Nano-immunosafety: issues in assay validation

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Abstract. Assessing the safety of engineered nanomaterials for human health must include a thorough evaluation of their effects on the immune system, which is responsible for defending the integrity of our body from damage and disease. An array of robust and representative assays should be set up and validated, which could be predictive of the effects of nanomaterials on immune responses. In a trans-European collaborative work, in vitro assays have been developed to this end. In vitro tests have been preferred for their suitability to standardisation and easier applicability. Adapting classical assays to testing the immunotoxicological effects of nanoparticulate materials has raised a series of issues that needed to be appropriately addressed in order to ensure reliability of results. Besides the exquisitely immunological problem of selecting representative endpoints predictive of the risk of developing disease, assay results turned out to be significantly biased by artefactual interference of the nanomaterials or contaminating agents with the assay protocol. Having addressed such problems, a series of robust and representative assays have been developed that describe the effects of engineered nanoparticles on professional and non-professional human defence cells. Two of such assays are described here, one based on primary human monocytes and the other employing human lung epithelial cells transfected with a reporter gene.

1. The importance of assessing immunosafety of engineered nanomaterials
Assessment of nanosafety, with particular regard to nanomedicines and health-related nanomaterials, should consider the possible effects on the immune system with great care. The immune system is responsible for defending the integrity of our body and maintaining its health. Possible alterations of the normal functions of the immune system, as a consequence of interaction with nanoparticles, must be avoided as they can cause severe pathological derangements. It is expected that nanotechnologies applied in medicine (nanodrugs, nano-imaging tools, nano-delivery systems, nanodevices) will greatly improve the quality of the diagnostic and therapeutic intervention in the near future, \textit{e.g.}, allowing us to target with great precision secluded organs or tissues (brain, metastatic tumours, etc.). In order to
speed up the development of such nanotools, the safety of the nanomaterials should be evaluated at every phase of development (from synthesis of the raw materials to the final formulation). To this end, it will be necessary to design and implement robust and representative methods able to predict the immune-related risk of developing diseases. Available standardised immunological assays are not suited in most cases for detecting nanoparticle effects, and should therefore be custom-adapted or re-designed to this end.

A major issue in immunosafety assay design is the need for standardisation and ease of applicability of the validated assays. In this context, \textit{in vitro} assays are greatly preferred because of their high reproducibility, in addition to avoiding the use of large numbers of experimental animals. To increase relevance, the possibility of employing \textit{in vitro} models of human primary cells, rather than animal tissues or transformed/tumour cell lines, is of great importance.

2. Set up and validation of nano-immunosafety \textit{in vitro} assays

A series of issues will have to be considered in order to set up appropriate immunosafety assays for testing nanoparticle effects.

The first is the biological target. What we really need to know is not only if nanoparticles are toxic for immune cells or if they can activate inflammation or an allergic reaction. This is indeed important, but it is an acute effect that can be easily identified with standard assays [1]. What is much more difficult to identify is the capacity of nanoparticles to interfere with immune responses, \textit{i.e.}, to affect the capacity of our immune system to react appropriately to dangers and protect our body’s integrity. For example, will our immune cells be able to fight and destroy meningococci nesting in our throat, or will they be too weak, and thus allow the bugs to become virulent, invade our body and cause meningitis? If our immune cells are loaded with nanoparticles, even if perfectly alive, will they be able to efficiently fight the danger, \textit{i.e.}, doing the right thing at the right moment in the right place?

Another major issue is the heterogeneity of the immunological status of the human population. It is well known that babies and elderly people have major alterations of the immune response (immature \textit{vs.} senescent). For instance, the “normal” immune response in an 80-year old person is very different from that of a healthy adult, as the senescence of the entire body has driven a different “basal” reactivity in the immune system, to cope with the increased need of scavenging dying/anomalous cells without causing unwanted inflammation [2]. On the other hand, babies have an immature immune system, which make them almost completely unresponsive to certain types of stimuli (\textit{e.g.}, polysaccharides). Pregnant women are a third group, besides babies and aged people, with a differently regulated “normal” immune responsiveness. Pregnant women have enhanced resistance to infections (to protect the development of the fetus) and at the same time experience a sort of immunosuppression to allow persistence and growth of a foreign tissue without rejecting it. It is evident that the impact of nanoparticles on such immune systems, which have very different regulation checkpoints, cannot be the same as in normal adults.

