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CHAPTER 5

GNPs decorated with ovalbumin-derived epitopes:
effect of shape and size on T-cell immune responses

5.1 Abstract

Due to their ease of detection, unique optical properties, and large surface areas, gold nanoparticles (GNPs) are attractive nano-sized delivery vehicles. They have been employed to study multiple biological processes including cellular uptake, immune response, nanoparticle-mediated cytotoxicity, and apoptosis. As GNPs can be manufactured in various shapes and their size is programmable, the effects that their size and shape impose on biological processes can be studied. However, there is currently no clear evidence that a certain shape or size of nanoparticle is beneficial. To address this issue, we have utilized peptide amphiphile-coated GNPs and gold nanorods (GNRs). GNPs of 15 and 40 nm in diameter and 40 by 15 nm GNRs were coated with thiolated peptide amphiphiles and functionalised with model epitopes derived from chicken ovalbumin (OVA₂₅₇₋₂₆₄ and OVA₃₂₃₋₃₃₉). These particles were evaluated for their cytotoxicity *in vitro* in bone marrow-derived dendritic cells (BMDCs), and the cellular uptake was visualized using transmission electron microscopy (TEM) and quantified with inductively coupled plasma mass spectrometry (ICP). Subsequently, epitope-decorated GNPs and GNRs were tested for their immunogenicity in an *ex vivo* assay using transgenic ovalbumin mice (OT-I and OT-II). By using two distinct epitopes, it was possible to draw conclusions regarding the impact of shape and size of the nanoparticles on different aspects of the immune response.

Our findings indicate that the shape and size of GNPs and GNRs does indeed influence immune response. We were able to draw the following conclusions: (i) there is a size threshold below which antigen delivery efficacy drops (~15 nm in diameter), and (ii) there is a mass (or volume) threshold below which no effective antigen presentation *via* the major histocompatibility

complex class I (MHC-I) is possible (~40 nm in diameter). We believe these findings will have implications for vaccine development, and lead to a better understanding of cellular uptake as well as antigen egress from lysosomes into the cytosol.

5.2 Introduction

The field of nanomedicine focuses on the use of nanoparticles to diagnose, monitor and treat disease. Within this field, there is a particular focus on using nanoparticles as drug delivery vehicles or as vaccine platforms.¹⁻⁵ One reason that nanoparticles are suited to this purpose is that their nanometer size range imposes certain interactions between the particles and biological systems, for instance cellular uptake pathways, recognition by immunocompetent cells and clearance from the blood flow.⁶⁻⁸ However, researchers have reported contradictory data on the effects of nanoparticle size and shape on immune response,⁹ or effects of nanoparticle charge on cellular uptake, clearance, and immune response,¹⁰ and cytotoxicity.¹¹⁻¹⁵ Often, these reports do not provide sufficient information on the antigen loading or particle concentration. Literature may also lack normalization of the obtained results to a common parameter to aid comparison. Moreover, it is not always possible to find only one determinant parameter attributed to the biological effect of the studied nanoparticles. Parameters including size,¹⁶ shape,^{2, 17} charge,^{11, 18} rigidity,⁶ drug or antigen loading,^{1, 2} particle concentration,^{19, 20} total mass of nanoparticles,^{12, 21} and mean surface area available for contact^{2, 19} have all been proposed to affect the biological fate of studied particles.

Studying the effect of all these parameters on vaccine efficacy is an enormous undertaking. In order to make a new system suitable for screening the effects of the abovementioned parameters, a new approach is needed. This approach has to allow for thorough control over one parameter while other parameters, such as size, shape, charge, and/or surface chemistry are fixed. Gold nanoparticles (GNPs) can be synthesized in a range of sizes and

shapes therefore they are ideal for studying the shape and size dependency of a biological response. Through the use of different sizes and shapes of GNPs, the dependence of cellular uptake,^{17, 20, 21} cytotoxicity,^{12, 17} and immune response²² on physico-chemical properties can be studied. In addition, the surface chemistry of GNPs can be easily modified, and as a result, low toxicity and modulation of chronic inflammation risk caused by the antigen delivery particles are expected. Another advantage is that antigens are displayed on the GNP surface and cannot be compromised by non-specific interactions with the delivery vehicle itself. For example, peptide-decorated liposomes can show partial encapsulation of the displayed peptides *via* engulfment into the lipid membrane.²³

