Nanoimmunotoxicity: *in vitro* and *in vivo* approaches

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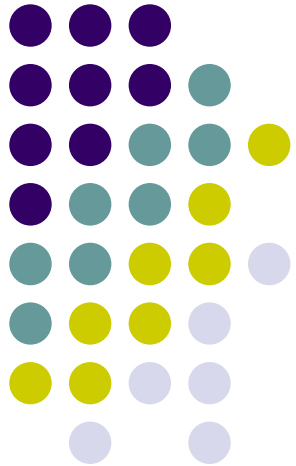
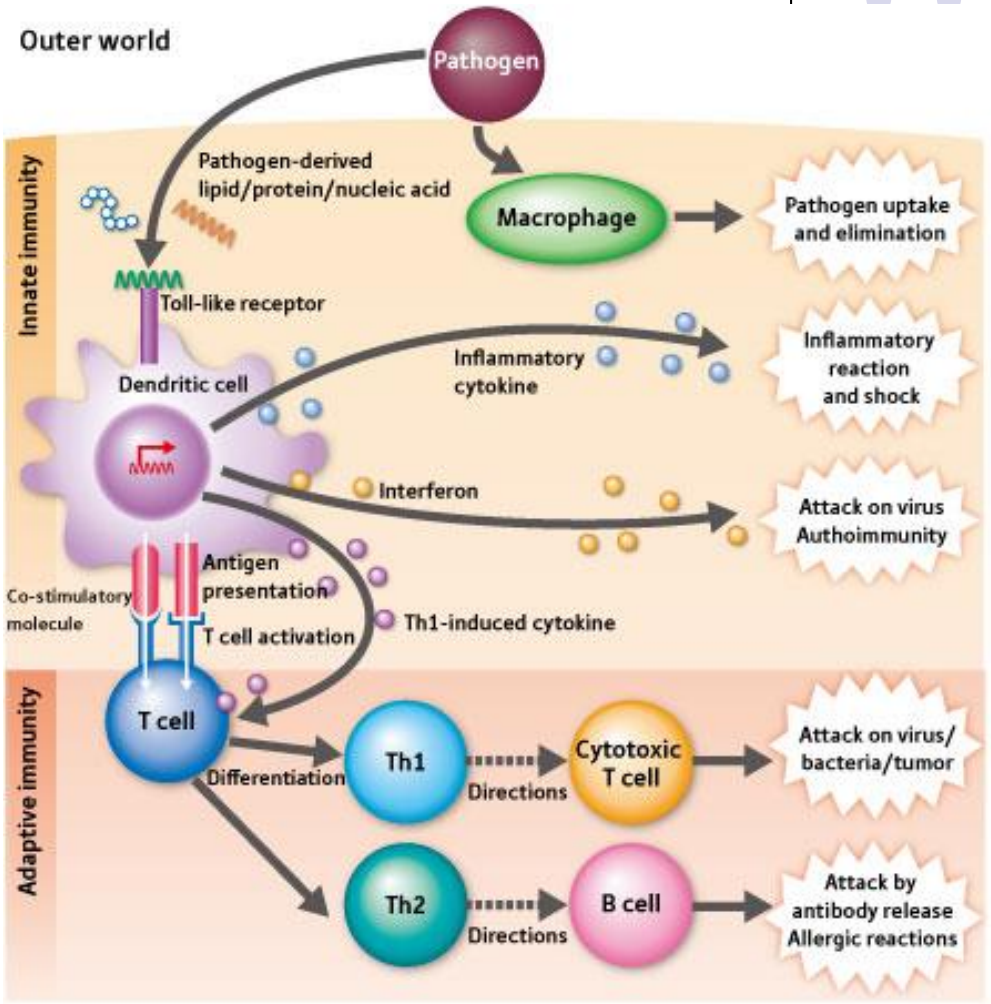


Immune System (IS)

- Defence against virus, bacteria, parasites, toxins, foreign antigens, etc.

