with PDGF, EGF, HGF and TNF- α , induce the downregulation of epithelial markers (E-cadherin and β -catenin) and tight junctions (occluding) and the overexpression of mesenchymal markers including Vimentin, Slug, Snail, Fibronectin, zinc-finger E-box binding homeobox 1 (ZEB1), ZEB2, and α -smooth muscle actin. As a result, the cells acquire migration and proliferation ability (Aras and Zaidi 2017; Fan et al. 2014). In malignant mesothelioma, EMT correlates with a more aggressive phenotype (Fassina et al. 2012). The use of neutralizing TGF- β antibody was shown to revert the EMT profile of mesothelial MeT-5A cells restoring the physiological expression of E-cadherin and fibronectin (Turini et al. 2019) (Figure 2).

TAMs stimulate angiogenesis in order to provide oxygen, nutrition and the elimination of cellular waste from cancer cells (Figure 2) (Noy and Pollard 2014). TAM stimulate angiogenesis through VEGF production by expressing (Hughes et al. 2015), TGF- β and TNF and contribute to the generation and maintenance of vascular network to supply the tumor microenvironment (Fallah et al. 2019). TAMs can also produce angiogenic growth factor such as VEGF (Lin and Pollard 2007) and

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angiogenic chemokines such as CXCL8 and CXCL12 by themselves (Marcuzzi et al. 2018). Regarding malignant mesothelioma many clinical studies confirmed that angiogenesis can be a good therapeutic target. Indeed, there is a strong correlation between blood vessel density and high serum levels of VEGF and a bad prognosis in mesothelioma patients (Edwards et al. 2001; Tsao et al. 2019). Antiangiogenic therapies are currently in study to be used in combination with the standard therapies (cisplatin/pemetrexed) for malignant mesothelioma treatment.

Different therapies targeting the axis involved in TAM recruitment are currently in study for many solid tumors (Jahchan et al. 2019). Regarding mesothelioma, high levels of CCL2 were measured in pleural effusions (Gueugnon et al. 2011) and in serum (Kishimoto et al. 2019) of mesothelioma patients and blocking CCR2/CCL2 axis decreases TAM accumulation, mesothelioma growth and tumor volume in murine models (Fridlender et al. 2010). Similarly, M-CSFR inhibition using the CSF-1R kinase inhibitor PLX3397 was successfully tested in a murine model of mesothelioma. PLX3397 alone or in combination with dendritic cell vaccination reduces the number of recruited TAM and the amount of angiogenesis and it improves the patient overall survival (Dammeijer et al. 2017).

6.2.3. Final Conclusions: Mutual Role of Inflammatory PAM, Immunosuppressive PAM and TAM in Mesothelioma Development

Several works published in the last 30 years evidenced clear *in vitro* and *in vivo* carcinogenic effects of asbestos and CNT related to their physico-chemical characteristics and their capacity to dysregulate the immune system. Here, we propose a new model explaining the development of mesothelioma, based on the continuum between inflammatory and immunosuppressive macrophages related to mesotheliomagenic particles before established mesothelioma.

A well described pro-inflammatory environment is first orchestrated by inflammatory particle-associated macrophages (PAM) and by their early release of mediators mainly including IL-1 β , TNF- α , and radical species. These molecules contribute to the transformation and proliferation of aberrant mesothelial cells, which can initiate tumor development. However, an early immunosuppressive environment ruled by immunosuppressive PAM further supports the development of mesothelioma. Indeed, the synthesis and secretion of immunosuppressive molecules (TGF- β , IL-10 and PGE₂) by these polarized macrophages limit inflammation and its chronic character but suppress T cell activity essential in tumor cell removal.

The survival and progression of mesothelioma is ensured at a later stage, by the influx of tumor-associated macrophages (TAM) which guarantee oxygen and nutrition to tumoral cells by promoting angiogenesis and tumor invasion. TAM, also, modulate immune check-points and recruit additional immunosuppressive cells, thus quenching the activation of the immune system towards mesothelioma.

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The detailed comprehension of immune mechanisms underlying the early phases of mesothelioma pathogenesis could represent a powerful tool to improve our catalog of bioassays measuring carcinogenic activity of new particles. New strategies based on the dual state of macrophage activation can be suggested for treating patients developing mesothelioma and lung cancer. We also propose that the prolonged and continue balance between the two opposite PAM sub population and their related environments could explain, at least in part, the long latency period of mesothelioma development.