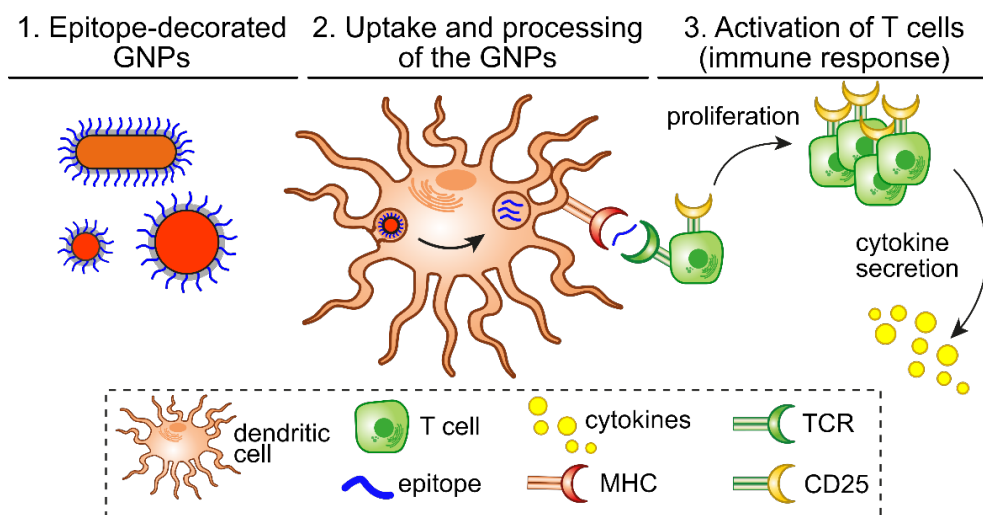
In this chapter, we studied the effect of nanoparticle shape and size on antigen processing and presentation. For this, gold nanorods (GNRs) of 40 by 15 nm in size and spherical GNPs of 15 and 40 nm in diameter were synthesized (**Scheme 5.1** and **Scheme 5.2**). The choice of these sizes was dictated by multiple lines of evidence showing that nanoparticles of 40-50 nm in diameter exhibit more efficient cellular uptake^{7, 20} and immune response compared to other sizes.^{2, 9} GNPs with a diameter of 15 nm are often used in literature^{3, 16, 18, 24} and were chosen to serve as a reference for GNRs' width. GNRs were introduced to elucidate the effect of shape and their length matched the diameter of the larger GNPs. Incorporation of two spherical particle sizes matching the GNRs dimensions was intended to decouple the effects of shape and size.

A protective coating on the gold surface is required to maintain particle stability in biological media, to prevent aggregation, and to eliminate potential cytotoxicity.^{11, 18, 24-27} It was previously shown that, depending on the aggregation state, endocytosis pathways may vary for the same nanoparticle

formulation.²⁸ In addition to stabilizing molecules, antigens can be attached to the GNP surface to modulate antigen processing in dendritic cells (DCs).¹ Using a common stabilizing molecule for the three selected nanoparticle types results in an identical surface chemistry allowing for a fair comparison of the elicited cellular responses. Previously, we reported the use of thiolated peptide amphiphiles as stabilizers for both GNPs and GNRs (**Chapters 3 and 4** of this thesis).²⁹ These coatings insulate the gold surface, prevent particle aggregation under harsh conditions (up to 3 M NaCl, or 1 M competing thiol), and provide GNPs with surface chemistry described as “protein-like”.³⁰ Published data suggests that active moieties displayed on the surface of peptide-capped GNPs preserve their biological function and their availability.³⁰⁻³³ Several reports suggest that GNPs do not impair immune functions of DCs or B cells.^{18, 25, 26} Unlike most other metallic nanoparticles, GNPs do not give rise to reactive oxygen species (ROS) *in vitro*, hence oxidative stress and ROS-related cytotoxicity are less common.²⁴

In this study, 15 nm and 40 nm GNPs and 15 by 40 nm GNRs were coated with a stabilizing peptide amphiphile,²⁹ and model epitopes derived from the chicken ovalbumin (OVA) protein (OVA₂₅₇₋₂₆₄ or OVA₃₂₃₋₃₃₉, **Scheme 5.2**). The peptide amphiphile stabilizer, herein referred to as the **base** molecule, comprised a doubly-thiolated C11 alkyl chain conjugated to a KVVVAAEEEE peptide sequence *via* the N-terminus and the side-chain of the lysine. The two bioactive peptide amphiphiles were bound *via* Au:S bonds to form epitope-decorated GNPs and GNRs. Both peptide amphiphiles comprised a thiolated C11 alkyl chain, a VVVVAAEEEE peptide sequence, a tetraethyleneglycol spacer, and one of the two epitope sequences. The bioactive peptide amphiphile coupled to OVA₂₅₇₋₂₆₄, the major histocompatibility complex class I (MHC-I) restricting epitope, was

abbreviated as **1**, and the peptide amphiphile coupled to OVA₃₂₃₋₃₃₉, the major histocompatibility complex class II (MHC-II) restricting epitope, was denoted as **2**. Since the same coating procedure was applied to all chosen GNPs, their immunological effects could be compared on the basis of antigen loading or available area.



