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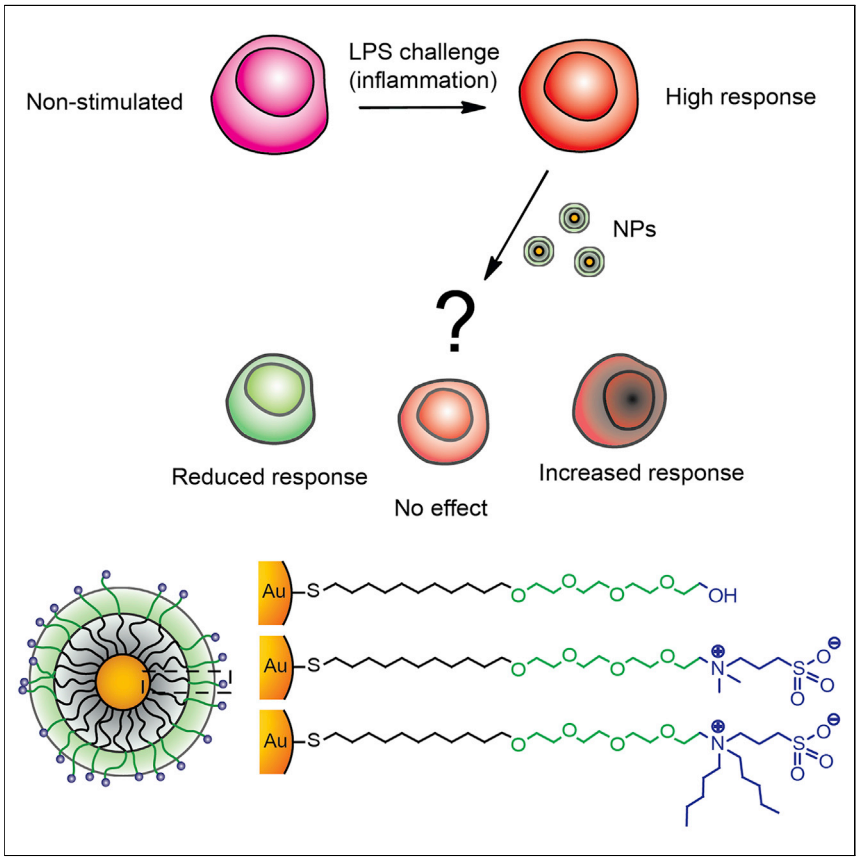
Immunomodulatory Effects of Coated Gold Nanoparticles in LPS-Stimulated In Vitro and In Vivo Murine Model Systems



Gold nanoparticles with different surface functionalities provide immunomodulatory properties and have potential applications as adjuvant or anti-inflammatory agents.

Daniel F. Moyano, Yuanchang Liu, Furkan Ayaz, ..., Bradley Duncan, Barbara A. Osborne, Vincent M. Rotello

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HIGHLIGHTS

Functionalized AuNPs modulate the immune response under the LPS challenge

ZDiPen AuNPs boost inflammatory response

ZDiMe AuNPs generate minimal immunological response

TEGOH AuNPs generate a significant anti-inflammatory response

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Daniel F. Moyano,[1,3] Yuanchang Liu,[1,3] Furkan Ayaz,[2] Singyuk Hou,[1] Premsak Puangploy,[1] Bradley Duncan,[1] Barbara A. Osborne,[2] and Vincent M. Rotello[1,4,*]

SUMMARY

The ability of nanoparticle surface functionalities to regulate immune responses during an immunological challenge (i.e., inflammation) would open new doors for their use in non-prophylactic therapeutics. We report here the use of functionalized 2-nm-core gold nanoparticles to control the immunological responses of in vitro and in vivo systems presented with an inflammatory challenge. Our results show that nanoparticles bearing a hydrophobic zwitterionic functionality boost inflammatory outcomes, whereas hydrophilic zwitterionic nanoparticles generate minimal immunological responses. Surprisingly, tetra(ethylene glycol) head groups generate a significant anti-inflammatory response both in vitro and in vivo. These results demonstrate the ability of simple surface ligands to provide immunomodulatory properties, making them promising leads for the therapeutic usage of nanomaterials in diseases involving inflammation.

INTRODUCTION

The ability to control immune responses by tailoring the chemical identity of nanoparticle (NP) surfaces has opened new avenues for therapeutics.[1–4] New vaccine formulations have been developed with the use of specific antigens at the NP surface[5] or by the induction of NP recognition for adjuvancy.[6] These applications rely on the capability of enhancing immune responses (adjuvancy) by either delivering the antigen of interest or enhancing antigen presentation.[7] With this prophylactic approach, vaccines against cancer,[8] hepatitis B,[9] and other diseases have been developed.[10] However, much less understood is the behavior of nanomaterials in challenged systems, for example, when an inflammatory event is already present (remedial approach, Figure 1A). Controlling the immunological profile of inflammatory responses in these activated systems is important for the treatment of diseases characterized by excess inflammation,[11] such as arthritis,[12] atherosclerosis,[13] and fibromyalgia.[14]

NP surface chemistry plays a central role in the recognition and type of response that is triggered by the immune system toward these materials.[1–5] For example, the presence of surface charge greatly increases the adsorption of opsonins, forming a protein corona with concomitant recognition by the mononuclear phagocytic system.[15] This phenomenon can be overcome by the use of "stealth" functionalities (such as poly(ethylene glycol) and zwitterions) that inhibit the non-specific adsorption of proteins.[16] Interestingly, these functionalities trigger other types of immune responses on their own, such as activation of the complement system.[17,18] Similarly, by decorating the NP surface with hydrophobic moieties, specific types of innate

The Bigger Picture

Nanoparticle-based immunotherapies have to date been aimed toward the development of prophylactic therapeutics such as vaccines. However, nanomaterials have the potential to reduce or enhance immunological challenges already present in the body. Here, we use engineered nanoparticles to demonstrate how pre-existing inflammatory effects can be reduced or boosted by the chemistry of the nanoparticles surface. These findings open new avenues for non-prophylactic applications of nanoparticles, a critical step toward the development of a new generation of immunotherapies.