A final source of variability in the human immune response is disease. What is safe for healthy people may not be so for people with a disease. For instance, nanoparticles may not have effects on the immune response of a healthy adult, but they could exacerbate the hyperreactivity reaction to allergens in an asthmatic person. Conditions of frailty (\textit{e.g.}, chronic infections, chronic diseases, malnutrition, environmental conditions), which deeply affect the immune reactivity, can also influence the possible impact of nanoparticles on the immune response.

The immunosafety assays that we aim at designing for testing the impact of nanoparticles should answer to such questions, as well as to the questions of chronic and/or cumulative exposure. These issues are summarised in the Table 1.
2.1. Selection of relevant endpoints/biomarkers
How to select phenotypic, molecular or functional endpoints predicting risk of developing disease is a major issue in immunosafety at large, not only for nano-immunosafety. Correlates of disease/risk must take into account the above considerations on the heterogeneity of the immune responses in different population groups, which would set different thresholds for discriminating between normal and (pre)-pathological levels of certain markers. An example is for instance the circulating levels of the inflammatory cytokine IL-18, one of the accepted early markers of renal failure [3]. IL-18 is present at measurable levels in the serum of normal individuals, and is significantly increased in renal disease, but also in several other inflammatory and autoimmune diseases, often preceding disease development [4]. Indeed, increased IL-18 may be considered as a general prognostic marker of inflammation and inflammatory disease. However, it should be noted that the circulating levels of IL-18 are different in different age groups, increasing significantly at older ages even in healthy individuals [5]. Thus, the threshold value discriminating between normality and risk of developing an inflammatory disease is not a fixed value, but depends on the age of the subjects, as well as on their general health status.

2.2. Selection of relevant cellular systems
The immune system encompasses several cell types and a multifaceted array of reactions, from early innate/inflammatory defensive reactions (e.g., production of reactive oxygen species, release of inflammatory cytokines) to late, more sophisticated and specific adaptive responses (e.g., antibody production, generation of memory). In vitro models, as we aim at developing, should be as simple as possible and able to provide surrogate markers of risk. We propose here that early defence responses, which are very reproducibly triggered by danger signals even in human subjects (not only in genetically homogeneous inbred mice), be taken as relevant endpoints for nano-immunosafety evaluation. Consequent of this, cells that are of central importance in for detecting

### Table 1. Issues in setting up nano-immunosafety assays.

| Issue                                | Description                                                                 |
|--------------------------------------|-----------------------------------------------------------------------------|
| Biological target                    | Direct toxicity and direct immunological effects (induction of inflammation, allergy, etc.). |
|                                      | Interference with the normal development of the defensive immune responses (increased susceptibility to infections, tumours, autoimmune reactions, etc.) |
| Heterogeneity of the human population| Effects on immunologically different populations (children, elderly, pregnant women, genetically diverse populations, etc.) |
| Health vs. disease                   | Effects on people undergoing infections, chronic diseases (cardiovascular, pulmonary, autoimmune, chronic inflammatory, tumours, etc.), malnutrition, and other conditions of frailty, as compared to normal healthy adults |
| Acute vs. chronic or repeated exposure| Effect of continuous or cumulative exposure to low doses; effect of co-exposure to nanoparticles and other agents, both biological (bacteria, viruses, etc.) and non-biological (dusts, chemicals, etc.) |
innate/inflammatory activation can be identified in two major groups: professional defence cells such as leukocytes (in particular circulating monocytes and tissue macrophages) that first come in contact with exogenously administered nanoparticles (such as nanodrugs), and mucosal epithelial cells (such as pulmonary and gut mucosal cells) that are the mechanical and biologically active barrier lining of our inner body. It is obvious that, depending on the selected endpoint and biomarker (e.g. adaptive immunity markers), other cell types will be more relevant and should be analysed (e.g., Th, Treg, Tc, B cells, etc.) [6].

2.3. Challenges in assay development

Development of reliable and representative in vitro assays for measuring nano-immunosafety faces a series of challenges that must be solved. In fact, they must be proven representative of the real-life situation, and devoid of artefacts that can mislead the operator and lead to false positive or false negative results.

2.3.1. Nanoparticle characterisation. Among issues that must be addressed for the correct interpretation of assay results, it is necessary to consider particle aggregation/agglomeration in the presence of culture medium and biological fluids (e.g., serum), ageing of particles upon storage or in physiological media (particle dissolution and release of reactive ions), interaction of particles with biological molecules present in physiological media (formation of a protein or lipid corona), compatibility of particle solvents with the biological systems (chemical immunotoxicity), contamination of particles with biologically active compounds (such as bacterial endotoxin), and optical interference of particles with the assay readouts. Only particle batches devoid of secondary effects (freshly synthesized, endotoxin-free, monodispersed, suspended in biocompatible solvents at optically non-interfering concentrations) were used in the in vitro immunotoxicity assays hereafter described.