Scheme 5.1. Immune response to epitope-decorated GNPs was studied in 3 steps: (1) GNPs of different shape and size were synthesized and coated with epitope/base constructs. Two epitopes were chosen as models: OVA₂₅₇₋₂₆₄ and OVA₃₂₃₋₃₃₉. (2) Uptake of the epitope-decorated GNPs by dendritic cells (DCs) was studied. (3). Activation of specific T-cells by DCs *via* MHC/epitope/T-cell receptor (TCR) contact led to T-cell proliferation and release of cytokines.

Immunogenicity of the epitope-decorated GNPs and GNRs was studied using cells derived from the ovalbumin transgenic OT-I and OT-II mice. T cells of these transgenic mice express T-cell receptors (TCRs) that are specific for either OVA₂₅₇₋₂₆₄ (OT-1) or OVA₃₂₃₋₃₃₉ (OT-2) epitopes and pair with either CD8 or CD4 coreceptors.³⁴ Previously, TCR-ligand interactions, and the T-cell activation, as well as their function in the development of autoimmunity and tolerance were studied using these mouse lines.^{1, 16, 35-37} ‘Splitting’ the immune response into two components (OVA₂₅₇₋₂₆₄ modulates

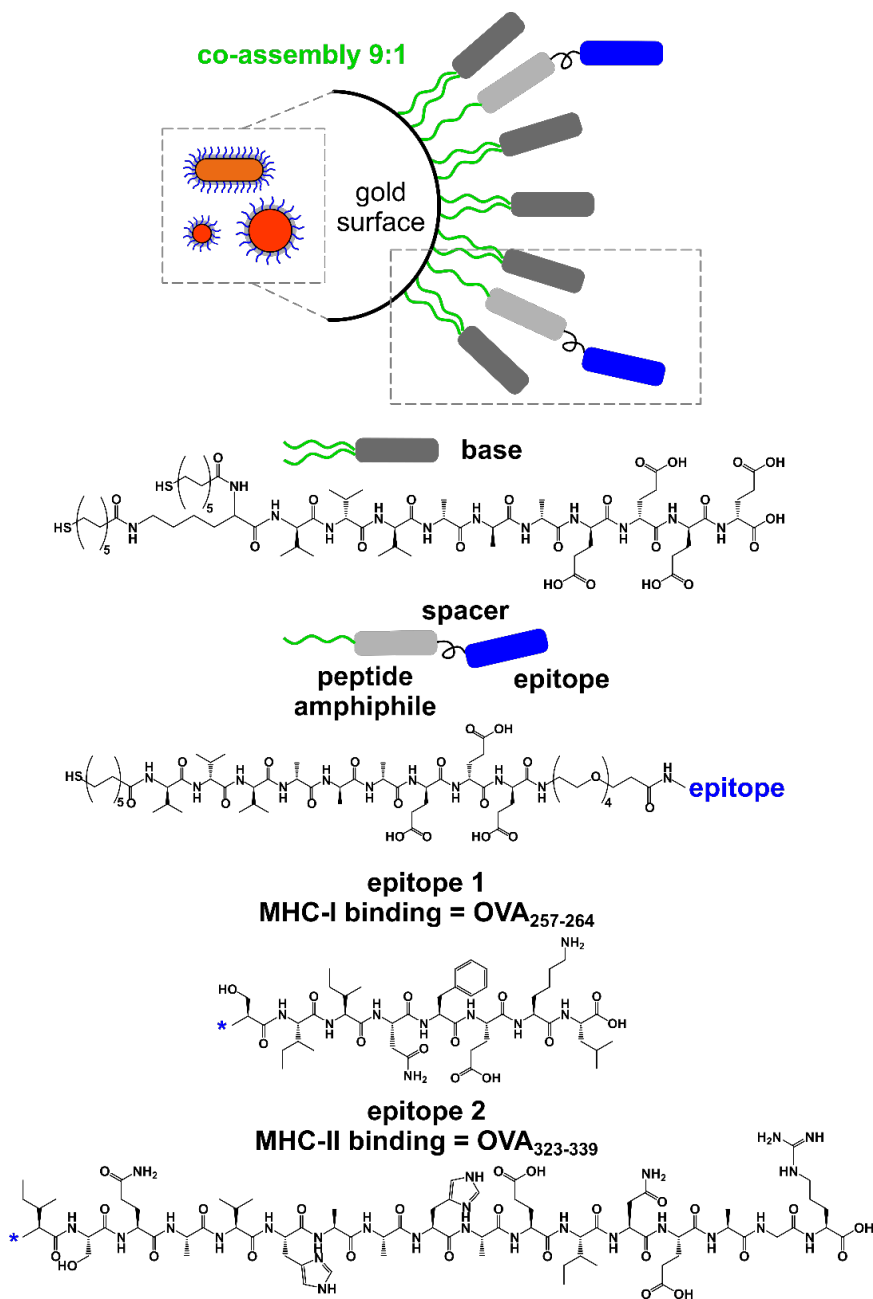
cytotoxic T-cell response, while OVA₃₂₃₋₃₃₉ – is a helper T-cell response) should provide more clarity as to how the immune system reacts to more complex antigens, depending on their size and shape.

