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Nanoparticle size influences the proliferative responses of lymphocyte subpopulations

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11 August 2015

Dear Editors,

We hereby submit our manuscript entitled "Nanoparticle size influences the proliferative responses of lymphocyte subpopulations" to be considered for publication as a communication in the RSC Advances. The manuscript presents new findings on how the immune response of dendritic cells, an important class of antigen presenting cells, is affected by particle size. 12 nm gold particles protected by the antioxidant peptide, glutathione, induced a cell-mediated response accompanied by the induction of inflammatory natural killer cells. We show that smaller gold particles (~2 nm), often referred to as nanoclusters, had very distinct effects to the 12 nm particles with a much higher cell uptake (a 67-fold increase) but without inducing significant DC maturation or lymphocyte proliferation. This study stresses how size variations in particles within a very small nanometric window (1-15 nm) can be tuned to give dramatically different effects on immune cells. This could be used to promote cytotoxic and inflammatory responses (12 nm particles) or as a potential antigen carrier (~2 nm particles).
We believe that this study is of great interest for the RSC Advances audience where the use of engineered nanoparticles for immunomodulation is considered a promising strategy for the treatment of cancer and infectious diseases.

The content of this manuscript has not been published previously and is not under consideration for publication by another journal. All figures should be published in colour.

If you have any questions, please do not hesitate to contact me:
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Thank you very much for your attention, I look forward to receiving your decision.

Yours faithfully,

Dr. Xavier Le Guevel

Reply to the reviewers

Referee: 1

Comments:
The manuscript wanted to understand the influence of gold nanoparticles' size on immune response. The idea is very interesting and important for immune-stimulation and immuno-therapy. However, the manuscript showed a very chaotic logical relationship, because relationship amongst various CDs, INF-□, and various ILs are very intricate. Therefore, the logical relationship of the manuscript should be reorganized, and the some details also should be adjusted. There are some comments should be considered and answered by authors as follow.

1. Based on the Figure S1, the TEM showed the obvious nanoparticles rather than nanoclusters with diameter less than 5 nm. Why did authors consider the 2 nm nano colloidal golds as nanoclusters rather than nanoparticles?

We used gold nanoclusters to refer to gold nanoparticles with sizes lower than 3 nm, as determined by TEM and dynamic light scattering methods. The TEM data confirmed the absence of particles with sizes above 3 nm using statistically-significant numbers of particles in different TEM images, however artefacts due to NC aggregation under the electron beam of the microscope mean we cannot use this technique to determine the exact size of the NCs. Previous work (X. Le Guevel et al, high photostability and enhanced fluorescence of gold nanoclusters by silver doping, Nanoscale (2012) 4, 7624) using PAGE electrophoresis and mass spectrometry confirmed a size around 10 kDa (i.e. smaller than 5 nm) for such fluorescent species.
The nanocluster (NC) terminology has been used by us and many other groups when describing the synthesis of such clusters (notably with glutathione as stabilising agents: X. Le Guevel et al., Nanoresearch (2012) 5, 319; J. Zhang et al, Scientific Reports (2015) 5, 8669) and published in many material science and multidisciplinary journals such as ACS Nano, Scientific Reports, Angewandte Chemie, Small, Biomaterials, Nanoscale, etc…
Thus, we think it is appropriate to name such particles as nanoclusters for the audience of RSC Advances. However, if the reviewer considers it preferable to alter the nomenclature and describe both species as nanoparticles, we would be happy to make this change.

2. Based on Figure 1(b), the cell uptake of NC was significant higher than that of NP. However, the following results on immune response of lymphocyte subpopulations, such as CD 80&86 expressions of DCs, showed inverse trends, why?

We completely agree with the referee that these opposing trends between strong NC uptake but weak effects on DC maturation/cytokine production are both surprising and very interesting. It suggests that the two types of particles are interacting with DCs in fundamentally different ways. In fact it has been previously observed that particle uptake, DC maturation and cytokine

production are not always linked (K. Fytianos et al. Uptake efficiency of surface modified gold nanoparticles does not correlate with functional changes and cytokine secretion in human dendritic cells in vitro. Nanomedicine. (2015) 11:633) and (E. Seydoux et al. Size-dependent accumulation of particles in lysosomes modulates dendritic cell function through impaired antigen degradation. Int J Nanomedicine. (2014) 9: 3885). The molecular basis for the different interactions with DCs by NCs/NPs is unknown but one possibility is that it is related to differences in the endocytic pathways involved in uptaking the two classes of particle. We have now highlighted these opposing trends and their possible significance in the modified manuscript.

3. Because 2 nm colloidal gold nanoparticles have been utilized widely in penetration of nuclear pore complex. It is apparently that 2 nm NCs show a certain potential on distribution in cell nucleus and influencing immune response of lymphocyte.

Indeed, some studies have shown intranuclear accumulation of those ultrasmall particles (Huo et al., Ultrasmall Gold Nanoparticles as Carriers for Nucleus-Based Gene Therapy Due to Size-Dependent Nuclear EntryACS Nano (2014) 8, 5852), but this behaviour is strongly dependent of the cell type, cell experiment and more importantly on the nature of the ligand protecting the nanoclusters (or ultra-small nanoparticles). We have worked extensively on gold nanoclusters protected by various ligands such as polyethyleneglycol, carbohydrate, zwitterion, glutathione) (Biomaterials, (2015) 43, 1; Nano Research (2012) 5: 379; Nanoscale (2015) 43, 1; ACS Applied & Interfaces (2015) 10.1021/acsami.5b06541) and we never detected the accumulation of those particles in the nucleus using repeated electron and optical microscopy experiments. In addition, several cytotoxicity experiments have indicated that NCs do not induce cell death or morphology changes at any NC concentration used in this work. However, the movement of some NCs across the intranuclear barrier cannot be completely excluded and could be difficult to visualise if such species do not accumulate in the nucleus.
From our experience of glutathione-stabilised gold nanoclusters and previously published works from other groups, we think it's highly improbable that very low levels of nuclear-localised NCs (if any are present) have a significant influence on the immune response of DCs or lymphocyte proliferation.

4. The most serious problem is the chaotic logical relationship through the manuscript. Although authors characterized the expression of different immune response proteins, it is hard to determine the influence depending on NPs and NCs completely, because different immune response proteins can generate cross influence. For example, expression of INF-□, it can be secreted by DC and other types of lymphocyte, then up-regulate the expression of IL-12和IL-10. And IL-12 can down-regulate the expression of IL-4和IL-13. Meanwhile, the immune responses of NK cells are always associated with IL-12 expression. Generally, immune-competence of CD56dim NK cells are better than that of CD56bright NK cells, however, IL-12 can enhance immune-competence of CD56bright NK cells beyond that of former. Based on

the complexity of different immune response proteins, authors must arrange their data logically to demonstrate their idea.

We agree with the reviewer's comment that the order the results were presented was a little bit confusing. Thus, we have modified their organisation to explain both the results and discussion more clearly.
We now describe the experiments from DC maturation onwards sequentially according to the order of the in vivo immunological process.
1) The cytotoxic effects of NCs/NPs on DCs.
2) Differences in DC uptake of NCs and NPs.
3) Effect of NCs/NPs on DC maturational status and cytokine secretions.
4) Subsequent immunological effect on the proliferation of different lymphocyte subpopulation including T (CD4 and CD8) and NK cells ($CD56^{bright}$ NK cells and $CD56^{dim}$ NK cells) and their cytokine secretions following incubation with DCs pre-treated with NCs/NPs.
We have also improved the introduction of each section to improve readability and make it easier to understand the relevance of the results to specific immunological processes.

Referee: 2

Comments:
Even the interaction of AuNPs with DCs has been studied (PLoS One. 2014; 9(5): e96584.), this manuscript focused studying the inactions of DCs with the naked GSH-modified Au NPs with two different sizes and the subsequent activation phenotyping of the lymphocytes. Applying nanotechnologies to immunotherapies is certainly of great importance.

1- My major concern is that the authors merely studied the naked GSH-modified AuNP. As mentioned in the 2nd paragraph of the introduction, DCs are for the uptake and process of antigens. Therefore, the purpose of the NPs is to deliver the antigens, peptides, proteins or glycoconjugates. If GSH-modified AuNPs are covalently linked with various antigens, how much that will change the interactions of AuNPs and DCs and the phenotype and activation of lymphocytes. I certainly understand that authors probably would submit the manuscript to other journals with this data, but I sincerely believe that the antigen-loaded AuNPs would dramatically uplift the importance of this work.

As the referee mentioned, other studies like Tomic et al (cited in the article) have reported the effect of NP size on the immune response of dendritic cells with particle sizes around 10-15 nm inducing stronger inflammation than bigger nanoparticles. Compared to most of the previous work done on NP-Dendritic Cell interactions, we have demonstrated how a change in particle size in a narrow window (2-12 nm) can drastically affect interactions between the particle and the cell in terms of uptake, DC maturation and lymphocyte proliferation. This is the critical aspect as the understanding of how the physico-chemical parameters of NPs influence the immune response is still far from being completely understood especially for ultra-small particles (<3 nm), which behave very differently from their larger counterparts due to their high surface property.

Although adding antigens onto NC/NP surfaces may be a logical step for other researchers (as we mention in our discussion), it is not something we are currently planning to look at ourselves. Our focus is specifically on the how the size of these very small particles affects their interaction with immune cells. The addition of large proteins or antigens with very different sizes to the NCs, would lead to a vastly altered nanosystem that would induce a completely different set of interactions with immune cells, and require a new set of experiments on address it. We feel this aspect is beyond the scope of the current paper.
Nevertheless we feel the data reported in this study will be of value to other researchers and contribute to the development of future applications toward using ultra-small particles for enhanced-cell uptake, and small particles to modulate NK cell responses. Therefore, we consider our findings to be highly relevant for the audience of RSC Advances, a multidisciplinary journal.

2- Some abbreviation used in the figures are very confusing. What's "IM" in the y axis of Figure 2a? Are they the percentages of CD80+ or CD86+ in the DCs? Why not just showing the percentage of CD80+CD86+ DCs? What's "PI" as the y axis in some figures? Percentage?
IM is a typo we changed for MI that stand for Maturation Index as described in the Dendritic cell Maturation section of the ESI. This index was calculated as the ratio between the percentage of stimulated and non-stimulated cells expressing different markers. We consider it necessary to use this index to make comparisons in order to avoid intra-individual variations on immature dendritic cell status. In this way we can better compare the effect of different nanostructures using cells from several patients.
This abbreviation has been now modified throughout the text and figures.

Similarly in the proliferative studies used a proliferation index (PI), calculated as follows:

$$\frac{\left[\%CD3^{+}CFSE^{dim}\ stimulated\ (lymphocytes + DCs)\right] - \left[\%CD3^{+}CFSE^{dim}\ unstimulated\ (lymphocytes + DCs)\right]}{\%CD3^{+}CFSE^{dim}\ unstimulated\ (lymphocytes)}$$

This is described in Lymphocyte proliferation section of the ESI.

3- On page 3, line 3-5, it says that "Our results indicate nonspecific proliferative responses by Treg and Th17 populations after stimulation with the different particles (Figure S7)." However, the error bars are so big that I don't really see much proliferation.
The results in Figure S7 show the percentage of Th17 and Treg in the CD4 proliferating cells. We used a statistical analysis based on a non-parametric test for related samples (Wilcoxon test) in addition to a Bonferroni correction. This allows us to make comparisons between different conditions from the same subjects and thus minimise inter-individual differences (represented as error bars) in the expression of the different markers at basal conditions.
Although we found an increased percentage of Th17 and Treg in the presence of NPs, NCs or free ligand compared to non-stimulated cells

(Lymph-iDC), statistical comparison in three distinct experiments indicated no statistically significant differences ($p<0.025$) as illustrated by the very high error bars.