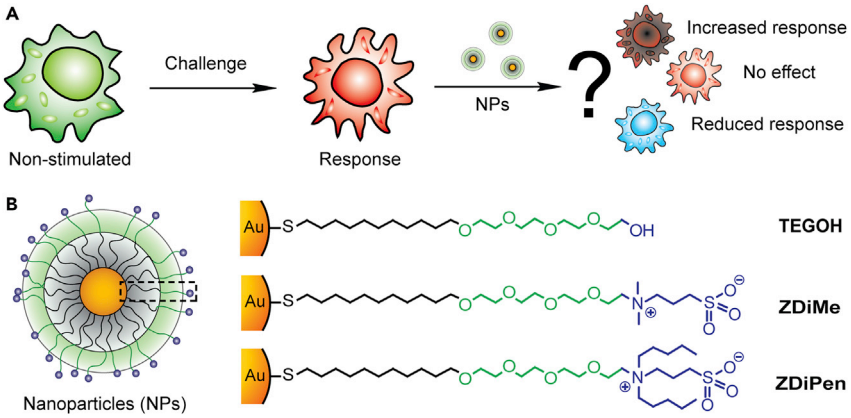

Figure 1. Rationale behind the Design of NPs and Their Chemical Structure

(A) Cartoon depicting the non-prophylactic therapeutic approach used for studying the inflammatory challenge. For our non-prophylactic studies, the challenge comprised stimulation with LPS, which induced a strong inflammatory response.

(B) Chemical structure of the NPs (2-nm diameter gold core) bearing different chemical groups while maintaining a net neutral charge.

immune responses can be triggered.[19,20] Despite these observations of relationships between NP surface chemistry and immune responses in unchallenged conditions, it is not clear how or which chemical functionalities can be used to control the immunological profile of challenged systems.[21]

We recently developed a series of uncharged NPs with specific surface functionality and variable hydrophobicity while avoiding the formation of a protein corona. These particles with zwitterionic and non-ionic tetraethylene glycol (TEG) ligands were engineered for the direct study of relationships between biological activities and NP surface chemistry.[22] Here, we report the use of these uncharged NPs to elucidate immunological responses when an inflammatory challenge is presented. In both in vitro and vivo studies with murine systems, we observed a reduction in inflammatory responses for neutral TEG particles, dramatically increased inflammation for NPs bearing hydrophobic zwitterionic functionality, and an unaltered response for hydrophilic zwitterionic particles. Our findings indicate that these particles are promising leads for use in adjuvant therapy (hydrophobic NPs) and as anti-inflammatory agents (neutral NPs).

RESULTS AND DISCUSSION

Three different types of surface-chemical functionalities were selected for our immunomodulation studies (Figure 1B), and a 2-nm Au core was used as the scaffold for their presentation.[23] TEGOH is structurally based on poly(ethylene glycol) coatings, which are commonly used in nanomaterials and can be recognized in vivo by different components of the immune system, such as the complement system.[16] Likewise, ZDiPen was developed with the premise that hydrophobic NPs are capable of triggering various types of innate immune responses and has been used previously in the development of vaccines.[24] This NP was selected as the most hydrophobic zwitterionic family member that maintained solution stability. Finally, hydrophilic structures (such as ZDiMe) have been shown to maintain a stable pro-/anti-inflammatory balance.[20] We engineered all of these NPs to bear a neutral charge, which dramatically decreases non-specific adsorption of proteins and thus reduces interference and allows the study of immune responses intrinsic to the chemical