This study covers three distinct stages in the immune response pathway summarized in **Scheme 5.1**. Before studying the elicited immune responses, the peptide amphiphile-coated GNPs and GNRs were characterized using UV-Vis spectroscopy and transmission electron microscopy (TEM). Next, cellular uptake and cytotoxicity of these GNPs and GNRs were evaluated in a population of DCs derived from mouse bone marrow (bone marrow-derived dendritic cells, BMDCs). After incubation with BMDCs, the cellular uptake by these DCs was studied with TEM and inductively coupled plasma mass spectrometry (ICP-MS). Upon cellular uptake, antigen-decorated nanoparticles are delivered into the cells. To initiate an immune response, antigens are cleaved and processed into epitopes inside lysosomes or in the cytosol to be later presented in the groove of MHC-II (inside lysosomes) or MHC-I (in the cytosol).³⁸ Next, an immune response is triggered by contact of the epitope-loaded MHC with a TCR of specific T cells and is accompanied by cytokine release and upregulation of cellular markers.³⁹ In this study, DCs activation by OVA epitope-decorated GNPs and GNRs and antigen processing were quantified using enzyme-linked immunosorbent assay (ELISA) and flow cytometry. The capability of epitope-decorated GNPs to mediate effective antigen processing and MHC/epitope/TCR binding, as well as cause CD25 upregulation (a T-cell activation marker) was assessed using flow cytometry.

5.3 Results and Discussion

5.3.1 Characterization of peptide-gold conjugates.

In order to keep the surface chemistry identical, GNPs of different shape and size were coated with a mixture comprising 9 molar parts of the **base** peptide and 1 molar part of the active peptide **1** or **2** (**Scheme 5.2**). The **base** composition was inspired by previous work (see **Chapters 3** and **4** of this thesis) and comprised a double thiolated C₁₁ chain conjugated to a KVVVAAAE₅ peptide domain *via* the N-terminus and the lysine side-chain.²⁹ The thiol group enabled coordination of the ligands to the gold surface *via* an Au-S bond. This peptide amphiphile formed a self-assembled monolayer on the gold surface and stabilized GNPs in the 20-100 nm size range. The active peptides comprised a single thiolated C₁₁ chain conjugated, *via* the N-terminus, to a peptide with the sequence V₃A₃E₃ while the epitopes were coupled to the C-terminus of this peptide sequence *via* a PEG₄-spacer. Epitope sequences corresponded to OVA-derived T-cell epitopes (SIINFEKL, OVA₂₅₇₋₂₆₄, is an MHC-I binding epitope, and ISQAVHAAHAEINEAGR, OVA₃₂₃₋₃₃₉, is an MHC-II binding epitope). The active peptides **1** and **2** were conjugated to a single alkyl chain instead of a double chain to aid solubility of the resulting compounds.



Scheme 5.2. Schematic representation of the shell composition (**base** peptide and epitope-bound peptides **1** and **2**) used to stabilize GNPs and GNRs. **Base** peptide was mixed with either **1** or **2** at a 9:1 molar ratio to form shells around GNPs and to provide near-identical surface chemistries.