We now mention the statistical analysis used in the figure legend of Figure S7.

4- On page 2, line 13, “CD8o” should be “CD80”
This change has been made as suggested by the reviewer.

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Nanoparticle size influences the proliferative responses of lymphocyte subpopulations

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12 NM GOLD NANOPARTICLES INDUCE CELL MEDIATED RESPONSES ACCOMPANIED BY INFLAMMATORY NATURAL KILLER CELL STIMULATION, WHEREAS 2 NM GOLD NANOPARTICLES ARE MORE EFFICIENTLY UPTAKEN WITHOUT INDUCING DENDRITIC CELL MATURATION OR LYMPHOCYTE PROLIFERATION.

The use of engineered nanoparticles for immunotherapy has been extensively investigated, notably towards the development of novel vaccines[1-5]. By tailoring their physicochemical properties such as size, shape, surface chemistry, nanoparticles are able to positively or negatively modulate immune responses via antigen presenting cells (APCs) [2-4, 6, 7]. This strategy promises the development of novel nanovaccines that are able to modify adaptive immune responses for the treatment of cancer or auto-immune diseases[6, 8-10]. For example, increased nanoparticle surface hydrophobicity has been shown to progressively induce acute inflammation in both *in vitro* and *in vivo* models[7]. Nanoparticle shape can also affect immune responses, for example rod and star shapes elicit enhanced immune responses, although their clearance may have toxic effects[11, 12]. Another parameter that has been studied is the influence of spherical particle sizes from 40 nm to one micron with larger nanoparticles favouring the induction of cell-mediated responses and a higher cell uptake for particles smaller than 500 nm[13, 14].

In this study we chose gold particles with two different sizes at the lower end of the nanosize window: 2 nm so-called nanoclusters (NCs) and 12 nm nanoparticles (NPs) both protected by the same glutathione (GSH) tripeptide ligand. GSH is an antioxidant which offers the advantage of improving the colloidal stability of particles in complete medium, while its free carboxyl groups allow molecules to be conjugated onto the surface [18]. Dendritic cells (DCs), an important type of APC, modulate both effector and tolerance responses by presenting MHC Class II-bound antigens to T lymphocytes while secreting co-stimulatory factors that depend on their maturational state[19-21]. Our aim was to determine if size (<15 nm) affects particle uptake by dendritic cells, and whether it alters their effects on the immunological system as judged by changes to DC maturation and the inflammatory responses of different lymphocyte subpopulations. DC maturation was evaluated by cell surface markers and cytokine secretion, while inflammatory responses was followed by looking at the proliferation of different lymphocyte subpopulations including CD4+ and CD8+ T and Natural Killer (NK) cells, as well as cytokine production.

Particles synthesis was conducted in aqueous solution using gold salt and GSH, with sodium borohydride also used for the larger particles (see ESI for details). Particle size was determined by dynamic light scattering (Figure 1a) and electron microscopy (Figure S1) indicating high monodispersity for NCs (∅ ~2 nm) and NPs (∅ ~12 nm). We used thermal analysis to estimate ligand content per particle (based on the ligand's organic moiety) finding around 56% and 19% for NCs and NPs, respectively. No significant aggregation of NCs or NPs was observed after incubation in complete medium (RPMI1640 + 10% serum) for 48 hours. Absorbance analysis revealed that NPs possess the typical plasmon band at 520 nm. In contrast, NCs exhibited strong UV absorption (Figure S2) and intense photoluminescence in the orange-red window (λ_{max}= 610 nm)[22] (Figure S3), which originate from the strong quantum confinement seen in species of this size and metal-ligand interactions [23-26].

NC and NP cytotoxicity was evaluated in human monocyte-derived dendritic cells (DCs) via specific Live/Dead cell staining and flow cytometry. Incubation for 48 hours with concentrations up to 25μg.mL^{-1} of NCs, NPs or equivalent concentrations of free GSH ligand had no significant effect on cytotoxicity compared to non-treated

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cells (Figure S4). Cellular particle uptake was quantified by inductively coupled plasma high-resolution mass spectrometry (ICP-HRMS) after incubating DCs with NCs or NPs (10 μg gold/ mL) for 48 hours (Figure 1b). We found that NC uptake in terms of gold particles per cell was 67 times higher than that of NPs, indicating highly efficient internalisation of NCs into DCs. This observation is in agreement with previous studies showing stronger uptake of small particles, most likely related to their high diffusion capacity[27, 28]. The internalisation of NCs and NPs in the cytoplasm and close to the membrane could be readily observed by the presence of dark accumulations under bright-field illumination, and by photoluminescent emissions upon two-photon excitation ($\lambda_{exc.}$= 720nm) (Figure S5).

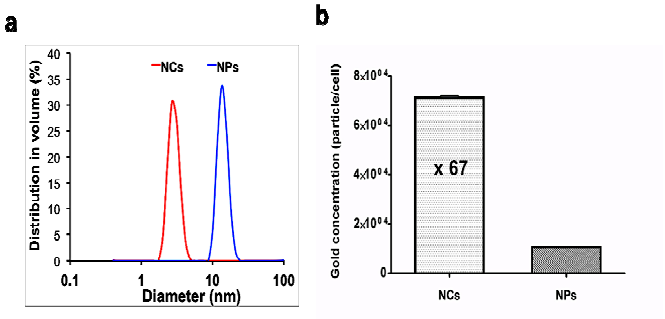

Figure 1. (a) Size analysis by dynamic light scattering of GSH ligand-stabilised NCs and NPs in aqueous solution. (b) Inductively coupled plasma high-resolution mass spectrometry (ICP-HRMS) measurements of DCs incubated with NCs or NPs (10μg/mL in complete medium) for 48 hours.

One of the first response steps in the immune response to an antigen by a dendritic cell is its maturation. Mature DCs are characterised by the loss of their ability to internalise antigens, while upregulating MHC Class II molecules and co-stimulatory factors such as CD80 and CD86, thereby acquiring the capacity to present antigens to naïve T-cells and inducing different immunological responses.[29-31]. Thus, to understand how particle size might affect DC-mediated immune responses, we incubated immature DCs with NCs and NPs (1 to 25 μg/mL of gold in complete medium) for 48 hours. The maturational status of DCs was then evaluated using flow cytometry-based detection of CD80 and CD86 surface antigens. Positive (lipopolysaccharide; LPS) and negative (free GSH ligand in eq. ligand concentration) controls were also included. Results depicted in Figure 2a show that incubation with NPs significantly increased the expression of both CD80 and CD86 in a dose dependant manner, suggesting the induction of DC maturation. In contrast, no DC maturation i.e. no increase in expression above the MI>2 threshold, was seen for cells cultured in the presence of NCs or with free ligand alone. We were surprised to observe no significant increase in DC maturation following incubation with NCs, especially as they were more strongly uptaken than NPs. Recent studies have shown that particle uptake does not always correlate with functional changes in human dendritic cells in vitro[32, 33]. Dissimilarities between the two classes of particle might be related to differences in the endocytic pathways involved in their uptake. Further studies will be required to understand the molecular basis for the different interactions between DCs and NCs/NPs.

DC maturation tends to induce high levels of MHC molecules and the secretion of sets of cytokines that play a key role in activating naïve T cells and the launch of primary immune responses[8]. To further characterise DC maturation following incubation with NPs or NCs, we measured the pattern of cytokine release into the culture supernatant in each case. Our analysis of cytokine production (IL12, IFNγ, IL4, IL13, IL10, IL17), depicted in Figures 2b and S6, detected significant increases in IL12, IFNγ and IL10 in response to NPs, in a dose dependent manner. Elevated expression of IL12 and IFNγ suggest that NPs induce an inflammatory DC response[20] while no significant cytokine production was detected using NCs or free ligand consistent with the absence of DC maturation following these treatments.

Our finding that particle size affects DC maturation suggests that it may also affect subsequent steps in the immunological response, where mature DCs interact with lymphocyte sub-populations. To examine this possibility we examined how DCs incubated with NCs or NPs can subsequently induce changes in lymphocyte proliferation and activity in terms of cytokine production. Different T lymphocyte and NK cell subpopulations have been reported to shape immunological responses in different ways. T helper cells can be divided into Th1, Th2, Th17 and Treg subpopulations depending on their cytokine production[34]. Th1 cells have been implicated in responses to infectious and inflammatory disorders[35]. Th2 cells are essential for the induction of allergy and asthma[36]. Th17 cells are related to neutrophil activation and implicated in several autoimmune diseases[34, 36]. Treg cells are involved in the regulation of inflammatory effector cells. Moreover, two NK cell subpopulations have been described: i) CD56dim cells (the main NK population in peripheral blood) expressing high levels of CD16 and perforin, which mediate cytotoxic activity, and ii) CD56bright cells usually associated with the inflammatory NK population[37] that produces high levels of IFNγ[38].

In this work, we carried out co-culture experiments where T and Natural Killer (NK) cell populations were incubated with NC or NP-pre-treated DCs to determine their antigen presenting activity by measuring proliferation and cytokine release. DCs were pre-incubated with NCs, NPs or free ligand at different concentrations (1 to 25 μg gold/mL in complete medium or equivalent ligand concentrations), washed and then co-cultured over 6 days. No significant cytotoxicity was observed regardless of the particle concentration used (Figure S4). Lymphocyte proliferation, expressed as a proliferation index (PI), was evaluated by measuring the frequency of cells with reduced levels of a fluorescent tracer ($CFSE^{dim}$ cells) together with specific subpopulation markers. Results indicated increases in the PI of T lymphocytes ($CD3^+$ $CFSE^{dim}$), T helper ($CD4^+$ $CFSE^{dim}$) and NK ($CD56^+$ $CFSE^{dim}$) cells following incubation with high NP concentrations (Figure 3a). No proliferative response increases (PI<2) were detected for cytotoxic T cells ($CD8^+$) incubated with NP-treated DCs, or following co-culture with DCs treated with NCs or free GSH.

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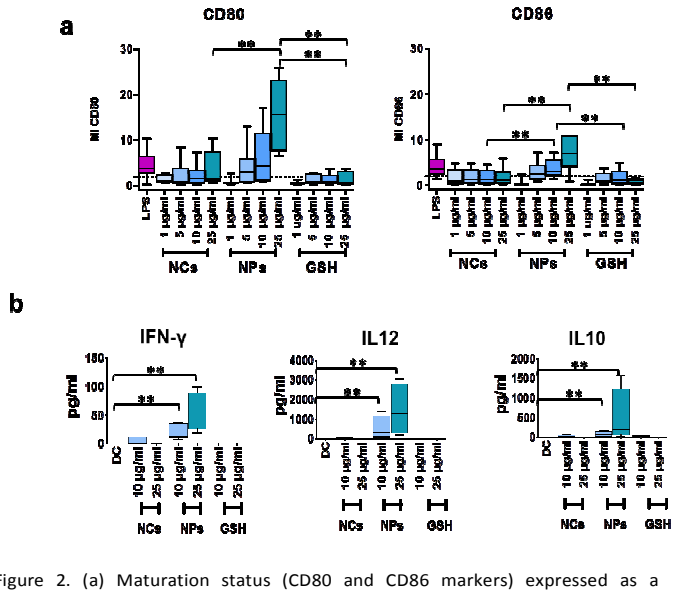

Figure 2. (a) Maturation status (CD80 and CD86 markers) expressed as a maturation index (MI), and (b) cytokine secretion* (IFN-γ, IL12, IL10) of DCs incubated with NCs, NPs, free GSH (1 to 25 μg/mL in complete medium) and LPS (Lipopolysaccharide) as positive control for 48h.* - tested at 10 and 25 μg/mL of NCs, NPs, or eq. conc. of free GSH. Statistical comparisons were performed using the Wilcoxon test for related samples. The Bonferroni correction was applied for comparison of three groups. Statistical differences were considered significant when $p<0.025$.