- Innate immunity
- Adaptive immunity

- Discrimination between self and not-self
- Auto-regulation to limit potentially dangerous inflammatory responses



First interaction of NMs with IS: uptake and internalisation

- Endocytosis/phagocytosis occurs primarily in specialised cells of the IS, also called professional phagocytes:
  - **macrophages**
  - **monocytes**
  - **dendritic cell**
  - **neutrophils**

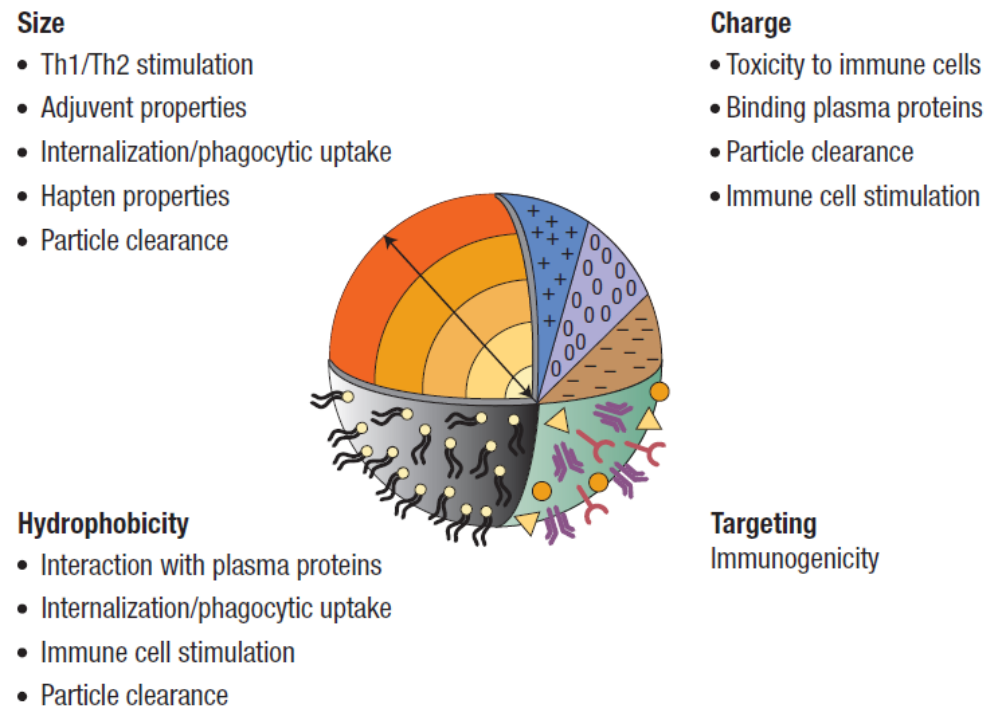
  } **fate of the NMs**

- Biodegradation or long-term persistence
- Relevance of the exposure/administration route (barriers)
- NM structure and properties

NM properties determine their interaction with IS: the effect of NM size, charge, hydrophobicity and targeting on immunotoxicity

- Widely heterogeneous molecular entities with highly variable physico-chemical characteristics

- Marked toxicological as well as immunotoxicological heterogeneity to be expected



(Dobrovolskaia and McNeil, Nature Nanotechnology, 2007)

Nanotechnology engineering can modify NMs to either avoid or specifically target the IS