The stabilizing effect of the **base** sequence was ascribed to increased hydrophobicity within the shell around the gold core and effective self-assembly (up to 73% β -content within the shell).²⁹ It was anticipated that addition of 10 mol % of single chain amphiphiles **1** or **2** to the double chain **base** amphiphile would not disturb peptide amphiphile self-assembly on the gold surface. The resulting assemblies (in the absence of gold) were probed with circular dichroism spectroscopy to confirm this assumption (CD, **Figure S5.1**).

Peptide amphiphiles were synthesized using protocols described in **Chapter 3** of this thesis, dissolved in 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP), mixed in a 9:1 ratio and the solvent was evaporated yielding a thin film. Upon rehydration with PBS (pH 7.2), supramolecular assemblies were formed. The **base** sequence in PBS adopted a typical β -structured profile as evidenced by the pronounced ellipticity minimum at 222 nm. Incorporation of 10 mol % of **2** did not affect the overall spectral shape, or position of the ellipticity minimum, indicating that **2** did not interfere with the self-assembly of the **base** amphiphile. However, **1** did influence the resulting assemblies to some extent: the ellipticity minimum did not shift, but the band became narrower and its intensity increased in comparison to the effect **2** demonstrated. This suggests a decrease in contributions from other secondary structures. Absence of unordered structures or significant changes in the CD spectra caused by addition of **1** or **2** implies that the mode of self-assembly was preserved, and it was not disrupted by interactions with the epitopes. Our previous study showed that the self-assembly behavior of these peptide amphiphiles in solution was translated well to self-assembly on the gold surface.²⁹ In this study, the peptide amphiphiles mixtures were deemed to follow the same trend. Thus, when the epitopes are displayed on

the GNP surface, they are expected to be available for interaction and not buried within the peptide amphiphile shell.

Citrate-stabilized GNPs of different sizes and CTAB-protected GNRs were prepared using standard protocols described in **Chapter 3** and **Chapter 4** of this thesis. GNPs were characterized with dynamic light scattering (DLS), ultraviolet-visible spectroscopy (UV-Vis), and transmission electron microscopy (TEM) to confirm their size and monodispersity (**Table 5.1**, **Figure 5.1**, **Figure S5.2**). Due to their rod-like shape, GNRs were not analyzed by DLS, instead only UV-Vis spectroscopy and TEM data were collected.

Table 5.1. GNPs size characteristics before/after modification using TEM and DLS.

Sample	15 nm GNPs	40 nm GNPs
Av. size of the core (TEM)	15.0 ± 1.4*	43.5 ± 3.3
Citrate	15.5 ± 0.2	40.4 ± 0.5
Base	26.8 ± 0.3	50.8 ± 0.1
1	25.9 ± 0.3	50.8 ± 0.8
2	26.6 ± 0.6	54.3 ± 0.2

To determine the core size, citrate-stabilized GNPs were imaged using TEM. *-standard deviation. The size of peptide amphiphile-coated GNPs was determined using DLS. Both average size and mean hydrodynamic diameter are given in nm. Peptide amphiphile-coated samples were prepared in PBS (pH 7.2).

Upon modification with the peptide amphiphiles, a slight increase in size for all peptide-gold conjugates was observed with DLS. The incorporation of the extended sequences **1** or **2** (10 mol%) into the **base** peptide shell did not lead to agglomeration of GNPs (**Figure 5.1**; for intensity size distributions see **Figure S5.2**). The UV-Vis spectroscopy supported these findings: the absence of plasmon band broadening for **15-base**, **15-1**, and **15-2** relative to **15-citrate** indicated that no aggregation occurred after the peptide coating

was applied (**Figure 5.1A**). The same was true for 40 nm GNPs and GNRs. The small (1-7 nm) red-shift of the plasmon band is related to substitution of citrate or CTAB on the gold surface with other molecules, which leads to a change in the dielectric constant.⁴⁰ Since neither DLS (**Figure S5.2A,B**) or TEM (**Figure 5.1B**) indicated aggregation, the peptide-coated GNPs and GNRs were presumed to be monodisperse. TEM imaging revealed particle clustering, which can be explained by drying effects during deposition of samples on a TEM grid. This clustering was also facilitated by the presence of salts, since all tested samples were prepared in PBS (pH 7.2).²⁹ Zeta potential values of as-synthesized citrate-capped GNPs and amphiphile-coated GNPs showed that particles had a negative surface charge (up to -30 mV, **Figure S5.2C-E**). A slight deviation of surface charge for GNPs coated with **1** and **2** from the samples coated with the **base** sequence was explained by a slight change in the surface chemistry when the active peptides were incorporated in the peptide amphiphile shell. The GNRs showed a zeta potential change from positive to negative, indicative of CTAB displacement from the GNR surface by the peptide amphiphiles (**Figure S5.2E**). A negative net charge for peptide amphiphile-coated GNPs and GNRs is advantageous for two reasons: negatively charged particles cause less cytotoxicity compared to positively charged counterparts, and better cellular uptake than neutral nanoparticles.⁷