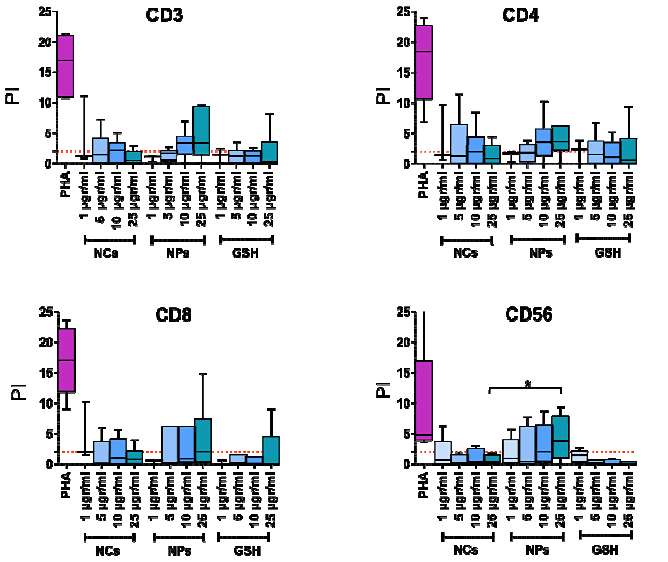

Figure 3. Proliferative responses of different lymphocyte subpopulations (CD3, CD4, CD8, and CD56 markers), expressed as a proliferation index (PI), with Peripheral Blood Mononuclear Cells (PBMCs) + DCs incubated with NCs, NPs or GSH (1 to 25 μg/mL in complete medium) for 6 days. Statistical comparisons were performed using the Wilcoxon test for related samples. The Bonferroni correction was applied for comparison of three groups. Statistical differences were considered significant when $p<0.025$.

To better understand the immunological effect of NP-stimulated DCs, we set out to identify the different subpopulations involved in T and NK cell proliferation. Our results indicate non-specific proliferative responses by Treg and Th17 populations after stimulation with the different particles since no differences in their proliferative response were found compared to GSH-stimulated cells (Figure S7).

The production of IFNγ by lymphocytes not secreting IL4 and IL13 (Figure S6) following the presentation of NP pre-treated DCs could also be associated with the induction of a proliferative CD4 sub-population corresponding to a cell-mediated response (Th1) and matches the cytokine release observed during NP-induced DC maturation.

Regarding NK cells, significantly increased proliferation of the $CD56^{bright}$ NK subpopulation combined with elevated IFNγ production was observed in response to co-culture with NP-treated DCs in a dose dependent manner (Figures 4a, b). Importantly, there was no increase in Granzyme B secretion, a cytotoxic mediator released from cytoplasmic granules in cytotoxic T and NK cells, following NP-treated DC co-culture (Figure 4c). These two observations are consistent with the presence of high levels of inflammatory NK cells ($CD56^{bright}$).

Our lymphocyte proliferative data indicate that the increase in Th1 lymphocytes and $CD56^{bright}$ NK cells is related to the production of IFNγ and IL12 combined with undetectable levels of IL4 and IL13 (Figure 2b and S6) following NP stimulation. These results suggest that NP treatment promotes innate immunity-type responses from NK cells.

The results obtained in this work are in agreement with a previous study suggesting crosstalk between innate and adaptive immune systems involving interactions between DCs and NK cells[39]. This concept is supported by the demonstration that DCs can activate NK cells, while activated NK cells can influence DC maturation or direct their cytotoxic-effects specifically towards immature DCs, which would hamper tolerant responses[40, 41]. Thus, crosstalk between DC and NK cell populations is likely to be a key factor in influencing the balance between tolerant and immunologic responses.

By employing the same surface chemistry for both particles, this study clearly demonstrates the strong influence of particle size on DC uptake, DC maturation, the proliferative response of T lymphocytes and the presence of inflammatory NK cells.

The ultra-small particles (NCs, ∅~2nm) were efficiently uptaken by DCs but did not induce maturation or lymphocyte proliferation. These observations seem to agree with studies demonstrating high passive targeting and clearance of NCs stabilised with GSH[17, 42]. The presence of functional GSH carboxyl groups on the NC surface should enable the conjugation of biomolecules such as antigens and, in the absence of carrier-associated immunogenic reactions, represents a good candidate antigen delivery system. In contrast, the slightly bigger nanoparticles (NPs, ∅~12nm) caused DC maturation and T lymphocyte proliferation associated with cell-mediated immunity-type responses and the production of inflammatory NK cells in a dose dependent manner. These results are of considerable interest as this subpopulation is a potential target for anti-tumour immunotherapy, inflammatory response limitation and the treatment of autoimmune disorders[43]. Indeed, this NK cell sub-type represents a relatively small proportion of Peripheral Blood Mononuclear Cells (PBMCs) but presents the advantage of being one of the most efficient cytokine producers[37] and is able to shape the

adaptive response. Indeed, Caliguri et al. undertook a comprehensive investigation of the role of NK sub-types, demonstrating the unique innate immunoregulatory role of the CD56^{bright} subpopulation by producing specific types of cytokines[20]. Because lowered NK activity in peripheral blood has been associated with elevated cancer risk in patients, strategies involving enhancement of NK cells for anti-tumour treatment are currently being investigated [44, 45].

As previously discussed by Drobrovolskaia et al.[46], it remains difficult to demonstrate clear relationships between the physicochemical properties of particles and their effects on immune responses due to the complex interactions between cytokines and numerous other cellular processes such as multiple cell uptake mechanisms. Different particle types have been shown to elicit Th1 responses but the contribution of inflammatory NK cells accompanied by high IFNγ levels in response to 12nm nanoparticles potentially opens new avenues for immunotherapeutic treatments of cancer or infections.

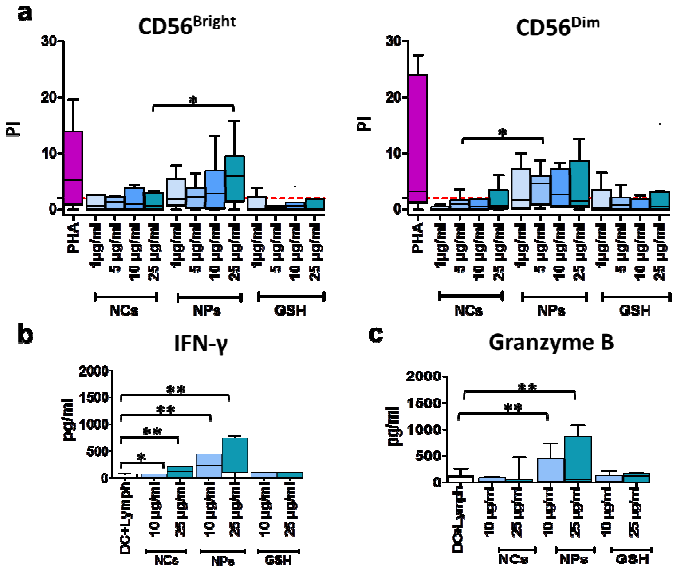

Figure 4. (a) Proliferative responses of different lymphocyte NK subpopulations (CD56^{dim} and CD56^{bright} cells); (b) Cytokine INFγ secretion levels during the proliferative lymphocyte experiments*, (c) Granzyme B assay on peripheral blood lymphocytes*. Proliferation positive control: PHA: Phytohemagglutinin.* only at 10 and 25 µg/mL of NCs, NPs, and eq. free GSH. Statistical comparisons were performed using the Wilcoxon test for related samples. The Bonferroni correction was applied for comparison of three groups. Statistical differences were considered significant when p<0.025.

Conclusions

In summary, we have shown how particle size in a narrow size range (1 to 15 nm) strongly influences cell uptake and immune responses with ultra-small size (<2 nm) leading to high cellular uptake without DC maturation and therefore lymphocyte proliferation. In contrast, bigger particles (12 nm) elicited DC maturation with a cell-mediated immunity pattern (Th1) and the proliferation of T helper lymphocytes in a concentration dependent manner. Moreover, we found that 12 nm NPs induced the proliferation of inflammatory NK cells with high levels of pro-inflammatory cytokine IFNγ secretion, suggesting that it might represent a tool for shaping and influencing adaptive immunity to fight cancer or infectious diseases. Our results underline the importance of examining different particle sizes in the 1-15 nm nanometric window in order to fine tune their effects on the immune system.

Acknowledgements

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Notes and references

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Electronic Supplementary Information (ESI) available: (synthesis and characterisation of the nanomaterial, experimental protocols, fluorescence microscopy, cytotoxicity and cytokine experiments). See DOI: 10.1039/c000000x/