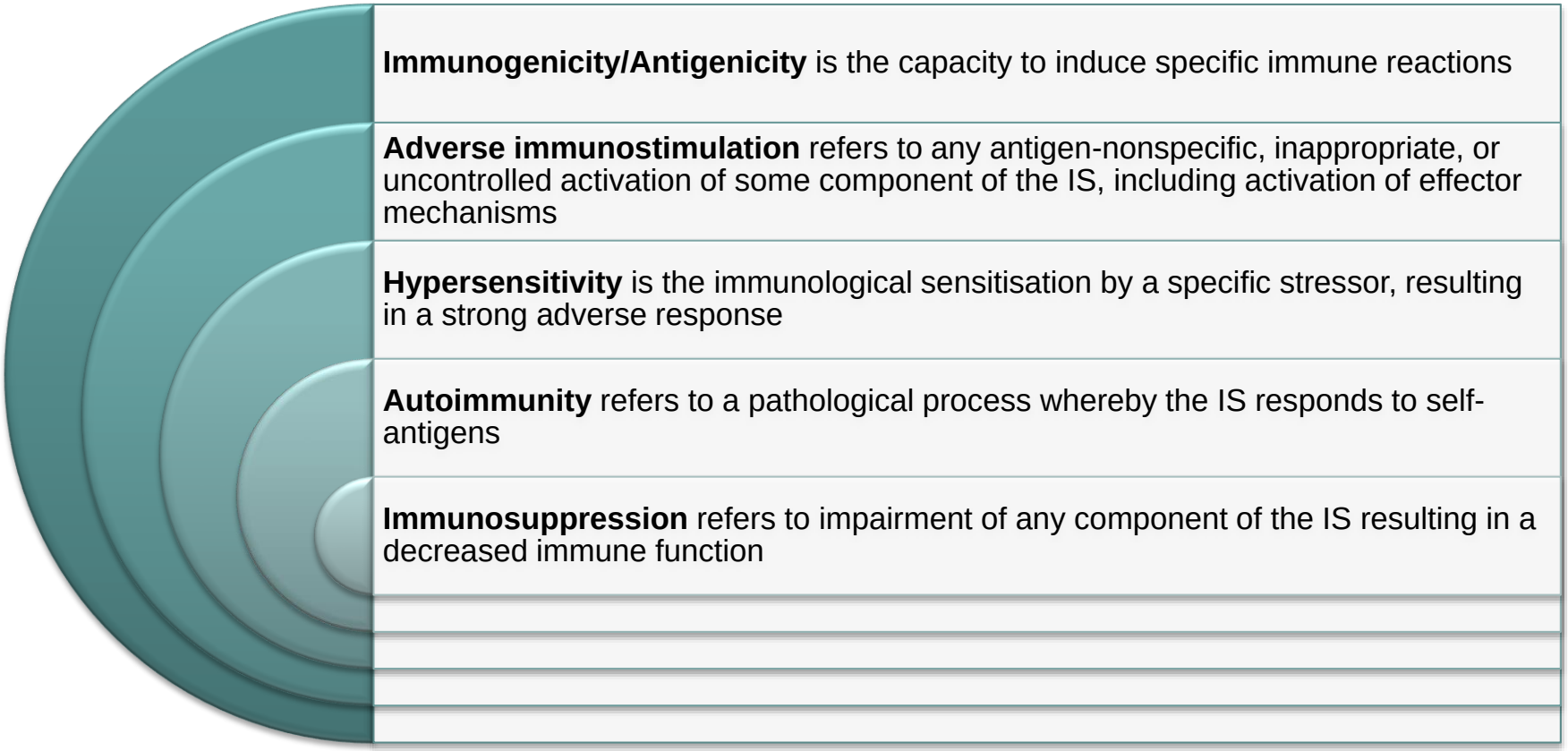
- **Avoiding IS**

  is desiderable for
  - industrial and environmental applications
  - medical applications not intended to stimulate or inhibit the IS (diagnosis)

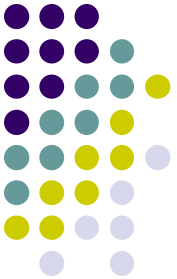
- **Interacting with IS**

  is preferred for
  - vaccination, drug delivery
  - anti-inflammatory, anticancer, and antiviral therapies
  - reducing immunotoxicity of traditional drugs

Immunotoxicity: any adverse effect on the IS that can result from exposure

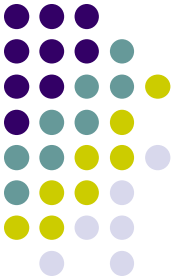


Nanoimmunotoxicity: state of the art



- Multiple endpoints and parameters must be considered
- Immunological effects must be discriminated from immunotoxicity
- Insufficient knowledge of the mechanisms involved to account for widely differing effects
- Lack of NM reference standards for immunotoxicity studies and of validated standard operative procedures (SOPs)
- No specific guidelines (only general guidance by EMA, ICH, WHO, OECD is available)

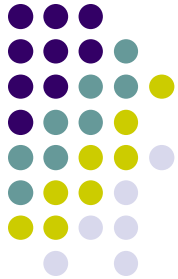
Need to develop specific approaches for immunotoxicity/immunosafety evaluation of NMs



In vitro studies

In vivo models

In vitro assays for immunotoxicity studies



Critical points

- Definition of measurable and predictive endpoints
- Choose of the immunological target (innate or adaptive responses)
- Selection of representative functional assays (feasibility, reproducibility)
- Use of reliable systems (*ex vivo* human or animal cells/tissues, cell lines)
- Set up of assay conditions (dose selection/metrics, exposure, time points)
- Characterisation of the NMs at different steps of the assays