TEM analysis revealed the presence of well-defined peptide shells surrounding the gold core (**Figure 5.1B**). The shell around the GNPs and GNRs ensured that particles were separated from each other with the interparticle distance corresponding to the shell thickness. This thickness of the shells (<5 nm) is in agreement with a peptide-amphiphile monolayer around the particles. It was previously shown that these peptide amphiphile monolayers are well-structured.²⁹ Notably, in the TEM images there is no

obvious difference in the shell thickness for samples with the functional peptides **1** or **2** incorporated, possibly due to a low epitope display density.

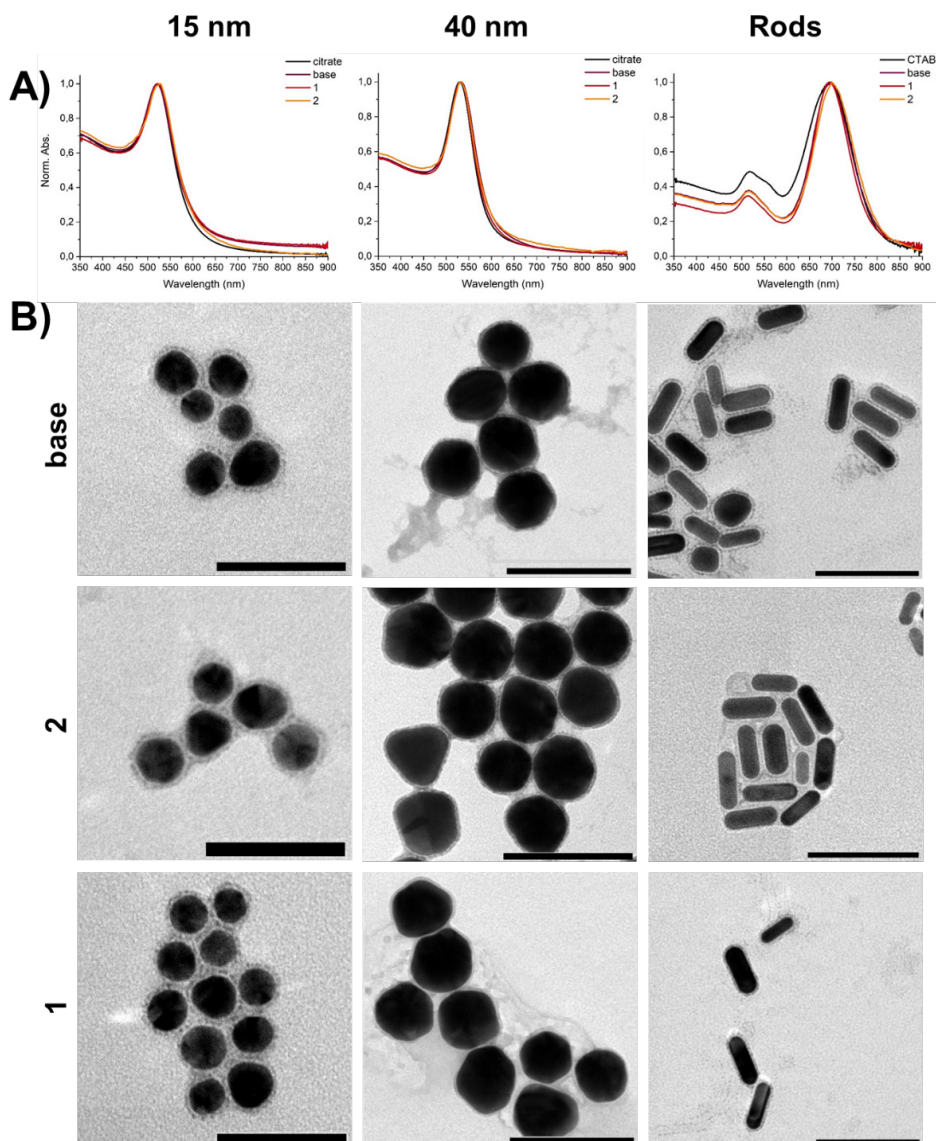


Figure 5.1. Sample characterization of the GNPs used in this study: (panel A) UV-Vis spectra for coated 15 nm GNPs, 40 nm GNPs, and GNRs; (panel B) TEM images of the peptide-gold conjugates evaluated in this work. Modified samples were prepared in PBS (pH 7.2). Scale bars: 50 nm for **15-base**, **15-1**, and **15-2**; 100 nm for others. Staining – 1% uranyl acetate. The visible shells are the peptide coatings.

According to our previous work, GNPs coated with these amphiphilic molecules are highly stable at elevated salt concentrations and are resistant towards thiols,²⁹ thus the GNPs and GNRs presented in this work were expected to be stable under biologically relevant conditions.

5.3.2 Number concentration and epitope dosage

To study biological effects elicited by nanoparticles of different size and shape, a defined parameter should be adopted to enable comparison. It can be particle number, particle area available for interaction,^{2, 19} or drug/active molecule loading/dosage.^{19, 20, 41} Unfortunately, reports with a normalization used to truly determine the effect of a particle parameter (e.g. of an antigen loading rather than particle mass) on biological response are scarce.^{2, 12, 21} To calculate the above-mentioned parameters, the number concentration of the nanoparticles should be known. For this, inductively coupled plasma mass spectrometry (ICP-MS) is the most appropriate technique. This technique has also been employed to determine the thiol-containing ligand coverage on GNPs,⁴² as well as metal content in cells and tissues.^{2, 12, 16, 18, 19, 24, 43}