2.3.2. Solvent contamination effects. A significant misleading effect that leads to false interpretation of assay results is the direct effect of chemical solvents in which nanoparticles are dispersed for maintaining them in colloidal suspension. Even with solvents that turn out to be completely non-toxic for immune cells, an effect in modulating the activities of immune cells can be present. For example (Table 2), when CaCo-2 gut epithelial cells were chronically exposed to Au nanoparticles (diameter 4 nm) and CoO nanoparticles (diameter 7 nm) there is a significant release of sICAM-1, at levels comparable to those induced by the inflammatory cytokine IL-1β (positive control). However, when the effect of the solvents in which the nanoparticles were suspended was examined, it turned out that the solvent in which Au NP were suspended (sodium citrate) was as active in inducing sICAM-1 as the Au NP themselves. On the other hand, the solvent in which CoO NP were suspended (TMAOH) was completely inactive in sICAM-1 induction. Thus, the correct interpretation of these results is that only CoO NP were able to induce sICAM-1 release, while the effect observed with Au NP was a false effect caused by the solvent. It should be noted that the modulatory effect of solvents can vary depending on the biological endpoint analysed. Indeed, taking again the example of the gut mucosal epithelial cells, while the sodium citrate solvent can induce sICAM-1 release, it was unable to inducing IL-1β or CXCL4, while TMAOH did not have effect on sICAM-1 nor IL-1β induction, but it was very active in inducing CXCL4 (Table 2). The bottom line is that solvents can be active as immunomodulators also in the absence of direct toxicity, and that such activity can be observed for certain biological endpoints, but not others. Thus, in order to evaluate the true NP-induced effects, a thorough analysis of the effects of contaminants and chemicals present in the working batches of NP must be performed on every single biological endpoint under study. In the absence of such controls, results can be misinterpreted and significant effects can be wrongly attributed to inactive nanoparticles.
Table 2. Solvent contamination can be responsible for immunomodulation.

| Treatment          | sICAM-1 | IL-1β   | CXCL4  |
|--------------------|---------|---------|--------|
| medium             | 0.00    | 0.10    | 10.39  |
| IL-1β              | 54.74*  | 20.28*  | 33.63* |
| solvent Au NP      | 46.47*  | 0.86 n.s.| 8.51 n.s. |
| solvent CoO NP     | 0.00 n.s.| 0.23 n.s.| 25.36* |
| Au NP (5.2 μg/ml)  | 58.58*  | 0.00 n.s.| 78.27* |
| CoO NP (3.5 μg/ml) | 43.58*  | 0.00 n.s.| 9.70 n.s. |

*a Differentiated human CaCo-2 gut mucosal epithelial cells were exposed for 15 days to culture medium (RPMI-1640 with 5% human AB serum) containing 9.1% of colloidal NP preparations, 9.1% of solvents without NP, or 10 ng/ml IL-1β as positive control.

*b Soluble factors in the supernatants of 15-day cultures were assessed by Proteome Profiler Antibody Array (R&D Systems), measured densitometrically, and expressed in Arbitrary Units.

*c Au NP were suspended in Na citrate 0.25 mM, oxidised NaBH₄ 0.3 mM, pH 8. Both NP and solvent were non-toxic.

d CoO NP were suspended in TMAOH 1 mM. Both NP and solvent were non-toxic.

*p<0.05 vs. medium control; n.s. not significant vs. medium control.