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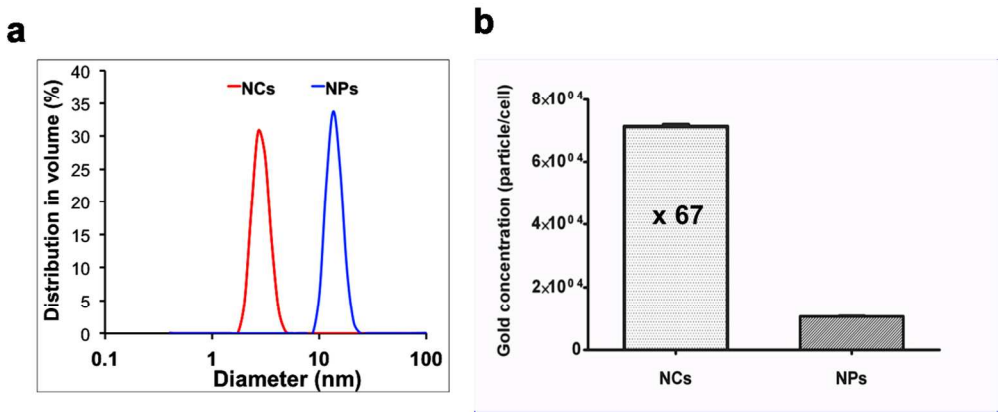
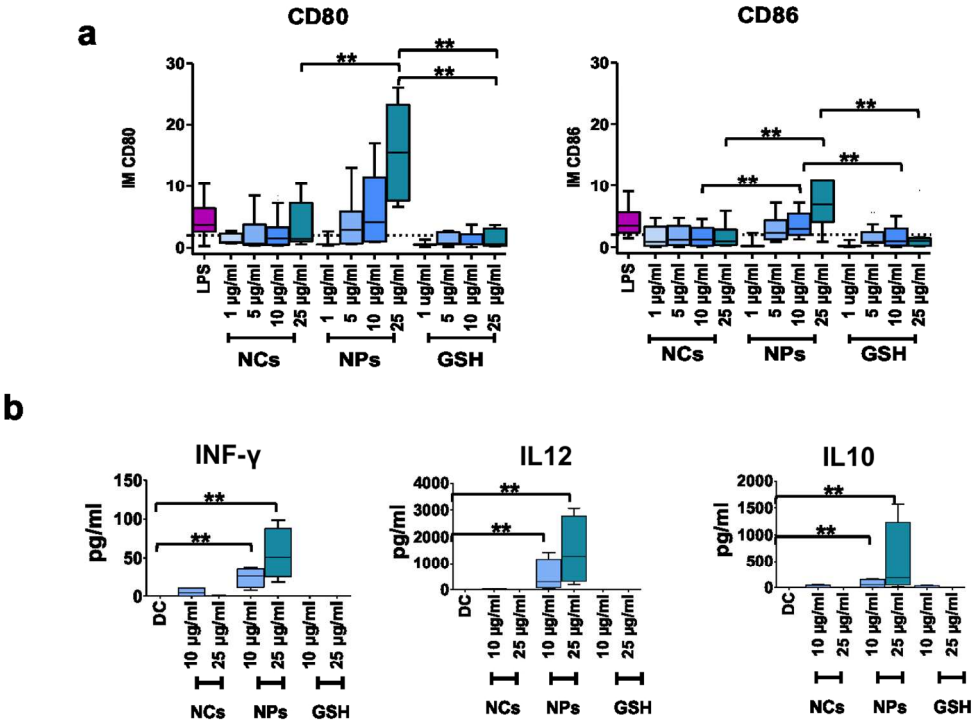
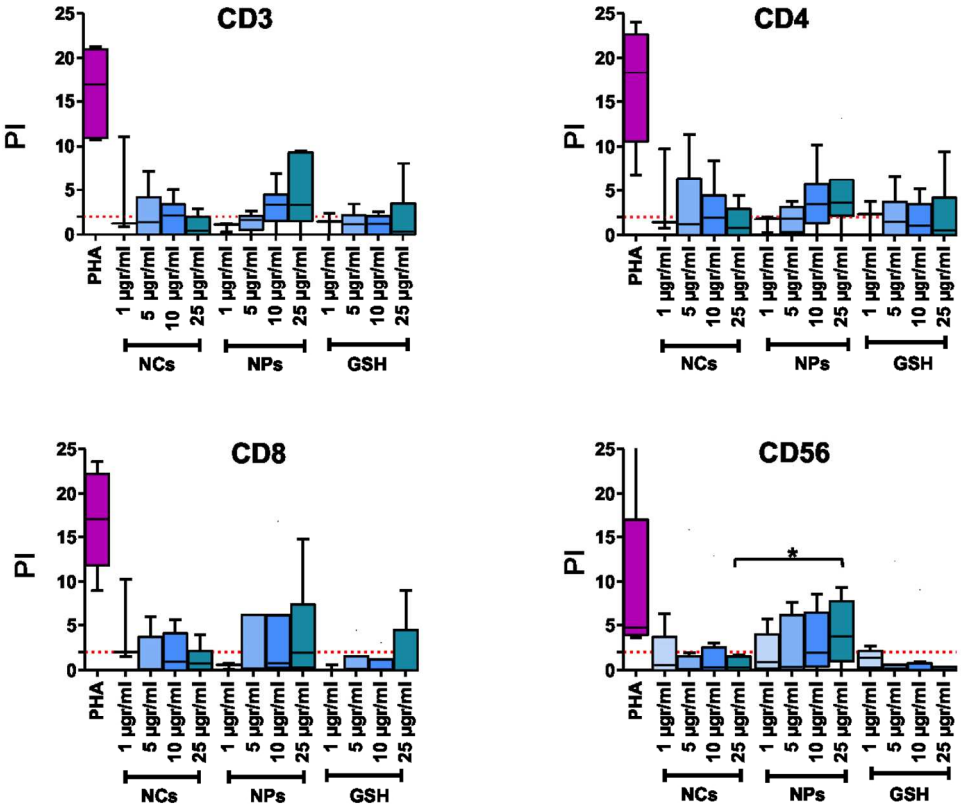


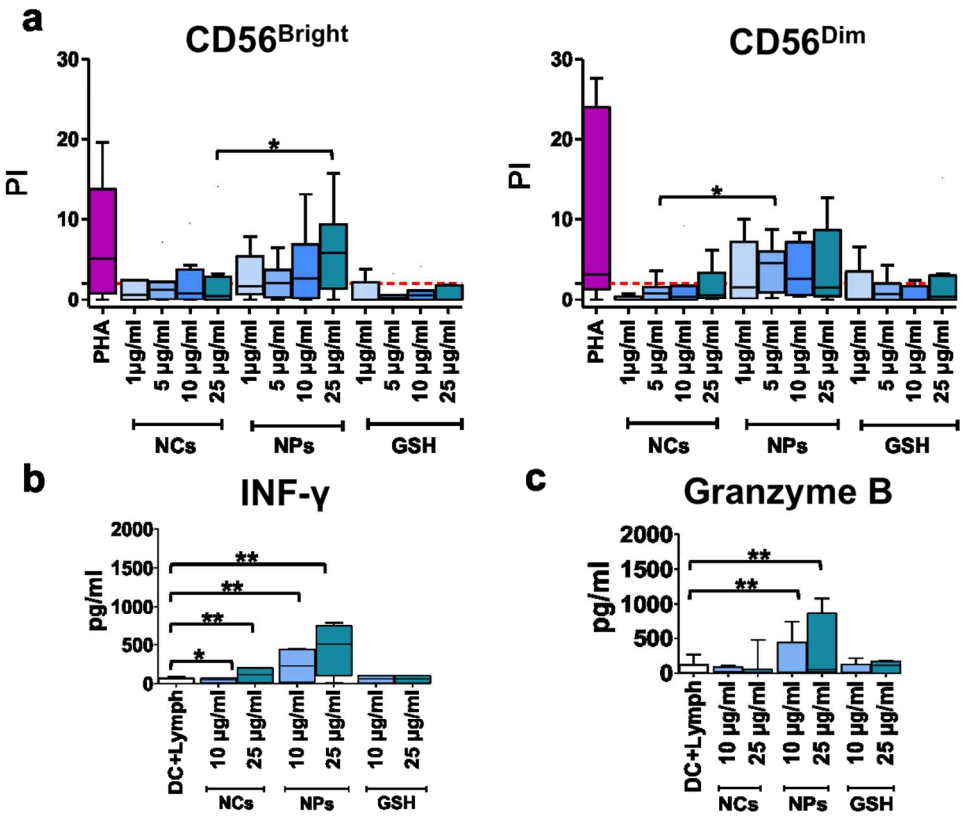
(a) Size analysis by dynamic light scattering of GSH ligand-stabilised NCs and NPs in aqueous solution. (b) Inductively coupled plasma high-resolution mass spectrometry (ICP-HRMS) measurements of DCs incubated with NCs or NPs (10μg/mL in complete medium) for 48 hours.



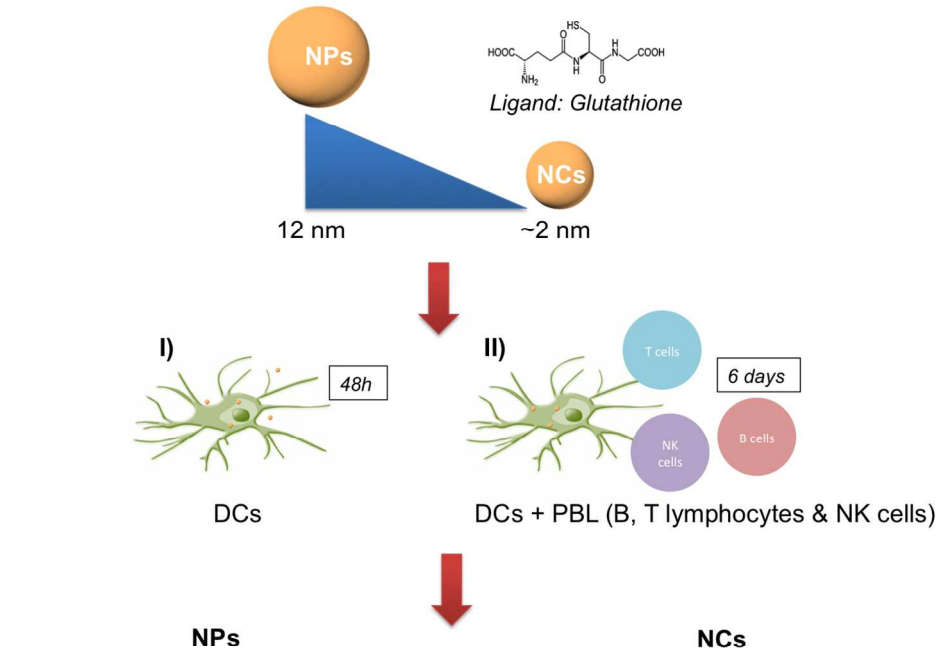
(a) Maturation status (CD80 and CD86 markers), and (b) cytokine secretion* (IFN-γ, IL12, IL10) of DCs incubated with NCs, NPs, free GSH (1 to 25 µg/mL in complete medium) and LPS (Lipopolysaccharide) as positive control for 48h.* - tested at 10 and 25 µg/mL of NCs, NPs, or eq. conc. of free GSH.



Proliferative responses of different lymphocyte subpopulations (CD3, CD4, CD8, and CD56 markers) with Peripheral Blood Mononuclear Cells (PBMCs) + DCs incubated with NCs, NPs or GSH (1 to 25 µg/mL in complete medium) for 6 days.



(a) Proliferative responses of different lymphocyte NK subpopulations (CD56dim and CD56bright cells); (b) Cytokine INFγ secretion levels during the proliferative lymphocyte experiments*, (c) Granzyme B assay on peripheral blood lymphocytes*. Proliferation positive control: PHA: Phytohemagglutinin.* only at 10 and 25 µg/mL of NCs, NPs, and eq. free GSH.



NPs

- DC maturation
- Cell mediated pattern (Th1)
- ↗ Natural Killer cells and INFγ

NCs

- No DC maturation
- No lymphocyte proliferation
- High cell uptake

ultra-small size particles (<2 nm) lead to high cellular uptake without DC maturation and therefore lymphocyte proliferation whereas 12 nm gold nanoparticles induce cell mediated responses and accompanied by inflammatory natural killer cell stimulation

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Nanoparticle size influences the proliferative responses of lymphocyte subpopulations

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12 NM GOLD NANOPARTICLES INDUCE CELL MEDIATED RESPONSES ACCOMPANIED BY INFLAMMATORY NATURAL KILLER CELL STIMULATION, WHEREAS 2 NM GOLD NANOPARTICLES ARE MORE EFFICIENTLY UPTAKEN WITHOUT INDUCING DENDRITIC CELL MATURATION OR LYMPHOCYTE PROLIFERATION.

The use of engineered nanoparticles for immunotherapy has been extensively investigated, notably towards the development of novel vaccines[1-5]. By tailoring their physicochemical properties such as size, shape, surface chemistry, nanoparticles are able to positively or negatively modulate immune responses via antigen presenting cells (APCs) [2-4, 6, 7]. This strategy promises the development of novel nanovaccines that are able to modify adaptive immune responses for the treatment of cancer or auto-immune diseases[6, 8-10]. For example, increased nanoparticle surface hydrophobicity has been shown to progressively induce acute inflammation in both *in vitro* and *in vivo* models[7]. Nanoparticle shape can also affect immune responses, for example rod and star shapes elicit enhanced immune responses, although their clearance may have toxic effects[11, 12]. Another parameter that has been studied is the influence of spherical particle sizes from 40 nm to one micron with larger nanoparticles favouring the induction of cell-mediated responses and a higher cell uptake for particles smaller than 500 nm[13, 14].

In this study we chose gold particles with two different sizes at the lower end of the nanosize window: 2 nm so-called nanoclusters (NCs) and 12 nm nanoparticles (NPs) both protected by the same glutathione (GSH) tripeptide ligand. GSH is an antioxidant which offers the advantage of improving the colloidal stability of particles in complete medium, while its free carboxyl groups allow molecules to be conjugated onto the surface [18]. Dendritic cells (DCs), an important type of APC, modulate both effector and tolerance responses by presenting MHC Class II-bound antigens to T lymphocytes while secreting co-stimulatory factors that depend on their maturational state[19-21]. Our aim was to determine if size (<15 nm) affects particle uptake by dendritic cells, and whether it alters their effects on the immunological system as judged by changes to DC maturation and the inflammatory responses of different lymphocyte subpopulations. DC maturation was evaluated by cell surface markers and cytokine secretion, while inflammatory responses was followed by looking at the proliferation of different lymphocyte subpopulations including CD4+ and CD8+ T and Natural Killer (NK) cells, as well as cytokine production.