Challenges in *in vitro* assay validation/standardisation: *interference, contaminants, artefacts*

- Optical properties of NM (interference with assay readout)
- Catalytic properties/biochemical activities of NM (effects on assay reagents)
- Immunoreactive contaminants (bacterial endotoxin)
- Toxic surfactants or synthesis byproducts within NM formulations
- Interaction with medium components (proteins, polysaccharides); changes in aggregation/agglomeration state of NM

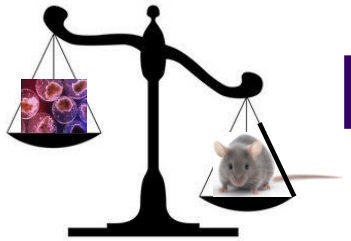
In vivo mouse models

Pro

- Wide availability of specific reagents
- Access to knock-out mice, or to over-espressing transgenic mice
- Genetic uniformity (inbred strains): standardisation of assays for regulatory purposes
- Knowledge of mouse genome
- Possibility of direct intervention (active immunisation; sampling of organs, tissues and cells)
- Easy and economic breeding and housing

Con

- Differences in IS components (TLR) and in IS plasticity
- Effect s of immune status (strain/genetic background) on the impact of NMs (uptake, clearance, blood persistence)
- Problems in the extrapolation of kinetic data (doses, time)
- Ethical aspects on animal testing



Need for *in vivo* models

- Adsorption, distribution, metabolism and excretion (ADME)
- Localisation and compartimentalisation in organs and tissues
- Complex mechanisms that cannot be extrapolated *in vitro*:
  - Immunogenicity/Antigenicity
  - Immunomodulatory potential on cell/antibody responses to antigens or pathogens

Take home message

The interaction NMs – IS may result in a complex panel of immunomodulatory effects

To explore this multifaceted picture, the combination of *in vitro* and *in vivo* models is recommended

NANoREG

Project funded by the EU Framework 7 Programme, contract no 310584

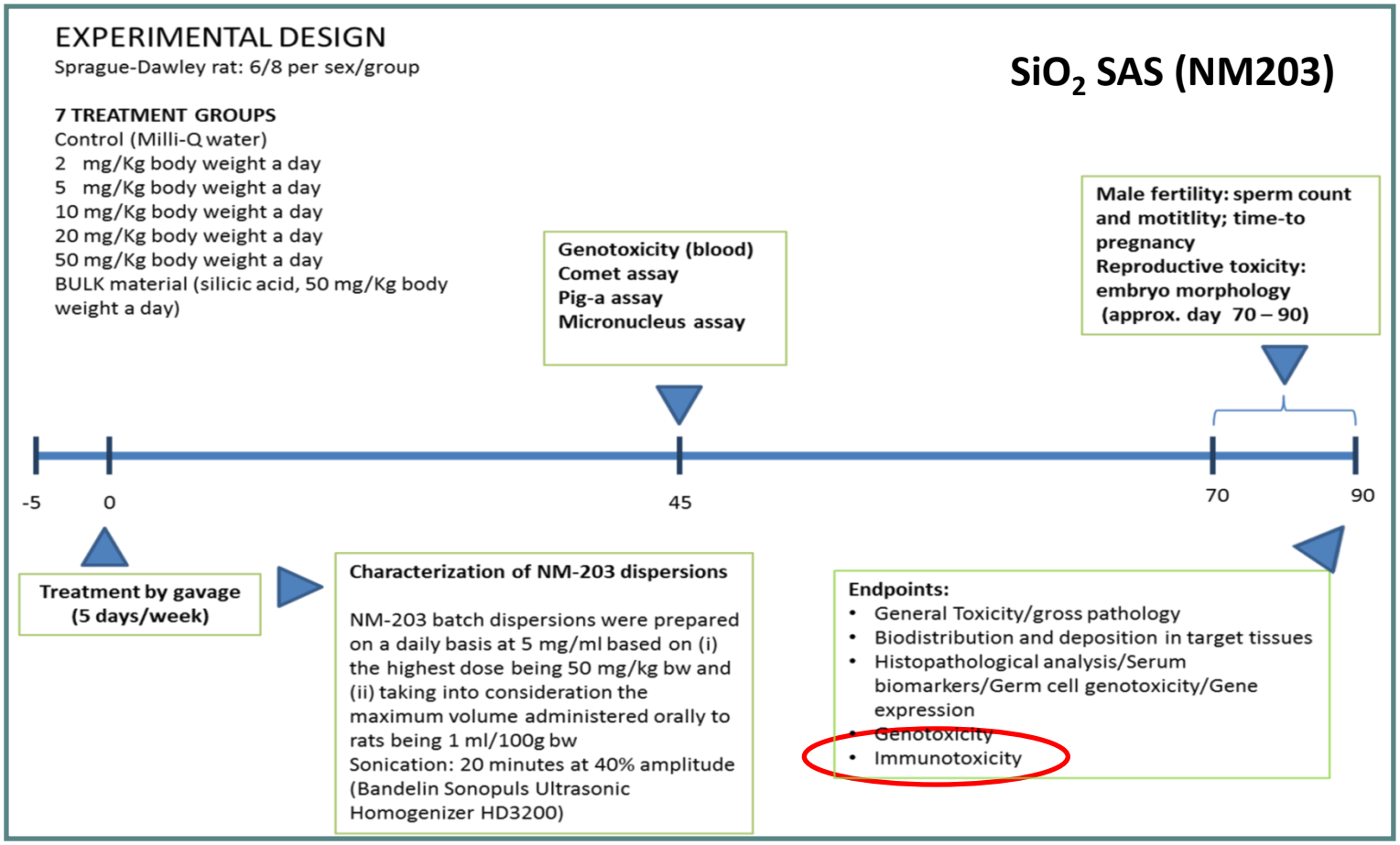
- "***NANoREG, A common European approach to the regulatory testing of nanomaterials***" (nanoreg.eu)
- *"The innovative and economic potential of Manufactured Nano Materials (MNMs) is threatened by a limited understanding of the related EHS (Environmental Health and Safety) issues. While toxicity data is continuously becoming available, the relevance to regulators is often unclear or unproven. The shrinking time to market of new MNM drives the need for urgent action by regulators. NANoREG is the first FP7 project to deliver the answers needed by regulators and legislators on EHS by linking them to a scientific evaluation of data and test methods."*