Peptide amphiphile-coated GNPs and GNRs were digested in *aqua regia* and analyzed with ICP-MS to determine gold mass concentration, which was later translated to particle number concentration (**Figure S5.3** and **Table S5.1**). Determined concentrations were linked to the optical density (OD) of the GNP or GNR suspension (**Table 5.2**). This enables accurate dosing of the GNP and GNR samples according to their OD, acquired with UV-Vis spectroscopy. We determined that a suspension of peptide amphiphile coated GNPs with an $OD_{LSPR} = 1$ corresponds to number concentrations within the 10^{11} - 10^{12} particle/mL range regardless of the

applied coating, which is in agreement with published data for citrate-capped GNPs.⁴⁴ Moreover, normalized number concentrations for GNPs with the same gold core but different peptide amphiphile coatings showed similar values, signifying that the employed coating strategy did not result in different optical properties arising from the applied stabilizer. Furthermore, incorporation of 10 mol % of the epitope-displaying peptide amphiphile **1** or **2** did not affect the optical properties either.

Table 5.2. Number concentrations and epitope dosage of GNPs and GNRs.

	Number concentration, particles/mL			Epitope dosage, µg/mL		
	15 nm GNPs	40 nm GNPs	GNRs	15 nm GNPs	40 nm GNPs	GNRs
base	4.03×10 ¹²	1.49×10 ¹¹	3.54×10 ¹¹	n/a	n/a	n/a
1	3.83×10 ¹²	1.25×10 ¹¹	4.06×10 ¹¹	2.5	0.4	0.6
2	3.46×10 ¹²	1.46×10 ¹¹	3.93×10 ¹¹	4.2	0.9	1.1

Samples were prepared in PBS (pH 7.2). For normalized mass concentrations and areas available for contact, see **Figure S5.3B,C**. The obtained mass concentrations were divided by the mass of one particle and normalize to OD_{LSPR} = 1.0.

To quantify the epitope dosage another peptide amphiphile was synthesized. Mercapto-undecanoyl-V₃A₃E₃G₅WG (denoted as **W**, see **Scheme S5.1**) was composed of a single C₁₁ alkyl chain, the same peptide domain as **1** or **2**, and a single Trp (tryptophan, W), conjugated *via* a Gly₅ spacer (glycine, G). GNPs and GNRs were coated with 9 molar parts of the **base** amphiphile and 1 molar part of **W**. The amount of the unbound peptide was determined through an absorbance measurement at A₂₈₀ (ε₂₈₀=5600 M⁻¹cm⁻¹) and by relating this to the Lambert-Beer law, the concentration of unbound (and hence bound) peptide could be calculated. From the amount of the bound **W** together with the known relationship between OD and number concentration,

the epitope loading per mL of a sample and/or epitope molecules per particle was calculated (**Table 5.2**).