2.3.3. Endotoxin contamination. The issue of endotoxin contamination of nanoparticles is a major source of artefacts and misinterpretation of assay results. Bacterial endotoxin is an ubiquitous contaminant of chemicals and glassware which cannot be eliminated upon sterilisation. Elimination of endotoxin can be obtained by incineration (depyrogenation), a procedure that can be applied only to few materials without risk of changing their physico-chemical characteristics. Endotoxin-free synthesis, i.e. by carrying out the full procedure with endotoxin-free glassware, materials and reagents, is the only reliable way of obtaining endotoxin-free nanoparticles [7]. Assessing the presence of trace amounts of endotoxin in every batch of engineered nanoparticles is an absolute requirement before testing them for immunotoxicity and immunomodulatory effects. Indeed, several immune cells are particularly sensitive to endotoxin, and either die in its presence or become strongly activated. This is for instance the case for human monocytes, which can react to minute amounts of bacterial endotoxin by up-regulating the expression of inflammatory genes (such as that of IL-1β). Thus, full monocyte activation may be achieved with nanoparticles containing traces of contaminating endotoxin, masking any possible effect/lack of effect due to the nanoparticles themselves [8]. The validated endotoxin detection assays used for the microbiological quality control of pharmaceuticals and medical devices are based on the Limulus Amoeocyte Lysate (LAL) and have replaced the previous in vivo test based on fever induction in the rabbit. The original LAL assay was based on endotoxin-induced gelification of the lysate; while the modern LAL assays measure the release of a dye from a substrate upon endotoxin-induced cleavage. Gelification and dye release are quantitatively correlated to the amount of endotoxin present. Assessing the presence of endotoxin in nanoparticle preparations must take into careful account the interference that optically active particles may have with the densitometric or colorimetric assay readout, as well as the possibility that nanoparticles may either directly induce or inhibit the enzymatic activity, thus mimicking or masking the presence of endotoxin [9].

3. Assay of innate/inflammatory reactivity of human primary monocytes

The innate immune system is an ancient mechanism of defence, which is quite reproducible and stable in its reactivity. In the attempt of setting up a fully in vitro assay for testing nano-immunosafety, we have chosen human primary blood monocytes, freshly isolated from the blood of adult healthy donors, and challenged them in culture with prototypical inflammatory stimuli (e.g., bacterial endotoxin) for
different times, to represent early vs. late inflammation. The assay was used to evaluate the ability of nanoparticles to interfere with the normal development of the innate/inflammatory reaction, by measuring inflammatory gene expression and cytokine production. It should be noted that, by intentionally stimulating monocytes with endotoxin, the possible contamination of nanoparticles with endotoxin would become biologically irrelevant. The experiment reported in Figure 1 was carried out with strictly endotoxin-free AgO nanoparticles, but in parallel trials the presence of a moderate contamination did not affect the results.

**Figure 1.** Differential modulation of gene expression in monocytes by nanoparticles. Human monocytes were exposed for 4 and 24 h to culture medium alone or containing 50 EU/ml endotoxin. At the beginning of the incubation, cells were exposed to endotoxin-free AgO NP (diameter 10-60 nm; 4.9 μg/ml). Controls were cells exposed to culture medium alone or to solvent (no detectable difference). Gene expression was assessed by real-time PCR, using β-actin as housekeeping gene. * p<0.05 vs. corresponding treatment in the absence of NP.

While nanoparticles were unable to elicit any significant reaction in quiescent monocytes (measured as expression of two inflammation-related genes, those for the cytokine IL-18 and for its receptor IL-18Rα), in endotoxin-activated cells the presence of nanoparticles could selectively alter the course of the response (no effect on endotoxin-induced IL-18, but a significant effect on receptor expression, which is upregulated at 4 h and downregulated at 24 h as compared to endotoxin alone). A kinetical in vitro model of monocyte activation that reproduces all phases of the inflammatory response is currently being set up and evaluated by genome-wide transcriptomic analysis. The model proves to be very reproducible from donor to donor, and describes very precisely the evolution of the defensive response, thus it can be suitable for identifying and molecularly describing the possible interfering effects caused by nanoparticles.

**4. Assay of innate/inflammatory reaction of human lung epithelial cells**

Epithelial cells are considered as very important non-professional defence cells, not only as barrier lining and protecting our inner body, but also as active defence effectors of innate immunity/inflammation. As a consequence, anomalous activation of epithelial cells may cause
pathological derangements. For example, the interaction between bronchial epithelial cells and dendritic cells is strongly involved in the development of allergic asthma [10]. Given that bronchial epithelial cells are potentially exposed to inhaled nanomaterials, it is highly relevant, in the context of nano-immunosafety testing, including this cell type in the development of in vitro safety assays. The use of primary human bronchial epithelial cells is hindered by the difficulty of obtaining and isolating these cells. Therefore, the human lung epithelial cell line, A549, was used instead, after validation of the selected endpoints by comparison with primary cells. A series of reporter cell lines, based on the A549 cell line, proved useful in detecting the effects of nanoparticles in a rapid and sensitive fashion. These reporter cells react by producing a light signal (following luciferase production) upon stimuli that activate the promoters of the inflammatory cytokines TNF-α, IL-6 or IL-8, or are able to activate the inflammatory gene activator NF-κB. The effects of nanoparticles are different, depending on whether cells are quiescent or activated by inflammatory stimuli as it occurs during a defence response [11]. These reporter cell lines represent a reliable and standardised first-line screening method to determine the potential immunomodulatory capacity of novel nanomaterials. When effects are observed, further experiments have to be performed to determine the exact pathways and the effects on primary bronchial epithelial cells.