Particles synthesis was conducted in aqueous solution using gold salt and GSH, with sodium borohydride also used for the larger particles (see ESI for details). Particle size was determined by dynamic light scattering (Figure 1a) and electron microscopy (Figure S1) indicating high monodispersity for NCs (∅ ~2 nm) and NPs (∅ ~12 nm). We used thermal analysis to estimate ligand content per particle (based on the ligand's organic moiety) finding around 56% and 19% for NCs and NPs, respectively. No significant aggregation of NCs or NPs was observed after incubation in complete medium (RPMI1640 + 10% serum) for 48 hours. Absorbance analysis revealed that NPs possess the typical plasmon band at 520 nm. In contrast, NCs exhibited strong UV absorption (Figure S2) and intense photoluminescence in the orange-red window (λ_{max}= 610 nm)[22] (Figure S3), which originate from the strong quantum confinement seen in species of this size and metal-ligand interactions [23-26].

NC and NP cytotoxicity was evaluated in human monocyte-derived dendritic cells (DCs) via specific Live/Dead cell staining and flow cytometry. Incubation for 48 hours with concentrations up to 25μg.mL^{-1} of NCs, NPs or equivalent concentrations of free GSH ligand had no significant effect on cytotoxicity compared to non-treated

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cells (Figure S4). Cellular particle uptake was quantified by inductively coupled plasma high-resolution mass spectrometry (ICP-HRMS) after incubating DCs with NCs or NPs (10 µg gold/ mL) for 48 hours (Figure 1b). We found that NC uptake in terms of gold particles per cell was 67 times higher than that of NPs, indicating highly efficient internalisation of NCs into DCs. This observation is in agreement with previous studies showing stronger uptake of small particles, most likely related to their high diffusion capacity[27, 28]. The internalisation of NCs and NPs in the cytoplasm and close to the membrane could be readily observed by the presence of dark accumulations under bright-field illumination, and by photoluminescent emissions upon two-photon excitation ($\lambda_{exc.}$= 720nm) (Figure S5).

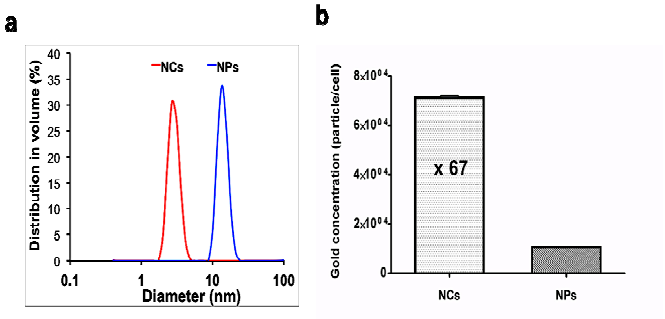

Figure 1. (a) Size analysis by dynamic light scattering of GSH ligand-stabilised NCs and NPs in aqueous solution. (b) Inductively coupled plasma high-resolution mass spectrometry (ICP-HRMS) measurements of DCs incubated with NCs or NPs (10µg/mL in complete medium) for 48 hours.

One of the first response steps in the immune response to an antigen by a dendritic cell is its maturation. Mature DCs are characterised by the loss of their ability to internalise antigens, while upregulating MHC Class II molecules and co-stimulatory factors such as CD80 and CD86, thereby acquiring the capacity to present antigens to naïve T-cells and inducing different immunological responses.[29-31]. Thus, to understand how particle size might affect DC-mediated immune responses, we incubated immature DCs with NCs and NPs (1 to 25 µg/mL of gold in complete medium) for 48 hours. The maturational status of DCs was then evaluated using flow cytometry-based detection of CD80 and CD86 surface antigens. Positive (lipopolysaccharide; LPS) and negative (free GSH ligand in eq. ligand concentration) controls were also included. Results depicted in Figure 2a show that incubation with NPs significantly increased the expression of both CD80 and CD86 in a dose dependant manner, suggesting the induction of DC maturation. In contrast, no DC maturation i.e. no increase in expression above the MI>2 threshold, was seen for cells cultured in the presence of NCs or with free ligand alone. We were surprised to observe no significant increase in DC maturation following incubation with NCs, especially as they were more strongly uptaken than NPs. Recent studies have shown that particle uptake does not always correlate with functional changes in human dendritic cells in vitro[32, 33]. Dissimilarities between the two classes of particle might be related to differences in the endocytic pathways involved in their uptake. Further studies will be required to understand the molecular basis for the different interactions between DCs and NCs/NPs.

DC maturation tends to induce high levels of MHC molecules and the secretion of sets of cytokines that play a key role in activating naïve T cells and the launch of primary immune responses[8]. To further characterise DC maturation following incubation with NPs or NCs, we measured the pattern of cytokine release into the culture supernatant in each case. Our analysis of cytokine production (IL12, IFNγ, IL4, IL13, IL10, IL17), depicted in Figures 2b and S6, detected significant increases in IL12, IFNγ and IL10 in response to NPs, in a dose dependent manner. Elevated expression of IL12 and IFNγ suggest that NPs induce an inflammatory DC response[20] while no significant cytokine production was detected using NCs or free ligand consistent with the absence of DC maturation following these treatments.

Our finding that particle size affects DC maturation suggests that it may also affect subsequent steps in the immunological response, where mature DCs interact with lymphocyte sub-populations. To examine this possibility we examined how DCs incubated with NCs or NPs can subsequently induce changes in lymphocyte proliferation and activity in terms of cytokine production. Different T lymphocyte and NK cell subpopulations have been reported to shape immunological responses in different ways. T helper cells can be divided into Th1, Th2, Th17 and Treg subpopulations depending on their cytokine production[34]. Th1 cells have been implicated in responses to infectious and inflammatory disorders[35]. Th2 cells are essential for the induction of allergy and asthma[36]. Th17 cells are related to neutrophil activation and implicated in several autoimmune diseases[34, 36]. Treg cells are involved in the regulation of inflammatory effector cells. Moreover, two NK cell subpopulations have been described: i) CD56dim cells (the main NK population in peripheral blood) expressing high levels of CD16 and perforin, which mediate cytotoxic activity, and ii) CD56bright cells usually associated with the inflammatory NK population[37] that produces high levels of IFNγ[38].

In this work, we carried out co-culture experiments where T and Natural Killer (NK) cell populations were incubated with NC or NP-pre-treated DCs to determine their antigen presenting activity by measuring proliferation and cytokine release. DCs were pre-incubated with NCs, NPs or free ligand at different concentrations (1 to 25 µg gold/mL in complete medium or equivalent ligand concentrations), washed and then co-cultured over 6 days. No significant cytotoxicity was observed regardless of the particle concentration used (Figure S4). Lymphocyte proliferation, expressed as a proliferation index (PI), was evaluated by measuring the frequency of cells with reduced levels of a fluorescent tracer ($CFSE^{dim}$ cells) together with specific subpopulation markers. Results indicated increases in the PI of T lymphocytes ($CD3^+$ $CFSE^{dim}$), T helper ($CD4^+$ $CFSE^{dim}$) and NK ($CD56^+$ $CFSE^{dim}$) cells following incubation with high NP concentrations (Figure 3a). No proliferative response increases (PI<2) were detected for cytotoxic T cells ($CD8^+$) incubated with NP-treated DCs, or following co-culture with DCs treated with NCs or free GSH.

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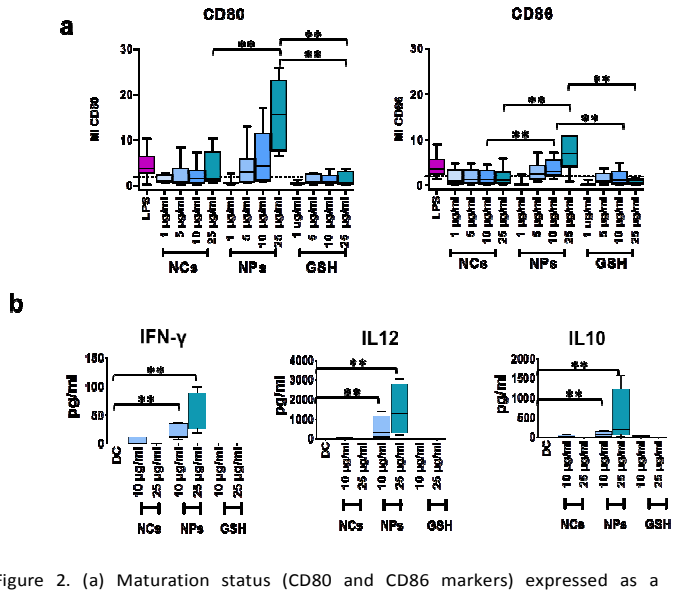

Figure 2. (a) Maturation status (CD80 and CD86 markers) expressed as a maturation index (MI), and (b) cytokine secretion* (IFN-γ, IL12, IL10) of DCs incubated with NCs, NPs, free GSH (1 to 25 µg/mL in complete medium) and LPS (Lipopolysaccharide) as positive control for 48h.* - tested at 10 and 25 µg/mL of NCs, NPs, or eq. conc. of free GSH. Statistical comparisons were performed using the Wilcoxon test for related samples. The Bonferroni correction was applied for comparison of three groups. Statistical differences were considered significant when p<0.025.

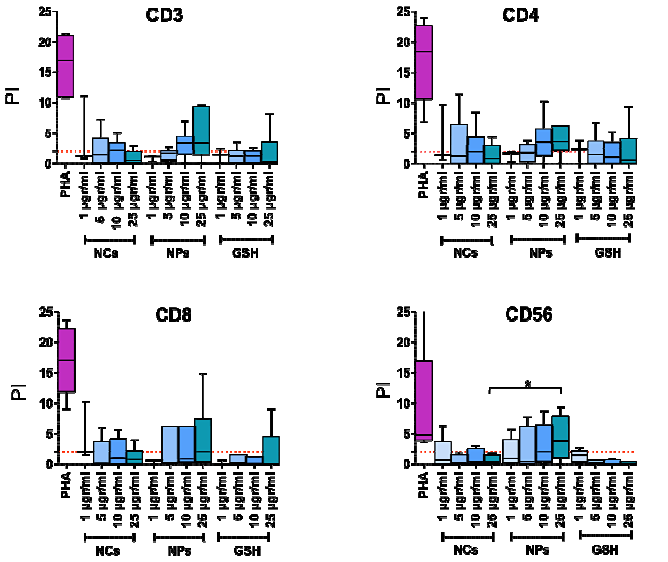

Figure 3. Proliferative responses of different lymphocyte subpopulations (CD3, CD4, CD8, and CD56 markers), expressed as a proliferation index (PI), with Peripheral Blood Mononuclear Cells (PBMCs) + DCs incubated with NCs, NPs or GSH (1 to 25 µg/mL in complete medium) for 6 days. Statistical comparisons were performed using the Wilcoxon test for related samples. The Bonferroni correction was applied for comparison of three groups. Statistical differences were considered significant when p<0.025.

To better understand the immunological effect of NP-stimulated DCs, we set out to identify the different subpopulations involved in T and NK cell proliferation. Our results indicate non-specific proliferative responses by Treg and Th17 populations after stimulation with the different particles since no differences in their proliferative response were found compared to GSH-stimulated cells (Figure S7).