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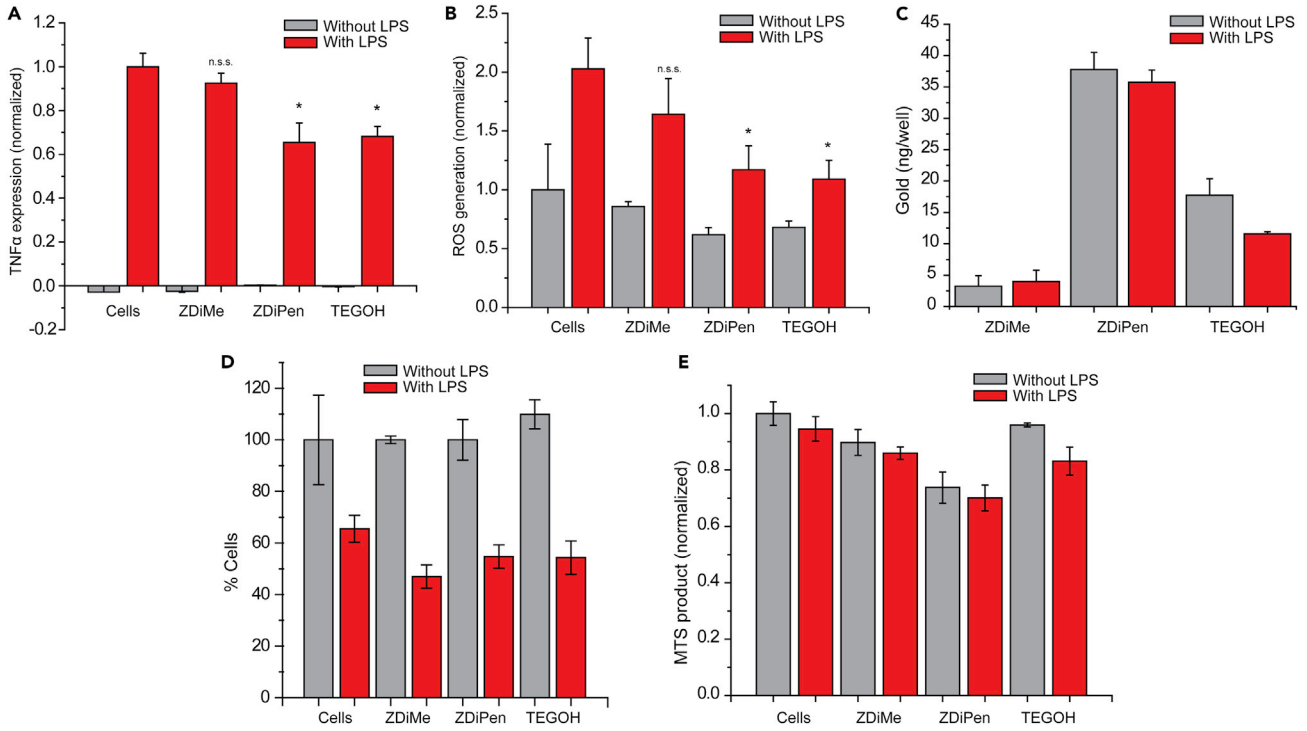

Figure 2. Performance of NPs in the J774.2 Cell Line

(A) TNF-α secretion of J774.2 cells in the presence of the NPs with and without LPS stimulation after 3 hr incubation. Values are normalized against the positive control (cell + LPS).

(B) Cellular uptake of the different NPs for both LPS-challenged and unchallenged conditions.

(C) ROS generation of J774.2 cells under the same experimental conditions (LPS challenge, 24 hr). Values are normalized against the normal cell response.

(D) Cell viability of J774.2 after 24 hr incubation with the different NPs and LPS was measured by trypan blue.

(E) MTS assay indicating the metabolism (vitality) of cells after 24 hr exposure with NPs and LPS. Values are normalized against the responses of untreated cells.

Error bars represent SDs from three repeats. Asterisks indicate a statistically significant difference, and "n.s.s." indicates the lack of a statistically significant difference in relation to the control (cells alone in gray or cell + LPS in red).

functionalities (Figure S1). Likewise, to ensure minimal interference originating from endotoxins, we performed a gel-clot test with Limulus amebocyte lysate to verify that all the NPs were free of endotoxins under the conditions of the study (below 0.03 endotoxin units/mL, 100 nM NP concentration).

We generated inflammation in our in vitro and in vivo studies by using lipopolysaccharide (LPS), a bacterial agent that triggers strong inflammatory responses.[25] As the readout, we measured the secretion of tumor necrosis factor α (TNF-α), a cytokine characteristic of pro-inflammatory cellular profiles.[26] TNF-α is the major target for many inflammatory diseases, and reduction of TNF-α secretion is a well-established clinical treatment for inflammatory diseases.[27]

Our initial in vitro studies used the J774.2 murine monocyte cell line, which is highly sensitive to LPS stimulation.[28] These monocytes are professional phagocytes, a type of cell that interacts strongly with nanomaterials.[29] NP ZDiMe did not affect the secretion of TNF-α in relation to the control (Figure 2A), suggesting that NPs coated with hydrophilic zwitterionic functionalities have minimal interaction with the cells,

an observation that is in agreement with the low cellular uptake of these NPs by J774.2 cells (Figure 2B) and consistent with previous studies showing long circulatory lifetime particles with this functionality.[30]

In contrast to hydrophilic zwitterionic particle ZDiMe, both TEGOH and ZDiPen decreased the secretion of TNF-α (Figure 2A), indicating a reduction of the inflammatory response to LPS. The cellular uptake of these two NPs was higher than that of ZDiMe, in agreement with previous reports depicting greater cellular uptake of hydrophobic NPs, possibly as a result of a stronger interaction with the hydrophobic portions of the cell membrane (Figure 2B). However, TEG NPs were not taken up as readily as ZDiPen, indicating that the anti-inflammatory effect depends on both cellular uptake and responses caused by the functionality per se. We wanted to verify whether these observations were valid in fully differentiated macrophages, a more specific type of cells that may not be sensitive to these molecular patterns. Thus, studies with all of the above particles were repeated with RAW 264.7 cells. As can be observed in Figure S2, similar results were obtained for these cells, indicating that this anti-inflammatory response is generalizable among different types of macrophages.