5. Conclusions and discussion
Immunosafety of engineered nanoparticles, with special reference to nanomedicines, is a relevant health issue that needs to be addressed by the Health Authorities through ad hoc guidelines and with appropriate investigation tools [12].

Given the high plasticity of the immune response, which can recognize minimal changes in foreign structures/molecules, the immunosafety of nanomedicines should be tested at each of the steps of pharmaceutical development, including the final formulation, since the safety of the single components of the formulation is no guarantee for the safety of their mixture.

The key issue in implementing nano-immunosafety, and in ensuring the efficient development of health-related nanotechnological products, is the availability of robust and reliable immunosafety assays. The commonly used immunotoxicity assays are ill-suited to testing nanomaterials, since the chemical-physical characteristics of the nanoparticles require in most cases to re-design the assay. Indeed, the particulate nature of the nanomaterial can cause a series of misleading artefacts resulting in false negative or false positive results. Thus, for each selected endpoint a robust assay should be selected/designed, with a readout that is not influenced by the particles. In any case, the concomitant use of two assays with different readouts for the same endpoint can usually solve the problem.

The design of in vitro immunosafety assays for nanoparticles requires a series of considerations. It is evident that in vitro assays may not fully replace the in vivo tests in animals. However, also by considering the global efforts in replacing animal testing with in vitro assays, the advantages of in vitro assays should be seriously considered. These include the clear focus (e.g., inflammation), which should not be considered as a limitation but as the possibility of a deeper evaluation of the effect and of its mechanism of action. Then, in vitro assays can be developed that are fully representative of the actual human exposure, by using normal human cells in culture rather than animals or animal cells, and continuous transformed cell lines. Eventually, in vitro assays can be standardised and simplified, and become available for screening in a much simpler and more rapid way than in vivo tests will ever allow.

The use of primary cells, as described here for the innate reactivity of human blood monocytes, may suffer of the problem of donor-to-donor variability, which is one of the reasons why transformed cell lines are often preferred, together with their better accessibility and availability. It is however important that primary human cells are used beforehand, to establish relevant endpoints and markers. In the case of human monocytes, the donor-to-donor variability is minimal, and accessibility relatively easy, which allows us to detect their reactivity to challenges in an exact fashion even when using cells from different donors. However, in many cases the use of human primary cells is unfeasible or time-consuming and with very low or variable yield (e.g., brain microglia, tissue macrophages, epithelial
cells). In such cases, but also with easier and abundant primary cells and blood leukocytes, the use of transformed cells may be of great help for designing simple, reproducible and standardised assays, once the endpoints selected have been validated as representative of the reactivity of normal primary cells.

The use of reporter cell lines, as those described here, is a very important possibility of developing rapid, easy to use assays suitable for large screenings. The innate/inflammatory reactivity of human non-professional defence cells (alveolar epithelial cells) is assessed by using a transformed cell line (the lung carcinoma line A549), which undergoes spontaneous differentiation in culture and reacts to stimuli in a fashion overlapping with that of primary lung epithelial cells, when considering the production of a series of inflammatory factors. The cell line is transformed with a luminescent reporter gene under the control of the promoter of one of these factors, to yield a reporter cell line representing the inflammatory reactivity of the normal alveolar epithelium.

The development of robust in vitro assays, custom-designed for assessing nanoparticle effects, will greatly help in the sustainable knowledge-based development of nanotechnologies applied to medicine. The same assays that are being developed now for assessing immunosafety could also provide valuable information on the features of the interaction between nanoparticles and human immune cells, helping us to design a future generation of nano-immunomodulatory drugs. In this view, it is of special interest the possibility of using nanoparticles for the direct targeting of immune cells for therapeutic/preventive strategies (delivery of drugs, delivery of vaccine antigens and adjuvants). Modifying the immune response in localised settings, a very difficult task nowadays, could also be addressed with the use of nanoparticles in medical fields such as oncology, autoimmunity, chronic inflammatory diseases, and neurodegeneration.

Acknowledgements
DB was partially supported by the Italian Ministry of University and Research (MIUR) within the PRIN 2007 project prot. 2007Y84HTJ on systems biology of human macrophages.

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