The production of IFNγ by lymphocytes not secreting IL4 and IL13 (Figure S6) following the presentation of NP pre-treated DCs could also be associated with the induction of a proliferative CD4 sub-population corresponding to a cell-mediated response (Th1) and matches the cytokine release observed during NP-induced DC maturation.

Regarding NK cells, significantly increased proliferation of the $CD56^{bright}$ NK subpopulation combined with elevated IFNγ production was observed in response to co-culture with NP-treated DCs in a dose dependent manner (Figures 4a, b). Importantly, there was no increase in Granzyme B secretion, a cytotoxic mediator released from cytoplasmic granules in cytotoxic T and NK cells, following NP-treated DC co-culture (Figure 4c). These two observations are consistent with the presence of high levels of inflammatory NK cells ($CD56^{bright}$).

Our lymphocyte proliferative data indicate that the increase in Th1 lymphocytes and $CD56^{bright}$ NK cells is related to the production of IFNγ and IL12 combined with undetectable levels of IL4 and IL13 (Figure 2b and S6) following NP stimulation. These results suggest that NP treatment promotes innate immunity-type responses from NK cells.

The results obtained in this work are in agreement with a previous study suggesting crosstalk between innate and adaptive immune systems involving interactions between DCs and NK cells[39]. This concept is supported by the demonstration that DCs can activate NK cells, while activated NK cells can influence DC maturation or direct their cytotoxic-effects specifically towards immature DCs, which would hamper tolerant responses[40, 41]. Thus, crosstalk between DC and NK cell populations is likely to be a key factor in influencing the balance between tolerant and immunologic responses.

By employing the same surface chemistry for both particles, this study clearly demonstrates the strong influence of particle size on DC uptake, DC maturation, the proliferative response of T lymphocytes and the presence of inflammatory NK cells.

The ultra-small particles (NCs, ∅~2nm) were efficiently uptaken by DCs but did not induce maturation or lymphocyte proliferation. These observations seem to agree with studies demonstrating high passive targeting and clearance of NCs stabilised with GSH[17, 42]. The presence of functional GSH carboxyl groups on the NC surface should enable the conjugation of biomolecules such as antigens and, in the absence of carrier-associated immunogenic reactions, represents a good candidate antigen delivery system. In contrast, the slightly bigger nanoparticles (NPs, ∅~12nm) caused DC maturation and T lymphocyte proliferation associated with cell-mediated immunity-type responses and the production of inflammatory NK cells in a dose dependent manner. These results are of considerable interest as this subpopulation is a potential target for anti-tumour immunotherapy, inflammatory response limitation and the treatment of autoimmune disorders[43]. Indeed, this NK cell sub-type represents a relatively small proportion of Peripheral Blood Mononuclear Cells (PBMCs) but presents the advantage of being one of the most efficient cytokine producers[37] and is able to shape the

adaptive response. Indeed, Caliguri et al. undertook a comprehensive investigation of the role of NK sub-types, demonstrating the unique innate immunoregulatory role of the CD56^{bright} subpopulation by producing specific types of cytokines[20]. Because lowered NK activity in peripheral blood has been associated with elevated cancer risk in patients, strategies involving enhancement of NK cells for anti-tumour treatment are currently being investigated [44, 45].

As previously discussed by Drobrovolskaia et al.[46], it remains difficult to demonstrate clear relationships between the physicochemical properties of particles and their effects on immune responses due to the complex interactions between cytokines and numerous other cellular processes such as multiple cell uptake mechanisms. Different particle types have been shown to elicit Th1 responses but the contribution of inflammatory NK cells accompanied by high IFNγ levels in response to 12nm nanoparticles potentially opens new avenues for immunotherapeutic treatments of cancer or infections.

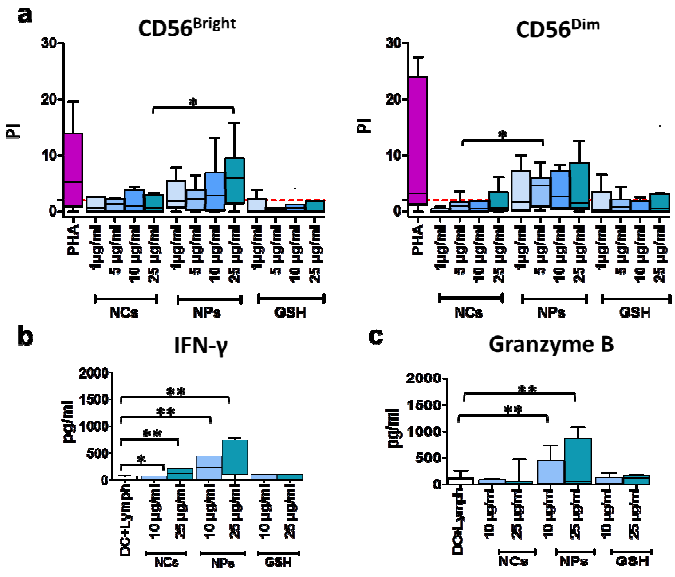

Figure 4. (a) Proliferative responses of different lymphocyte NK subpopulations (CD56^{dim} and CD56^{bright} cells); (b) Cytokine INFγ secretion levels during the proliferative lymphocyte experiments*, (c) Granzyme B assay on peripheral blood lymphocytes*. Proliferation positive control: PHA: Phytohemagglutinin.* only at 10 and 25 µg/mL of NCs, NPs, and eq. free GSH. Statistical comparisons were performed using the Wilcoxon test for related samples. The Bonferroni correction was applied for comparison of three groups. Statistical differences were considered significant when p<0.025.

Conclusions

In summary, we have shown how particle size in a narrow size range (1 to 15 nm) strongly influences cell uptake and immune responses with ultra-small size (<2 nm) leading to high cellular uptake without DC maturation and therefore lymphocyte proliferation. In contrast, bigger particles (12 nm) elicited DC maturation with a cell-mediated immunity pattern (Th1) and the proliferation of T helper lymphocytes in a concentration dependent manner. Moreover, we found that 12 nm NPs induced the proliferation of inflammatory NK cells with high levels of pro-inflammatory cytokine IFNγ secretion, suggesting that it might represent a tool for shaping and influencing adaptive immunity to fight cancer or infectious diseases. Our results underline the importance of examining different particle sizes in the 1-15 nm nanometric window in order to fine tune their effects on the immune system.

Acknowledgements

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Notes and references

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Electronic Supplementary Information (ESI) available: (synthesis and characterisation of the nanomaterial, experimental protocols, fluorescence microscopy, cytotoxicity and cytokine experiments). See DOI: 10.1039/c000000x/

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Nanoparticle size influences the proliferative responses of lymphocyte subpopulations

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Experimental section:

All chemical products were purchased from Sigma Aldrich (Spain). Ultra-pure Milli-Q water was used for the synthesis.{Hötzer, 2012 #5}

Synthesis.

NC synthesis was slightly modified from the preparation previously described[1]. Briefly, 326 μL of $HAuCl_4 \bullet 3H_20$ (50 mM) was added to 5 mL of glutathione (GSH) (1 mg/mL) and stirred at 70 °C. After 10 min, 50 μL of freshly prepared $AgNO_3$ (10 mg/mL) was added to the solution, changing colour from pale yellow to white and then black. The solution was left stirring for another 5 hours at 70 °C and then cooled down to room temperature. Afterwards, the solution was centrifuged at 10,000 rpm for 10 min to remove the big aggregates. The yellowish supernatant containing the fluorescent nanoclusters was filtered twice with 3 kDa cut-off Amicon filters to remove free glutathione and the pH was adjusted to 7. NC solution was kept refrigerated until use and was stable for months.

Gold nanoparticles coated with GSH (NPs) were prepared using the following protocol: 40 μL of $HAuCl_4 \bullet 3H_20$ (50 mM) was added to 10 mL of water under rapid stirring. Afterwards, 200 μL of $NaBH_4$ (6 mg/mL) was added dropwise and stirred for 30 min. Following this, 2 mg of GSH was added and stirred for another 90 min. The solution was then centrifuged at 13,600 rpm for 20 min to remove excess GSH and resuspended in water after adjusting the pH to 7.

Particle characterisation.

Particle size was determined by dynamic light scattering (DLS) using a Malvern instrument Nanosizer ZS. Thermal analysis of the samples were performed by Thermogravimetry (TG), and differential scanning calorimetry (DSC) using a METTLER TOLEDO model TGA/DSC 1 between 30 and 850°C at 10 °C/min with an air flux of 50 mL/min.

Electron Microscopy analysis. High-resolution transmission electron microscopy (HRTEM) measurements of the NCs was carried out with a JEOL 2200FS double aberration corrected FEG microscope, operated at 200 kV. TEM images of NPs were obtained on a FEI Tecnai G2 Twin TEM at 200 kV.

Optical measurement. Absorption spectra were collected on a UV-visible spectrophotometer Cary 100Bio (Varian) in the 190 nm - 900 nm range. Steady-state fluorescence measurements were obtained with a diluted solution of Au NCs using a Perkin Elmer LS45 Fluorescence Spectrometer.

Generation of Monocyte-Derived DCs. Fresh peripheral blood mononuclear cells (PBMC) obtained from 40 mL of blood from each individual were used for monocyte purification by means of anti-CD14 microbeads following the manufacturer's protocol (Miltenyi Biotec, Germany). The $CD14^-$ fraction was placed in 10% dimethyl sulfoxide and frozen for subsequent lymphocyte proliferation testing. To generate DCs, $CD14^+$ monocyte cells were incubated in complete medium (CM) containing Roswell Park Memorial Institute 1640 medium (Life Technologies, Invitrogen, USA) supplemented with 10% Fetal Calf Serum (FCS; Life Technologies, Invitrogen, USA), streptomycin (100 µg/ml), gentamicin (1.25 U/mL) as well as recombinant human rhGM-CSF (200 ng/mL) and rhIL-4 (100 ng/mL) (both from R&D Systems Inc., USA) for 5 days at 37°C and 5% CO_ENREF_3$_2$.

Cell toxicity analyses. The cytotoxic effects of the NCs and NPs on DCs were analysed by flow cytometry. Typically, 1×10^5 DCs were incubated with the NCs, NPs or free ligand GSH at 1, 5, 10 and 25 µg/mL concentrations in CM for 48 h at 37 °C and 5% CO_2. Untreated DCs were used as controls. After incubation, cells were stained with the Live/Dead NearIR kit (Life Technologies-Invitrogen, USA) for 15–20 min. Cells were then analysed using a flow cytometer (FACSCanto II flow cytometer, BD Biosciences, USA) according to the kit's instructions.

Dendritic Cell Maturation. DCs were incubated at 1×10^5 cells/well in 96-well plates (Nunc, Roskilde, Denmark) with NCs, NPs or the corresponding amount of free GSH at gold concentrations of 1, 5, 10 and 25 µg/mL in CM for 48 h at 37 °C and 5% CO_2. Between 7 and 15 experiments were performed with the different particle concentrations in order to obtain statistically-valid results. Treated DCs were then labelled with CD80 and CD86-specific monoclonal antibodies (BD Pharmigen, CA), analysed using a FACSCanto II flow cytometer (BD Biosciences, USA), and data processed with FLOWJO software (Tree Star, Inc, USA). Results are expressed as a maturation index (MI) calculated as the ratio between the percentage of stimulated and non-stimulated cells expressing the aforementioned

markers. Culture supernatants were collected and stored at –20°C for subsequent secreted cytokine analysis.