The secretion of reactive oxygen species (ROS) is an important aspect of inflammatory responses and is directly related to the activation of immune cells.[31,32] We measured endogenous ROS generation by J774.2 cells after exposure to NPs and LPS by using 2′,7′-dichlorodihydrofluorescein diacetate, a pro-fluorophore that is activated by ROS.[33] As shown in Figure 2C, both ZDiPen and TEG reduced the response of the cells significantly in comparison to the baseline (cells alone). These results are consistent with our TNF-α findings and indicate a decrease in the activation of the cells. We quantified live and dead cells after 24 hr of incubation by using trypan blue to determine whether the change in these responses was indeed a reduction of activity and not simply a decrease in the viability of cells. As observed in Figure 2D (and Figures S2E and S2F), J774.2 cells were viable for all the NPs under the conditions of the study, and live and dead assays were comparable to control assays, indicating no toxic effects of the particles. We further analyzed whether the viability of the cells was altered by the presence of the NPs by using (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2(4-sulfophenyl)-2*H* tetrazolium) (MTS). Interestingly, whereas ZDiMe and TEG had minimal or no effect on the metabolism of the cells, ZDiPen decreased the metabolic activity significantly, as shown in Figure 2E. From these studies, we can surmise that the decrease in immune response observed upon treatment with ZDiPen arose from a decrease in cell metabolism. The origin of the anti-inflammatory properties of TEG is more difficult to explain. Two possible mechanisms for immunomodulation by TEG have been identified. The first is that TEG acts as an antagonist for LPS, decreasing the response of the macrophage by blocking receptors. The second mechanism is that TEG binds to the LPS, blocking interactions between the LPS and the macrophage. Both options are viable in vitro; however, the latter mechanism would be unlikely in vivo (see below), where substantial anti-inflammatory activity is observed with TEG.

Given the promising activity of the NPs in vitro, we next examined the relevance of these results in vivo by using LPS-challenged mice under the oversight of the University of Massachusetts Institutional Animal Care and Use Committee. In particular, we wanted to observe whether TEGOH and ZDiPen maintained their capability to reduce inflammation and determine whether ZDiMe was not immunomodulatory. We first established the baseline of the response by measuring TNF-α blood levels (overall secretion) when C57BL/6 mice were challenged by intraperitoneal (i.p.)

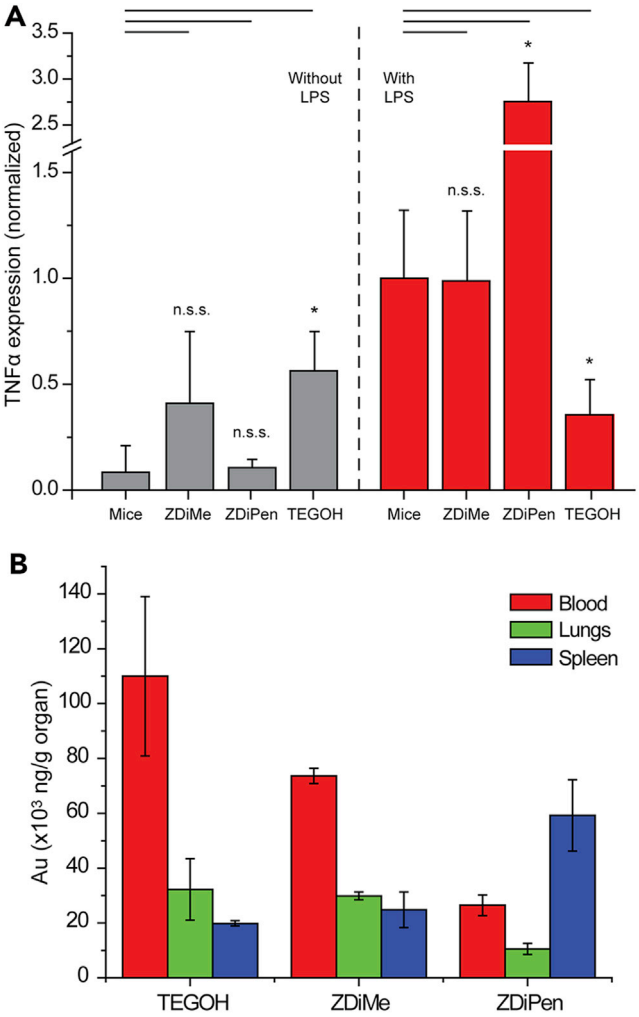

Figure 3. NP-Induced TNF-α Secretion and NP Distribution In Vivo

(A) In vivo TNF-α secretion 2 hr after ZDiMe, ZDiPen, and TEGOH (2.75 mg/kg) were injected in mice with and without the presence of LPS (0.01 mg/kg). Asterisks indicate a statistically significant difference, and "n.s.s." indicates the lack of a statistically significant difference in relation to the control.

(B) NP distribution in the blood, lung, and spleen of mice demonstrates the fast elimination of ZDiPen in comparison with the other two NPs. Accumulation in other organs is shown in Figure S3C. Error bars represent SDs from three repeats.

injection with different concentrations of LPS. Mice were killed after 2 hr of the treatment, and blood was extracted and processed as described in the Supplemental Experimental Procedures. A concentration of LPS of 200 ng/mice was chosen because this concentration was the lowest that induced a significant and robust TNF-α readout (Figure S3A). We then performed i.p. injections of LPS, followed by injection with TEGOH, ZDiMe, and ZDiPen (injection scheme in Figure S3B). In addition to measuring TNF-α levels, we also measured the concentration of gold in the different organs by inductively coupled plasma mass spectrometry to observe how different functionalities affect the biodistribution of these NPs. As shown in Figure 3A, no significant change in the inflammatory response was observed with ZDiMe in either unchallenged or challenged mice, mirroring the results in vitro. On the basis of these results, it can be concluded that hydrophilic zwitterionic structures do not perturb the immune system and do not trigger a significant response.