- **Istituto Superiore di Sanità (Partner 17)**
  - ***Dept. AMPP, Dept. MIPI, Dept. SPVSA, CSC***
  - *Immunotoxicity has been addressed in two Workpackages, with in vitro and ex vivo studies on immunotoxic and inflammatory properties of NM*

Immunotoxicity studies in NANoREG project

WP4 – *In vivo* studies (1)

In vivo 90-day oral toxicity study (M/F rats) OECD TG 408



In collaboration with Dr. F. Maranghi and coworkers, Dept. SPVSA, ISS

Immunotoxicity studies in NANoREG project

WP4 – *In vivo* studies (2)

Immunotox endpoints

- Organ weight (spleen, MLN)
- PHA-induced lymphocyte proliferation (spleen, MLN)
- Lymphoid population analysis by FACS (spleen, MLN)
- LPS-induced NO and cytokine production (peritoneal resident macrophages)
- Blood count
- Serum antibodies (isotypes) and inflammatory cytokines (leptin, TNF-alpha, IL-6)

Immunotoxicity studies in NANoREG project

WP4 – *In vivo* studies (3)

Results

- Reduced proliferative response to mitogen (MLN/males)
- Enhanced inflammatory response by macrophages (NO and IL-6 in males)
- Reduced numbers of circulating white blood cells, in particular lymphocytes and granulocytes (more evident in males)
- Limited effects on the levels of circulating antibodies (increased IgG levels in females)
- Undetectable (TNF-alpha) or low levels (IL-6) of serum inflammatory cytokines

Conclusions

- Gender differencies
- Difficulties in individuating a critical dose (effective in different parameters) and a dose-response relationship in the majority of immunological endpoints analysed

Hypothesis

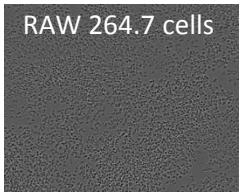
- Hepatic damage?
- Effect of sex hormones?