Lymphocyte Proliferation. $CD14^-$ PBMCs were labelled with 2 μM CFSE using the CellTrace CFSE proliferation kit (Life Technologies, Invitrogen, USA) at room temperature and darkness for 10 min. After washing, labelled PBMC (1.5×10^5) were co-cultured with 1.5×10^4 autologous DCs in 96-well plates (Nunc, Roskilde, Denmark) for 6 days at 37 °C and 5% CO2. DCs were pre-incubated with NCs or NPs at different concentrations as described above and washed several times (primed DCs). Between 5 and 10 experiments were performed with the different particle concentrations in order to obtain statistically-valid results . Co-cultured PBMCs were labelled for CD3, CD4, CD8 (to identify T lymphocyte subpopulations), CD56 (to identify NK cells), CD127, CD25, ROR-γt, Fox p3, and IL-17 monoclonal antibodies (BD Pharmigen, CA, USA) in order to detect the different lymphocyte subpopulations. Th17 cells were defined as $CD4^+$ ROR-γt^+ IL-17^+; and Treg as $CD4^+$ $CD127^{low}$ $CD25^{high}$ Fox $p3^+$. The percentage of proliferating cells with lower levels of CFSE fluorescence ($CFSE^{dim}$) was assessed by flow cytometry. Results are expressed as a proliferation index (PI), calculated as:

$$\frac{\left[\%CD3^+CFSE^{dim}\ stimulated\ (lymphocytes + DCs)\right] - \left[\%CD3^+CFSE^{dim}\ unstimulated\ (lymphocytes + DCs)\right]}{\%CD3^+CFSE^{dim}\ unstimulated\ (lymphocytes)}$$

PI was considered positive when greater than 2. Culture supernatants were collected and stored at –20 °C for subsequent secreted cytokine analysis.

Cytokine Secretion. Th1/Th2/Th17 cytokine and Granzyme-B production were determined in culture supernatant using the BD Cytometric Bead Array (CBA) human soluble protein Kit (BD Biosciences, CA, USA) following the manufacturer's protocol. Culture supernatants were obtained after incubating DCs primed with NCs or NPs at 10 or 25 μg/mL concentrations, alone for 48 h or mixed with PBMCs for 6 days. Cytokine measurements were conducted in 5 independent experiments. The results are expressed in pg/mL.

Ethical Statement. This study was approved by the institutional review board "*Comisión de Ética y de Investigación del Hospital Regional Universitario de Málaga*", and the

experiments were carried out in accordance with the Declaration of Helsinki. Oral and written informed consents for all the procedures were obtained from subjects included in the study.

Statistical Analysis. In this study, nonparametric analysis comparing obtained results with the different stimuli of the quantitative variables was performed using the Wilcoxon test for related samples. Maturation measurements were analysed using 17 patients; Lymphocyte proliferation was analysed for 8 patients and cytokine secretion for 5 patients. All reported *P* values represent 2-sided tests, with the level of significance set at the probabilities of $*P < 0.05$ or $** P < 0.01$. The Bonferroni correction was applied for comparison of three groups as two independent experiments and then statistical differences were considered significant when $p < 0.025$.

NC/NP quantification in DCs. Quantification of NCs or NPs in DCs was estimated by inductively coupled plasma high-resolution mass spectrometry (ICP-HRMS) using an ELEMENT XS (Thermo Fisher) system after digesting cells (an average of 70,000 cells per sample) with strong acid. Results are expressed as particles/cell calculated assuming average NC and NP sizes of 2 nm and 12 nm, respectively, as described by Lewis et al[3]. Briefly, the number of gold atoms per particle was estimated using the following formula:

$N_{atom} = (R_{particle} / R_{atom})^3$ with $R_{atom} = 0.137$ nm (R= radius)

and the number of gold atoms from ICP: N_{ICP}= mass of gold measured /M_{gold} *N_A

with $N_A=6.02x10^{23}$; $M_{gold}= 197$ g.mol^{-1}

Finally particle/cell = $N_{ICP.}/(N_{atom}*cell_{numb.})$ with $cell_{numb.}= 70,000$.

Confocal Microsopy

Au NCs and Au NPs stabilised with GSH ligand were analysed using a Leica DM6000 inverted microscope connected to a Leica SP5 confocal laser scanning and multiphoton system. Sub-membrane cortical actin in treated DCs, washed and fixed with 4% paraformaldehyde, was stained using fluorochrome-conjugated Phalloidin. NC-incubated

DCs were stained for ~20 minutes with 10 μM Atto488-conjugated Phalloidin (Sigma) with Atto488 and NC fluorescence visualised by confocal microscopy simultaneously with ~500-520 nm and ~570-650 nm emission detection windows, respectively. NP-treated DCs were stained with Alexa 647-conjugated Phalloidin and visualised by confocal microscopy using 633 nm laser excitation and a ~650-700 nm detection window. NP photoluminescence was detected sequentially using 720 nm two photon excitation and a ~550-650 nm detection window. Images were captured using 25x NA 0.95 water or 63x NA 1.4 oil immersion objectives.

Characterisations

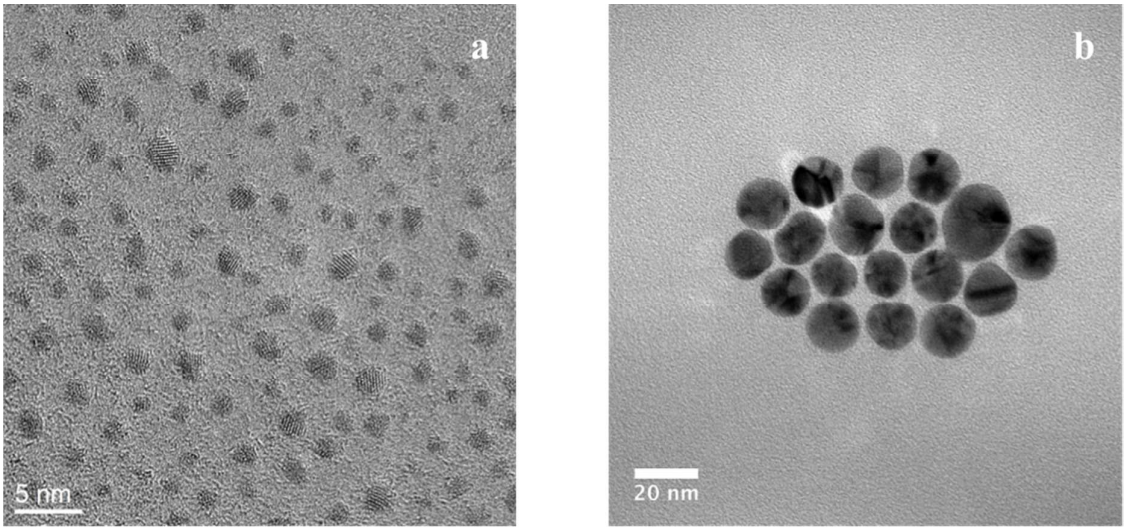

Figure S1. Transmission electron microscopy images of (a) NCs and (b) NPs.

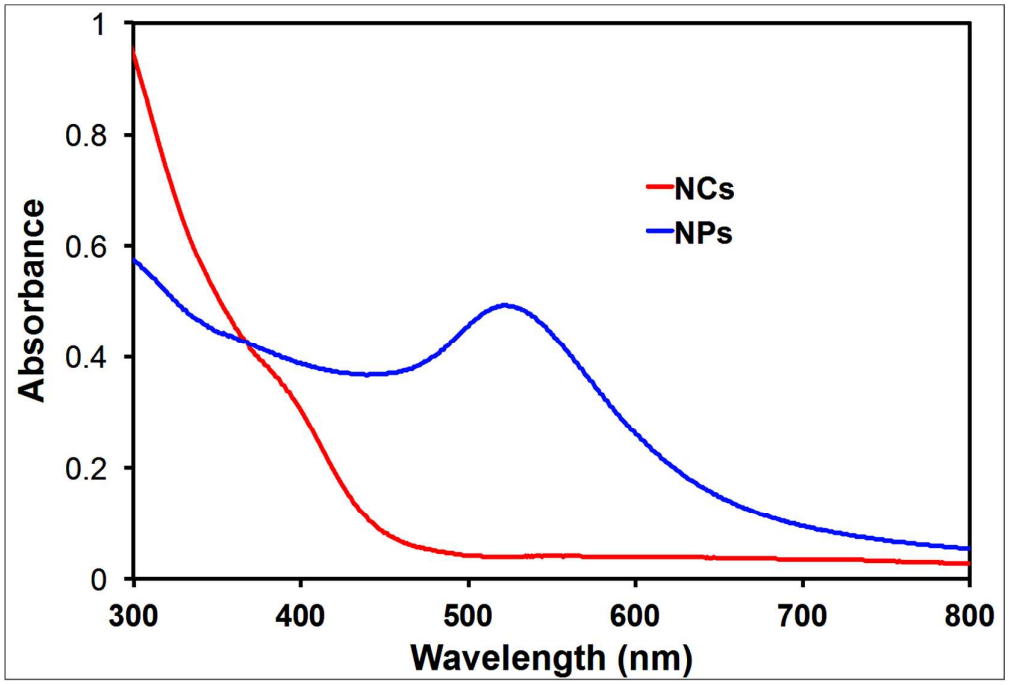

Figure S2. Absorption spectra of the NC (red line and NP (blue line) solutions. Measurements indicated strong UV absorption for the NCs and the plasmon band at λ= 520 nm for the NPs.

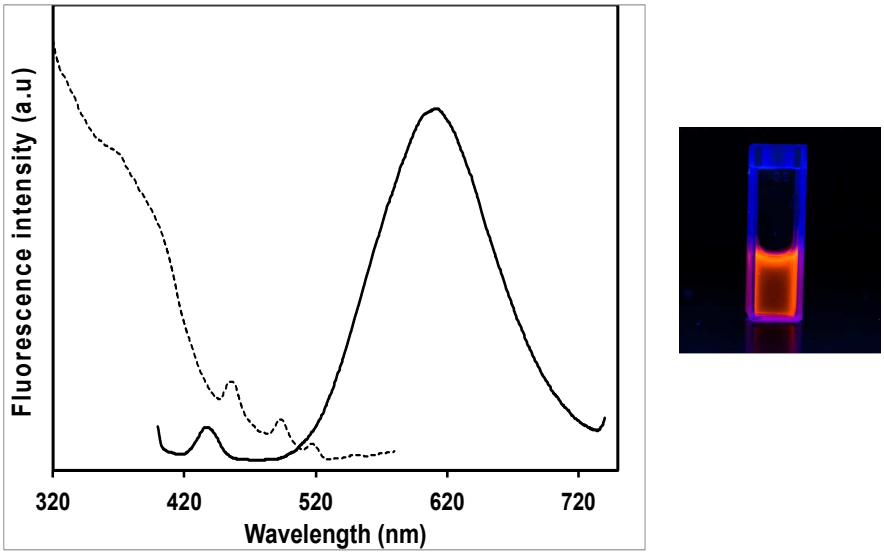

Figure S3. Excitation (dashed line) and emission (solid line) spectra of a diluted aqueous NC solution. NCs exhibited intense orange fluorescence under UV illumination (picture on the right).