This result, coupled with the fact that these structures have been shown to possess extended blood circulation times,[30] indicates the utility of hydrophilic zwitterions for the generation of non-immunogenic delivery vehicles.[17]

The results obtained for ZDiPen contrast strongly with those of ZDiMe, showing a strong pro-inflammatory response (Figure 3A). This outcome is particularly intriguing because this hydrophobic NP responded very differently in vitro, where an anti-inflammatory response was observed (Figure 2A). These in vivo results suggest the involvement of other components of the immune system, such as the complement system, a part of the immune system hypothesized to be triggered by hydrophobic moieties,[34] in the mechanism that orchestrates NP-induced inflammatory responses. However, the biodistribution profile was very distinct from that of ZDiMe (Figure 3B). Despite the fact that both NPs have the same surface charge and size, the presence of a hydrophobic head group strongly decreased the concentration of NPs in the blood while increasing levels in the spleen. This result, coupled with the strong interaction with cells observed in the cellular-uptake studies, suggest that the change in the in vitro and in vivo behavior possibly arose from differences in the interaction with other components of the immune system, such as the complement system or non-specific hydrophobic-driven interactions with other cells. The highly strong synergistic response obtained by the combination of LPS and ZDiPen, a level that cannot be achieved by either LPS or ZDi1en alone, indicates the potential utility of these functionalities for adjuvant therapies that would not require covalent conjugation between the antigen and the NP.[35]

Perhaps the most surprising result of the in vivo studies was the anti-inflammatory effect of TEGOH, which decreased the secretion of TNF-α in vivo (Figure 3A) in relation to cells + LPS, a result that mimics our findings in vitro. Interestingly, TEGOH triggered an inflammatory response in unchallenged systems on its own (an effect that has been observed in the past for similar NPs[36]), and these levels were maintained in the presence of LPS, suggesting a buffering effect of the stimuli. Likewise, the biodistribution of TEG was similar to that of the hydrophilic ZDiMe, indicating that this particle is not eliminated as quickly as the hydrophobic ZDiPen. This result suggests that the immunological response does not necessarily correlate with biodistribution in the same way that cellular uptake does not correlate with TNF secretion in vitro. Recent studies have reported the generation of antibodies against polyethylene glycol (PEG)-like structures when NPs are injected in the body, indicating an interaction between this type of functionality and components of the immune system involved in adaptive immunity (i.e., immune cells such as macrophages), a process that is related to the extent of the PEG chain and the size of the particle. Because in our case the immunological response was observed both in vitro and in vivo, however, a cell-mediated mechanism is probably directing the response. This mechanism is consistent with the potential role of TEG as an antagonist for LPS binding and activation, a possibility that is under exploration. Although binding of LPS to TEG and subsequent interaction cannot be ruled out, the separate injections of these two agents makes this possibility less likely. The fact that there is a decrease in inflammatory response indicates the potential of these NPs for use in anti-inflammatory therapies, a property that was not observed in the previous studies performed in the absence of an inflammatory stimulus.[35]

Conclusion

We have demonstrated that surface chemistry of NPs can be used to generate pro- and anti-inflammatory responses in cells and animals challenged with LPS.

These studies provide important clues for the therapeutic use of nanomaterials in diseases involving inflammation, as well as input for the design of non-immunogenic materials. Hydrophilic zwitterionic motifs provide minimally immunogenic coverage suitable for delivery applications. In contrast, hydrophobic zwitterionic particles are strongly pro-inflammatory in LPS-challenged systems, suggesting their utility in adjuvant applications. PEG-like structures have anti-inflammatory properties that emerge with challenged systems, providing a potential role for these motifs in combatting inflammation in diseases such as rheumatoid arthritis and atherosclerosis. These findings demonstrate the ability of chemically simple functionality to provide lead structures for the use of NPs in non-prophylactic applications, an important step toward the development of a new generation of immunotherapies.

EXPERIMENTAL PROCEDURES

Full experimental procedures are provided in the Supplemental Information.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and three figures and can be found with this article online at http://dx.doi.org/10.1016/j.chempr.2016.07.007.

AUTHOR CONTRIBUTIONS

Conceptualization, V.M.R., B.A.O., and D.F.M.; Investigation, Y.L., D.F.M., and F.A.; Recourses, S.H., P.P., and B.D.; Writing – Original Draft, D.F.M. and Y.L.; Writing – Review & Editing, B.A.O., and V.M.R.; Supervision, B.A.O. and V.M.R.

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Supplemental Information

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SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Nanoparticles synthesis and characterization:

- *Synthesis*: 2 nm diameter gold nanoparticles (synthetized by the Brust-Schiffrin methodology[1] and purified using to remove precursors[2]) capped with a monolayer of 1-pentanethiol were dissolved in methanol/DCM 95:5, and a solution of the respective ligands (synthesized according to the reported procedure[3]) in the same solvent system was added (5:1 ratio of ligand/nanoparticles w/w). The reaction was maintained with constant stirring and under an inert atmosphere (Nitrogen). After 3 days, the solvent was evaporated under vacuum without heating, and the resulting residue was washed several times with ethyl acetate and hexanes to remove the excess of the ligand and 1-pentanethiol. The residue was then dispersed in water and was put under dialysis (~10K MW membrane) for 5 days. The NP solution was then added to a cut-off filter (~10K MW membrane) and was centrifuged with endotoxin-free water several times to remove any other contaminants.

- *Characterization*: The final concentration of the nanoparticles was determined by UV following the reported procedure. Dynamic light scattering (DLS) was used to determine the hydrodynamic size and the zeta potential of the particles, and determined that all the NPs have similar size and charge (Figure S1). Limulus amebocyte lysate (LAL) gel cloth test was performed to determine that the NPs are free of endotoxins, using a Pyrosate® Kit from Associates of Cape Cod, Inc. with a limit of detection of 0.03 EU/mL. No visible clots were observed after incubating the lysate with NPs at a concentration of 100 nM during 50 min at 37 ºC.