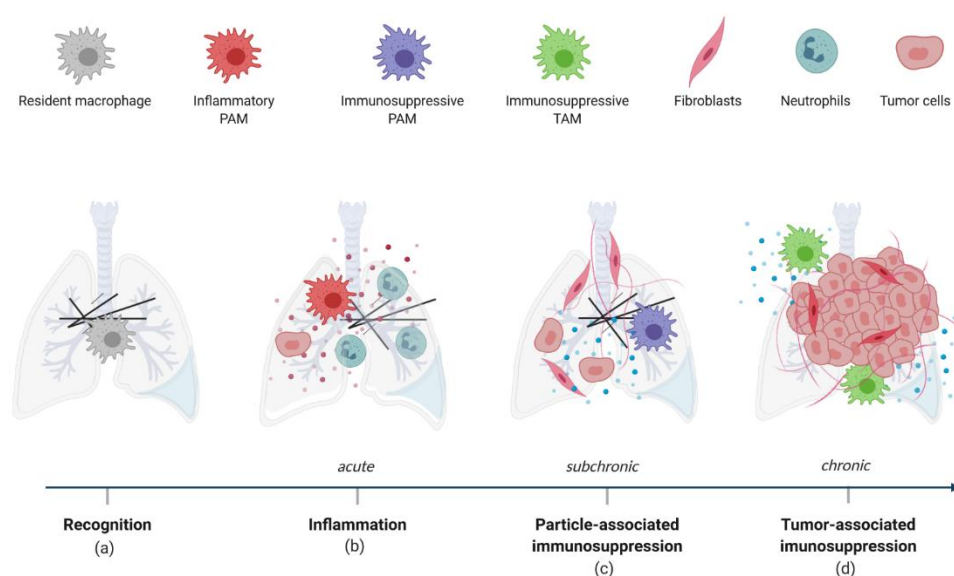


Figure 1: Particle- and tumor cell-polarized macrophages are the driven force of mesothelioma initiation and progression. Resident macrophages in the lung encounter, recognize, bind and internalize asbestos-like persistent particles (a). In attempt to phagocyte and eliminate carcinogenic particles, resident macrophages dye and release danger signals that alert the immune system and induce the recruitment of inflammatory neutrophils and particle-associated macrophages (iPAM). Inflammatory PAM release immunostimulating and oxidant mediators that contribute to amplify the inflammatory response and transform mesothelial cells (b). In an attempt to regulate inflammation, immunosuppressive particle-associated macrophages (immunosuppressive PAM) infiltrate inflammation-resulted tissue lesions and release TGF- β , IL-10 and PGE₂. The continuous release of these immunoregulatory mediators results in an early immunosuppressive microenvironment favoring the development of tumors consolidated with fibroblasts and matrix proteins (c). When mesothelioma is established, mesothelioma tumor-associated macrophages (TAM) and cancer-associated fibroblasts (CAF) accumulate in the tumor microenvironment creating an additional immunosuppressive microenvironment which prevents the natural T cell anticancer activity (d).

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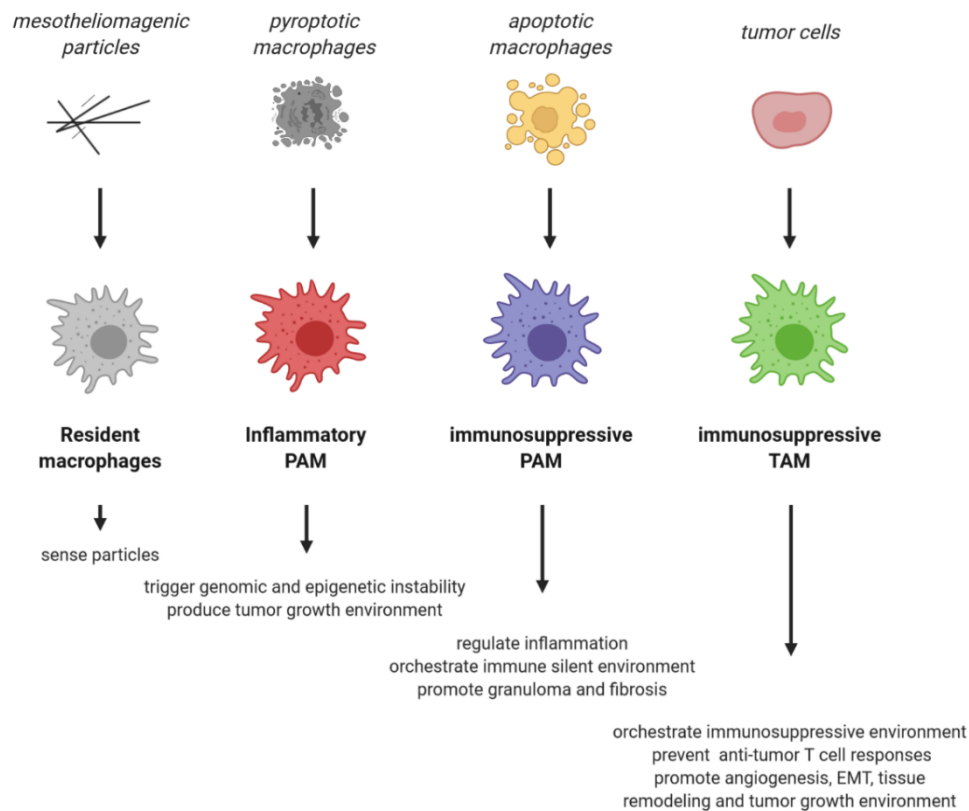


Figure 2: Particle (PAM) and tumor (TAM)-associated macrophage continuum and their pathological functions during mesothelioma development. Resident macrophages are very plastic cells which oversee to different functions depending on the external stimuli. Resident macrophages maintain homeostasis in the tissues adapting themselves to the environmental changes. Resident macrophages dye by pyroptosis after mesotheliomagenic particle phagocytosis and alert immune system. Inflammatory particle-associated macrophages (inflammatory PAM) are recruited to boost inflammatory reaction initiated by dying resident macrophages after the encounter with the particles. iPAM-related cytokines (IL-1 and TNF family), alarmins (HMGB1 and IL-1 α) and radicals (ROS/RNS) cause mesothelial cell transformation, survival and proliferation. Immunosuppressive PAM recruited by active efferocytosis (apoptotic macrophages) release TGF- β , IL-10 and PGE₂ which dampen inflammation, promote immune silent microenvironment and increase matrix protein

deposition. Tumor-associated macrophages (TAM) infiltrating mesothelioma inhibit antitumor T cell activities, coordinate the cancer cell and CTL metabolism and favor cancer cell survival and proliferation.

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