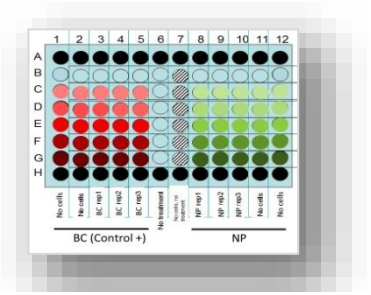
Immunotoxicity studies in NANoREG project

WP5 – *In vitro* studies (1)

In vitro immunotoxicity study (RAW 264.7 cell line)

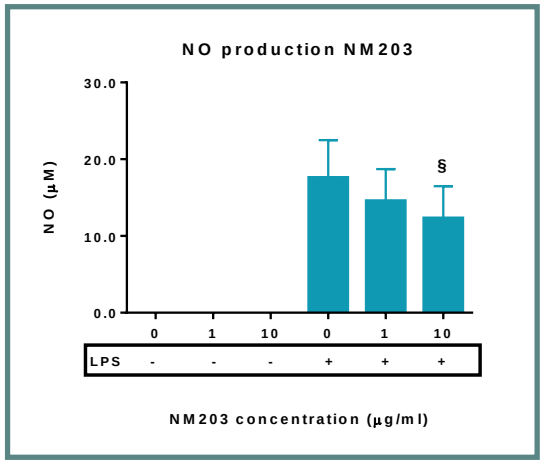
- **Mouse macrophage cell line RAW 264.7** (ECACC 91062702) derived from blood monocyte/macrophage cells, grown in semi-adherence for 4 days after thawing in DMEM complete medium
- Evaluation of NM toxicity to select **subtoxic concentrations** (MTS assay protocol from *NanoValid* project) for functional experiments
- Preparation of NM batch dispersion (protocol from *Nanogenotox* project); **DLS characterization** of batch dispersion and of the dilutions in culture medium at the beginning and the end of experiment
- **NMs:** **SiO_2** (NM200 and NM203); **TiO_2** (NM100 and NM101)
- **Immunotoxicity endpoints:**
  - **Apoptosis/necrosis** (24h)
  - **NO production** (w or w/o LPS as a positive control) (48h)
  - **Pro-inflammatory cytokine secretion** (IL-6 and TNFalpha) (w or w/o LPS as a positive control) (24h)



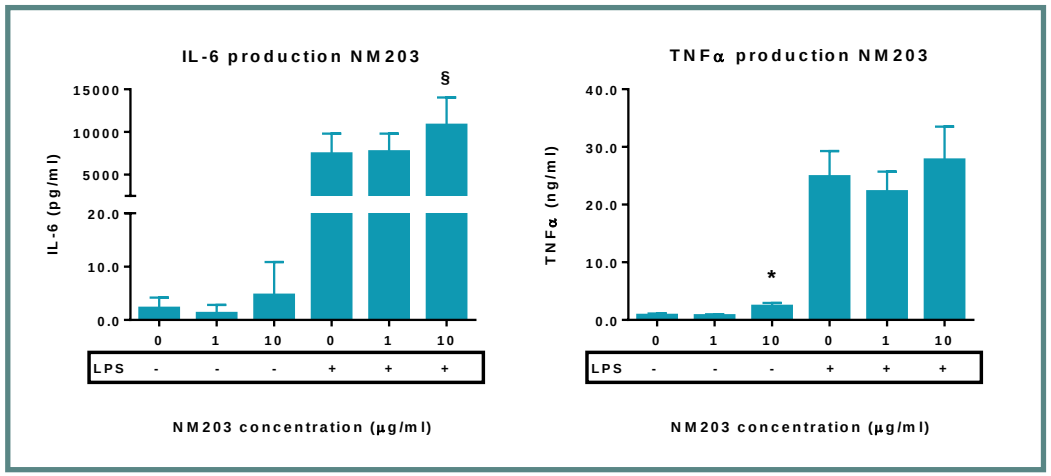


Immunotoxicity studies in NANoREG project

WP5 – *In vitro* studies (2)



No production of NO; significant reduction of NO production induced by LPS at higher dose



Significant production of TNFalpha at higher dose, without modulation of the LPS-induced production; significant increase of LPS-induced IL-6 production at higher concentration

Take home message (2)

The interaction NMs – IS may result in a complex panel of immunomodulatory effects

To explore this multifaceted picture, the combination of *in vitro* and *in vivo* models is recommended

In vivo studies are able to yield much more refined information on the global effects of chronic exposure to NMs

In vitro approaches allow simpler and reproducible evaluation of multiple parameters