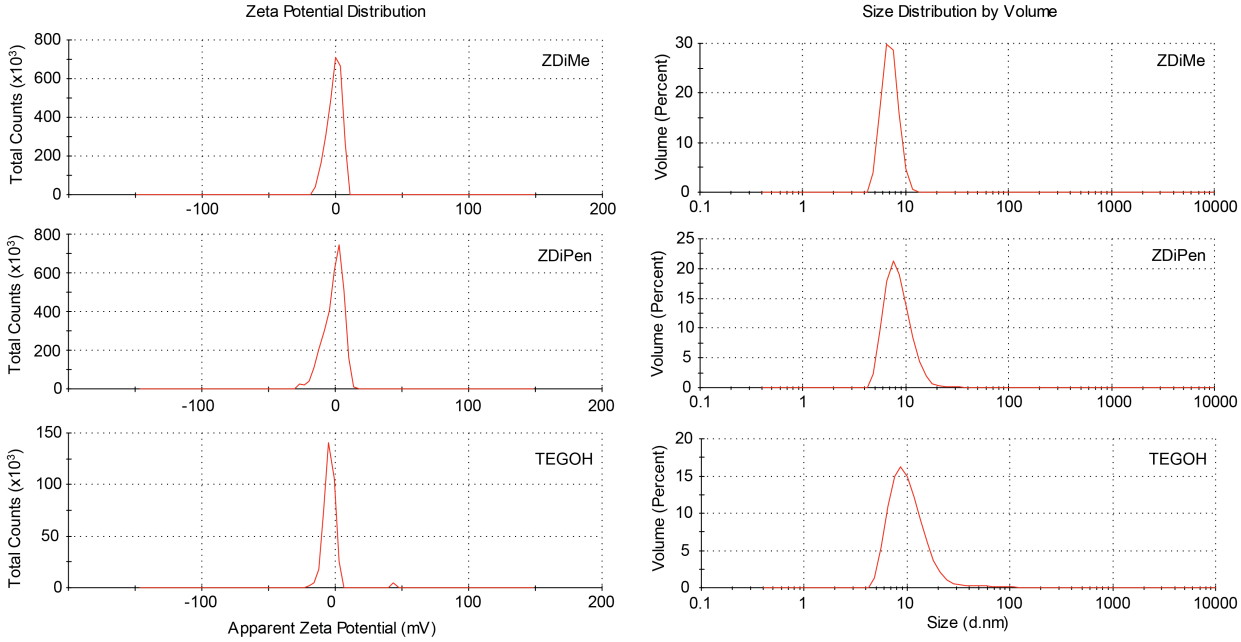

Figure S1. Hydrodynamic size and zeta potential of the nanoparticles

***In vitro* cell studies:**

- *Cell culture*: J774.2 mouse monocytes and RAW 264.7 macrophage cells were purchased from Sigma-Aldrich. Cells were grown in Roswell Park Memorial Institute media (RPMI 1640) supplemented with 10% fetal bovine serum, 1% antibiotics (100 µg/ml penicillin and 100 µg/ml streptomycin) and sodium pyruvate, and incubated at 37 ^{0}C under a humidified atmosphere of 5% CO_2. Under the above culture conditions, the cells were subcultured once every four days.

- *Nanoparticle and LPS treatment:* J774.2 and RAW 264.7 cells were seeded at 10^6 cells/well in a 24-well plate for 5 h (overnight for RAW 264.7) and then incubated with the nanoparticles at a concentration of

50nM, with or without 1µl of LPS (1mg/mL, Enzo Life Sciences, isolated and purified from *Salmonella minnesota* R595) in 1 mL of the described media. The cells were incubated during 3 h and 24 h at 37 ^{0}C under a humidified atmosphere and 5% CO_2. After the incubation time, the plate was centrifuged at 2,000 rpm and the media was collected for TNFα measurements. Experiments were done in triplicate.

- *TNFα secretion*: TNFα secretion was measured by enzyme-linked immunosorbent assay (ELISA) at 3 h and 24 h using TNFα kits (BD Biosciences, CA, USA) according to the manufacturer's instructions. Briefly, maxisorb 96 well plates (Krackeler) were coated with a solution of purified hamster anti-mouse TNF (0.5 µg/mL in bicarbonate buffer pH=9.5, 100 µL per well) overnight. The solution was then discarded and the plate was washed 3 times with a solution of 0.05% Tween 20 in PBS. 200 µL of blocking buffer (1% BSA in PBS) was then added to each well and the plate was maintained at room temperature for 3 h. The plate was washed again and 100 µL of the media samples were added to each well and kept overnight at 4 °C. The plate was then washed 3 times and 100 µL of a solution of biotin human anti-mouse TNF (0.5 µg/mL in assay diluent of 10% FBS in PBS) were added to each well and maintained at room temperature for 2 h. The solution was discarded and the plate washed 3 more times, followed by the addition of a solution of Streptavidin HRP in assay diluent and kept 2 h at room temperature. Finally, the plate was washed 3 times and 100 µL of a solution of TMB substrate (prepared according to the protocol of the manufacturer, BD OptEIA) was added, followed by the addition of 50 µL of 1 M sulfuric acid when the blue color developed. The adsorption at 450 nm was recorded and compared to a ladder of standards added and treated as described in the same plate. When required, samples were diluted in RPMI 1640 media. NPs at the concentrations of the study did not interfere with the readings. (Figure S2. a-d)

- *Cell viability*: After removing the supernatant media, 1 mL of PBS buffer was added to each well (of the 24 well/plate) and cells were redispersed carefully. 10 µL of the solution of cells was added to 90 µL of a solution of trypan blue (0.1 µM), and live/dead cells were counted with a hemocytometer. (Figure S2. e-f)