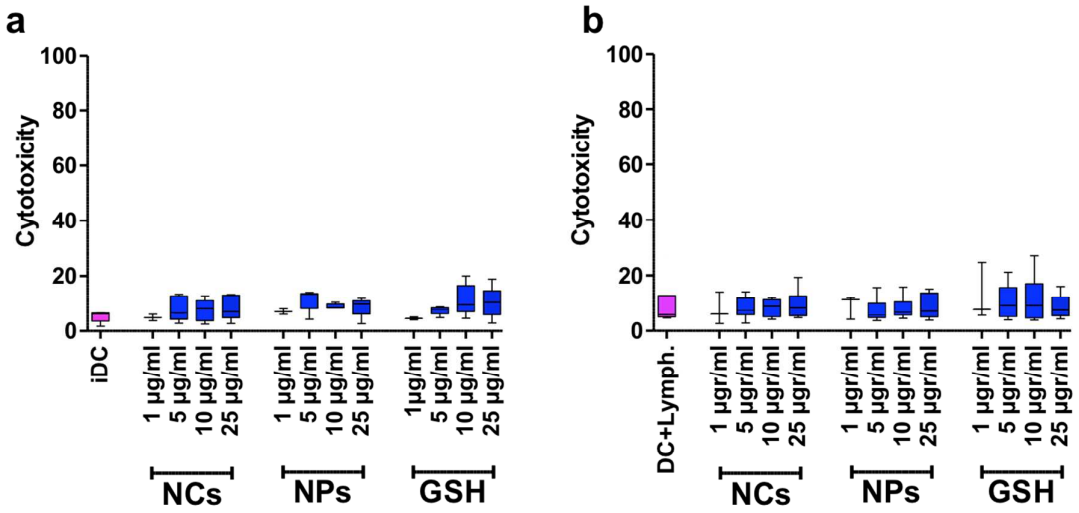

Figure S4. Cytotoxicity tests of (a) DCs for 48 hours in presence of NCs, NPs (1-25 µg/mL in RPMI1640 + 10% FCS) and GSH, and (b) for the lymphocyte proliferation experiments.

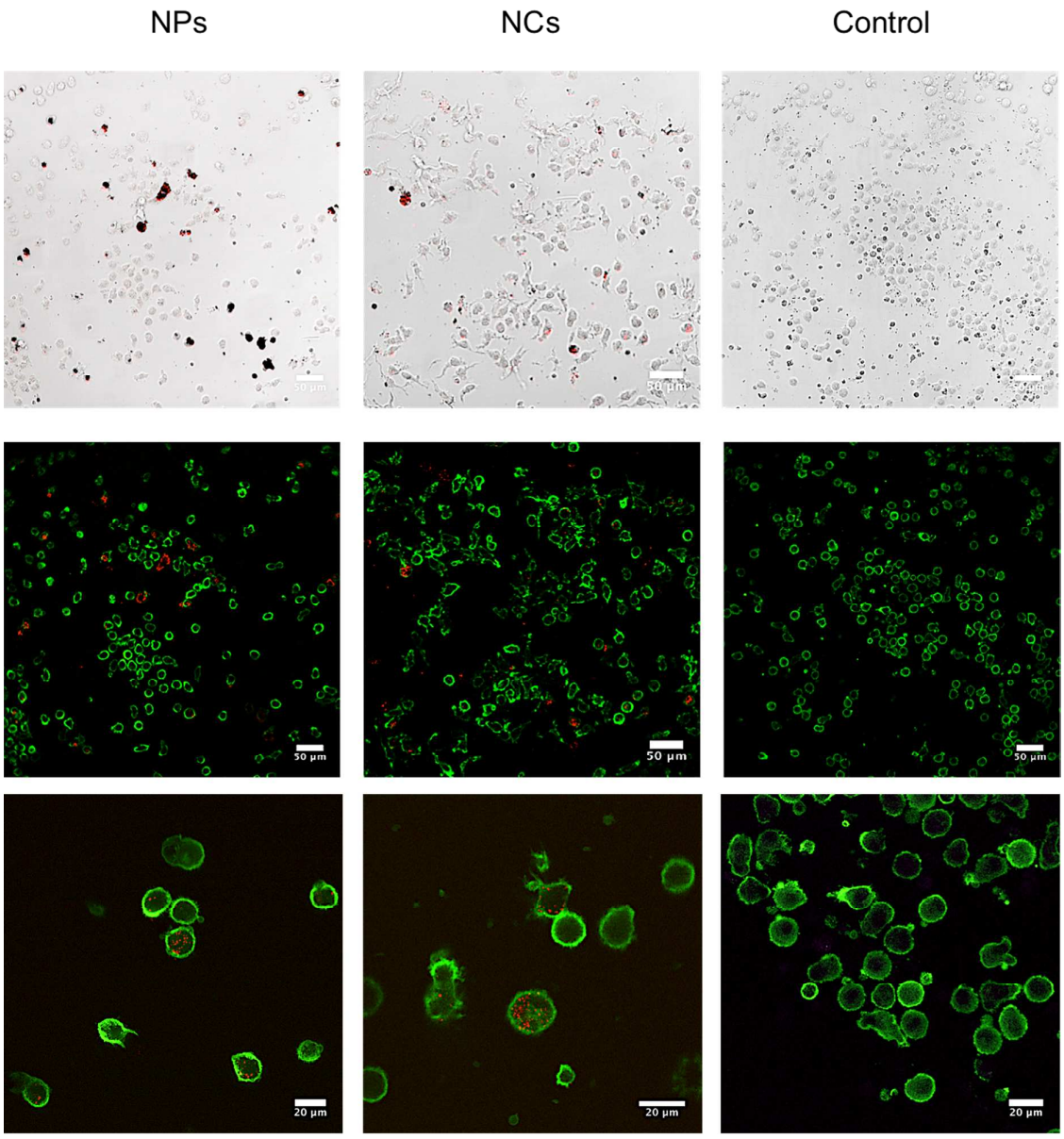

Figure S5. Dendritic cells (DCs) incubated with NCs, NPs (10 µg/mL in RPMI+10%FCS) and without particles (control) for 48 hours. Brightfield and fluorescence microscopy images show the internalisation of NCs and NPs (red) in DCs. Sub-membrane actin was stained (green) with Phalloidin-Alexa 647 (NPs) or Phalloidin-Atto 488 (NCs).

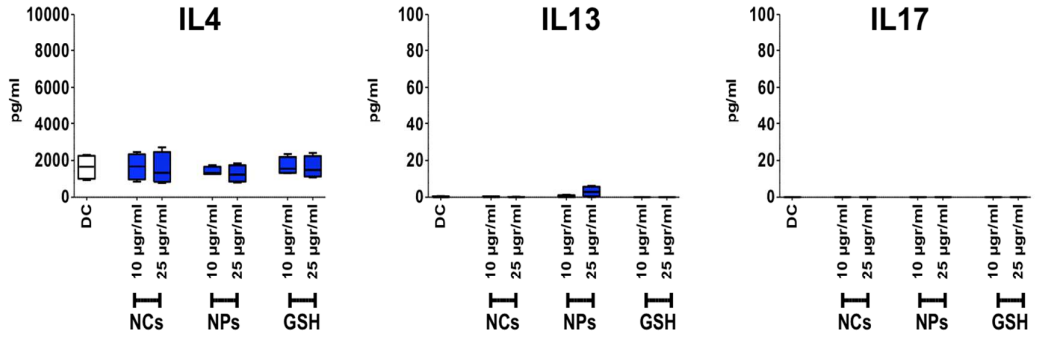

Figure S6. Cytokine secretion (IL4, IL13, IL17) after DCs were incubated with NCs, NPs (1-25 µg/mL in RPMI+10%FCS) and GSH for 48 hours.

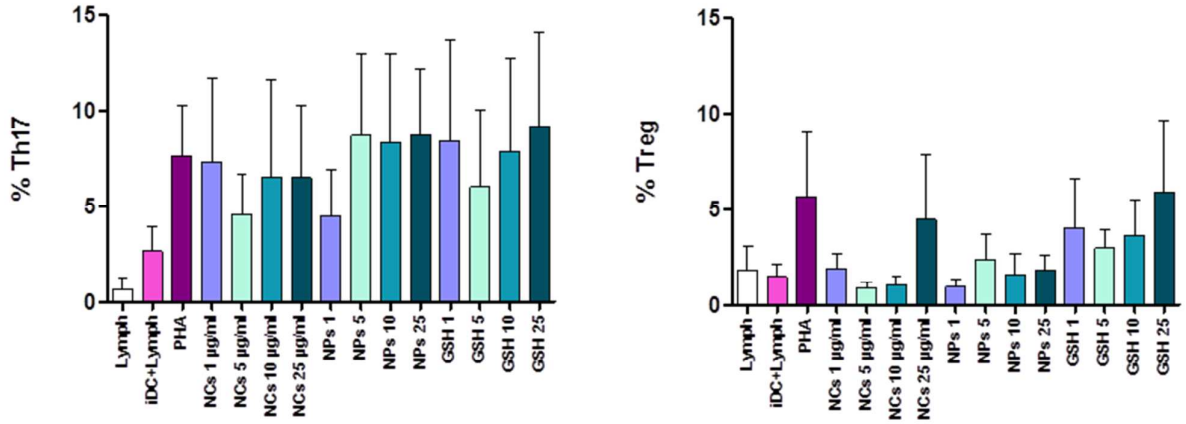

Figure S7. Percentage of different lymphocyte subpopulations as judged by the expression of Th17 or Treg markers in proliferating $CD4^{+}$T cells following a 6 day incubation of PBMCs with DCs pre-treated with NCs, NPs or free GSH. Statistical comparisons have been performed using the non-parametric test for related samples (Wilcoxon test). The Bonferroni correction was applied for comparison of three groups. Statistical differences were considered significant when $p < 0.025$.

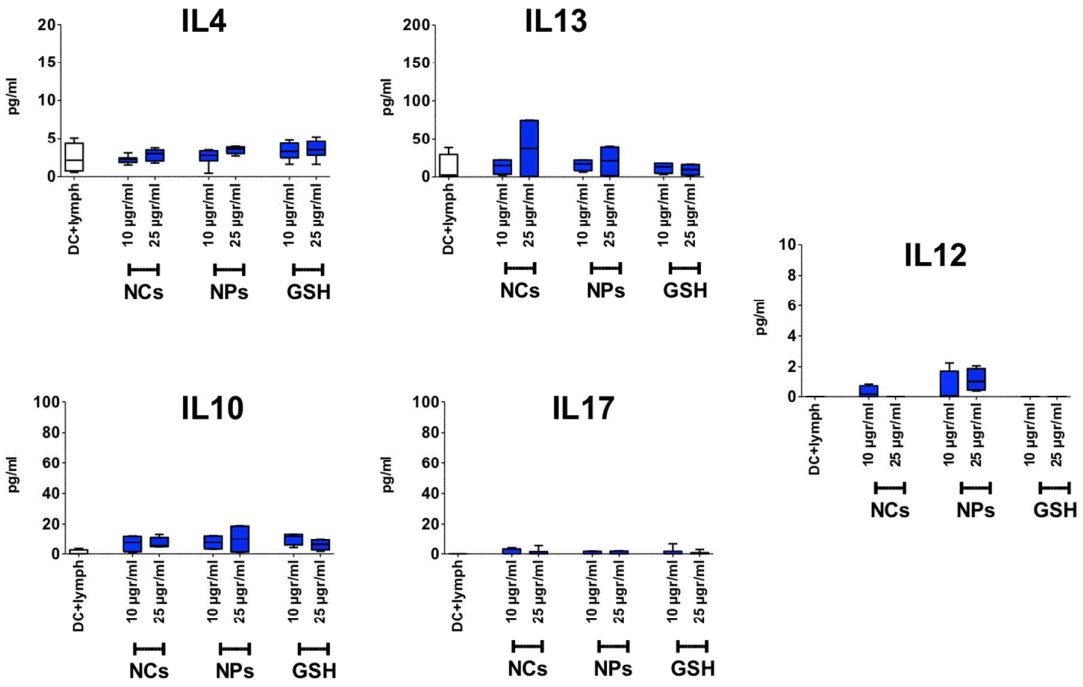

Figure S8. Cytokine production (IL4, IL10, IL12, IL13, IL17) following the pre-treatment of DC with NCs, NPs or free GSH and after several washing step incubated with peripheral blood lymphocytes for 6 days.

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