- *Cellular uptake*: After the cells were incubated with the gold nanoparticles and the supernatant media was removed, the remaining cells were carefully washed 3 times with PBS to remove NPs that were not uptaken. 250 µl of lysis buffer was then added to each well and the plate was maintained at -20 ºC for further treatment. The quantitative cellular uptake of gold nanoparticle was determined by ICP-MS. Briefly, the cells in one well were totally transferred to metal ion-free tube, and gold was dissolved by freshly prepared Aqua Regia solution (HCl: HNO_3= 1:3) for 15 min. Then, ultrapure water was added until 10 mL, and the content of gold in the samples was analyzed by ICP-MS. (Figure S2. g-h)

- *Reactive Oxygen species (ROS) generation*: ROS were determined by the use of 2',7'-dichlorodihydrofluorescein diacetate. Briefly, J774.2 cells were plated into a 96-well plate (10^5 cells/well) using similar conditions and time points as before. After the incubation period, cells were wash with PBS 3 times and were subsequently treated with the fluorescein dye (150 µL/well, 10µM). After incubating for 30 min, the cells were washed with PBS (3 times) and 150 µL of lysis buffer was added to each well. The solution was then transferred to a 96 black well plate and the fluorescence intensity, resulting from the oxidation of dye, was recorded (excitation/emission: 488 nm/520 nm).

- *Cell metabolism test (MTS):* J774.2 cells were plated in a 96 well plate using the same conditions as ROS. After washing the cells 3 times with PBS, 100uL of cell culture media with no serum and 20 µL of the MTS solution (Cell Titer 96R Aqueous One Solution Cell Proliferation Assay Kit - Promega, USA) were added to each well. The samples were then incubated for 1h at 37 °C in a humidified atmosphere containing 5% of CO_2. After the incubation time, the absorbance of the samples was recorded (490 nm).

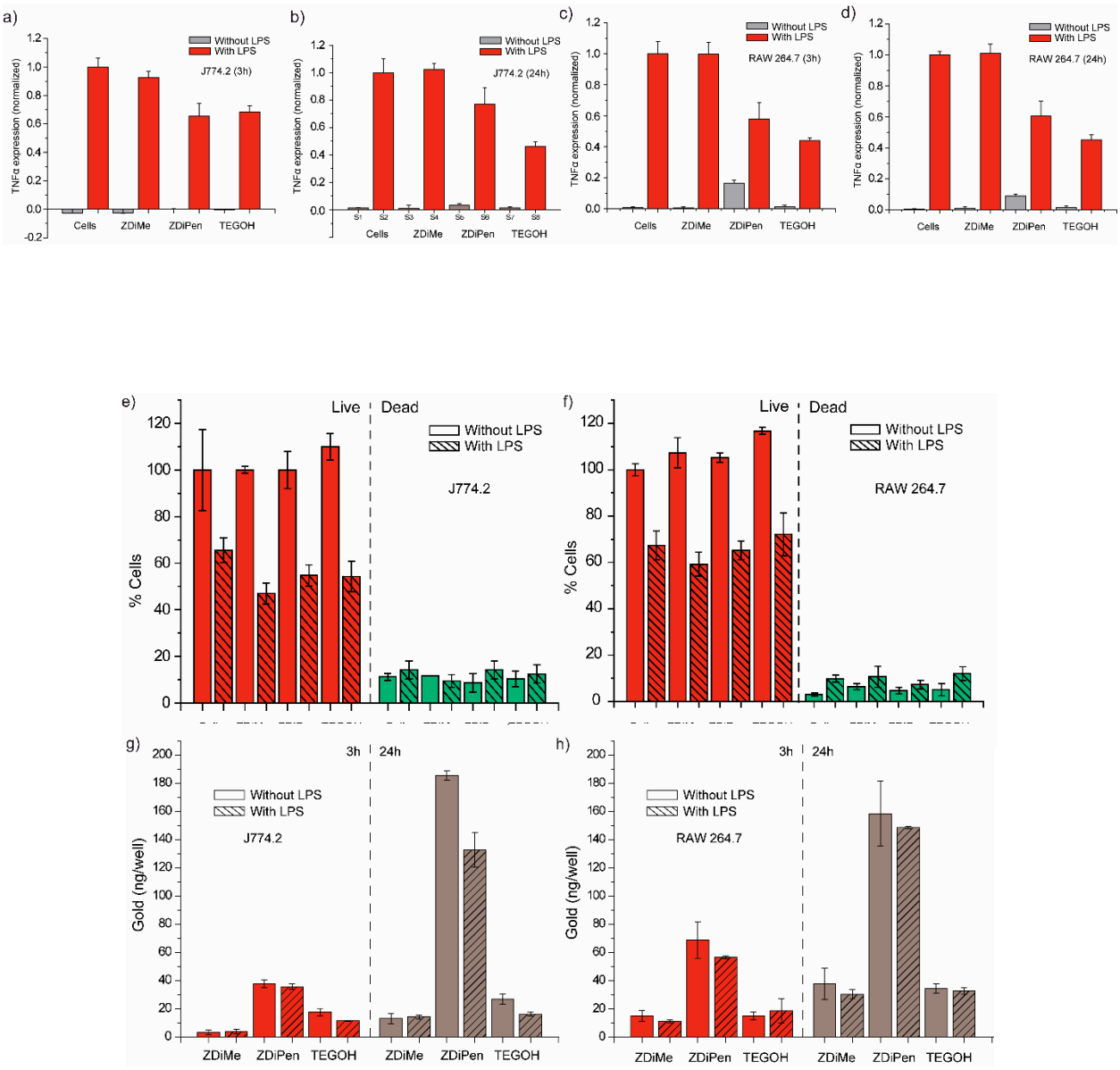

Figure S2. a-d) TNFα secretion of J774.2 and RAW 264.7 cells at 3h and 24h. e) Cell viability of J774.2 cells. f) Cell viability of RAW 264.7 cells. g) Cellular uptake of the NPs for J774.2 at 3 h and 24 h. h) Cellular uptake of the NPs for RAW 264.7 cells at 3 h and 24 h.

***In vivo* animal studies:**

- *Animal model*: C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and were allowed to rest at least one week in the animal facilities before any procedure was performed. All the animal procedures were performed according to protocol IACUC 2014-0060 at the animal facilities of the University of Massachusetts Amherst.

- *LPS dose study*: To assess sensitivity to lipopolysaccharide, solutions of different concentration of LPS in sterile PBS were injected via the peritoneum (IP), and 500 µL of blood were extracted by heart puncture after the animals were sacrificed (CO_2 chamber followed by cervical dislocation), 2 h after the injection. Blood was allowed to clot for 1 h at 37 °C and then overnight at 4 °C. Blood was then centrifuged to separate the clots and the supernatant serum was carefully removed and stored at -20 °C, until TNFα secretion measurements were performed following the ELISA procedure described previously

(*vide supra*). 3 mice per LPS concentration were used. Figure S3a depicts the TNFα secretion for different concentrations of LPS. Upon these results, 200 ng/mice was the selected dose for the studies.

- *Nanoparticle and LPS treatment:* Following the animal procedures described before, 100 µL of NPs (5 µM) were injected via the peritoneum, followed by an IP injection of 200 µL of a solution of LPS (1 ng/µL). As controls, untreated mice were sacrificed directly (negative), a group was injected only with LPS (positive), and 3 more groups were injected with NPs only (one group per nanoparticle, 3 mice per group), Figure S3b depicts the procedure for each group. After 2 h, mice were sacrificed and blood was extracted and treated as described before for TNFα detection. In addition, mice were dissected and the heart, lungs, pancreas, kidneys, spleen, intestine and brain were removed for biodistribution studies.

- *Organs treatment (biodistribution)*: the extracted organs were weighed and transferred to metal ion-free tubes, and the organs were dissolved by a solution of 1:3 H_2O_2 to HNO_3 overnight. The samples were then treated with Aqua Regia and diluted in ultrapure water as before, for further analysis of gold content by ICP-MS (Figure S3c).

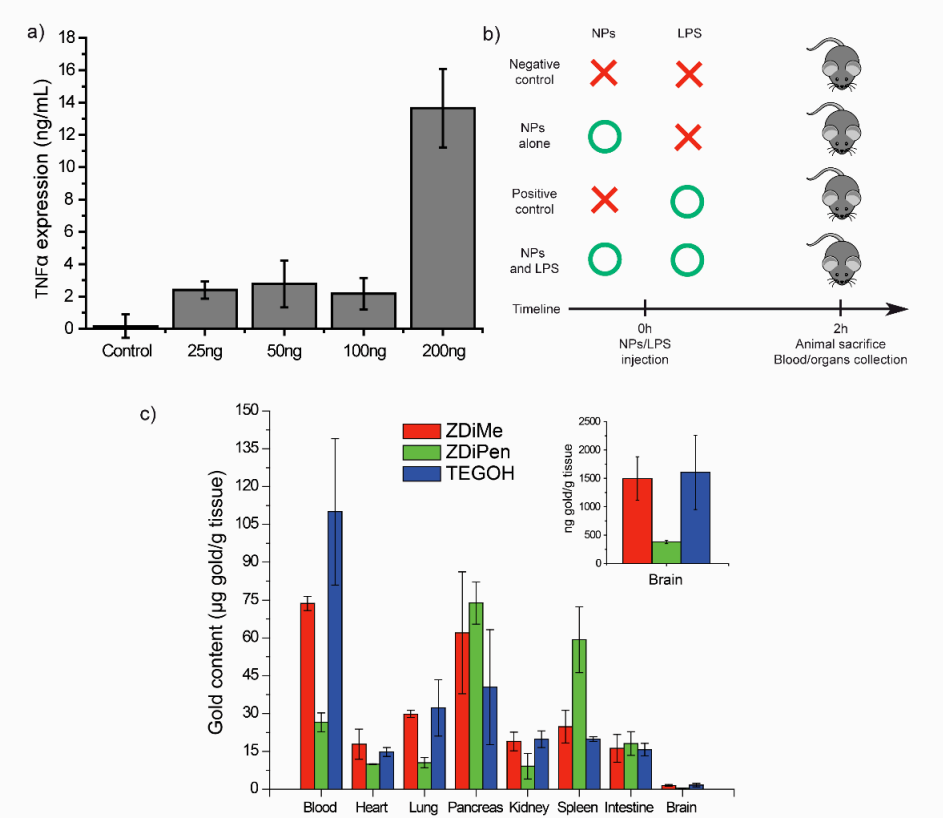

Figure S3. a) LPS dose study. b) Animal studies scheme. c) Bio-distribution of the different nanoparticles.

SUPPLEMENTAL REFERENCES

(1) Brust, M., Walker, M., Bethell, D., Schiffrin, D. J.,Whyman, R. (1994) Synthesis of thiol-derivatised gold nanoparticles in a two-phase Liquid–Liquid system. J. Chem. Soc., Chem. Commun, 801-802.

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