Due to high relative surface area and the high number concentration, 15 nm GNPs carry a significant epitope load, while carrying a low number of epitopes per particle. Consequently, 40 nm GNPs had a lower number concentration but higher antigen load per particle. Due to their shape, GNRs have a higher relative surface area and number concentration than 40 nm GNPs.

For *in vitro* experiments a defined concentration range was used in order to determine a dose-response effect of GNPs of different shape and size. The selection was based on the epitope loading, while the other parameters including particle number concentration and OD are shown in **Table S5.2**. The dosage window for GNPs and GNRs decorated with OVA₂₅₇₋₂₆₄ covered the 0.03-1.89 µg/mL range, while for OVA₃₂₃₋₃₃₉ it was 0.06-3.15 µg/mL (molar concentration ranges were identical). For studies of cytotoxicity and cellular uptake in bone marrow-derived dendritic cells (BMDCs) only the highest concentrations, and 4x, and 16x dilutions were tested. For OT-I and OT-II experiments a serial dilution (1x, 2x, 4x, 8x, and 16x) was used.

5.3.3 Cytotoxicity of coated GNPs and GNRs

An *in vitro* cytotoxicity test was performed prior to studying immune response, to ensure that the peptide amphiphile-coated GNPs and GNRs were not harmful to cells. BMDCs were used in this assay for two reasons. BMDCs, like other types of dendritic cells (DCs), are antigen-presenting cells and are the cell type most likely to first encounter pathogens. Moreover, the *ex vivo* assay using transgenic ovalbumin mice (OT-I and OT-II) to probe the immunogenicity require DCs treatment as the first step.

The assay consisted of the following steps: BMDCs were differentiated from bone marrow progenitor cells over the course of 10 days; BMDCs were exposed to peptide amphiphile-coated GNPs and GNRs at different concentrations for 4 hours; then the cytotoxicity was determined with a Lactate Dehydrogenase (LDH) assay. LDH is naturally present in the cytosol and is released into the cell culture upon cellular membrane damage caused by nanoparticle toxicity. The LDH level determined for BMDCs treated with PBS was taken as 0% cytotoxicity (negative control), while the LDH level determined for BMDCs treated with the assay lysis buffer was taken as 100% cytotoxicity (positive control).

The observed peptide-gold nanoconjugates cytotoxicity was found to be <10% after 4-hours of exposure and <20% after 24 hours for all tested GNPs and GNRs (**Figure 5.2** and **Figure S5.4**). This was true for all concentrations tested. Notably, **15-base** showed the highest toxicity, most likely attributed to the high number concentration of particles.^{19, 21} On average, cytotoxicity of spherical GNPs increased after 24 hours compared to 4-hours exposure for all tested particles. Moreover, there was no statistically significant difference between the cytotoxicity demonstrated by GNPs and GNRs after 24-hours exposure (only the highest concentrations were compared).

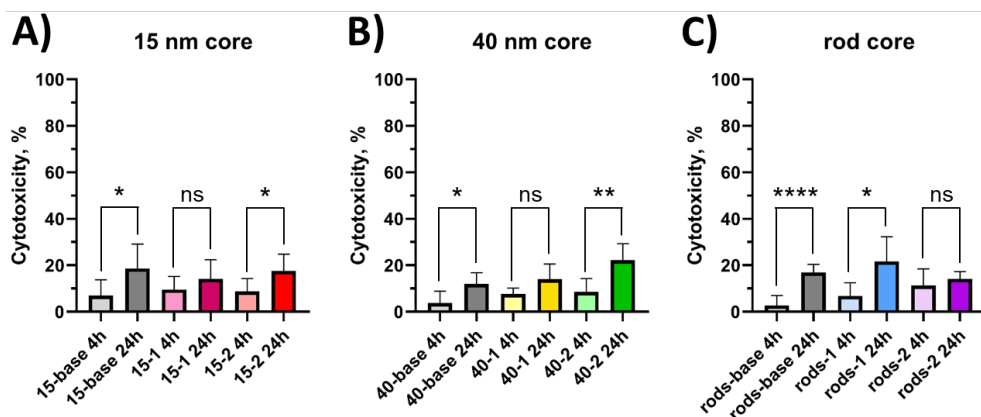


Figure 5.2. Cytotoxicity profiles for the peptide-coated GNPs and GNRs at the highest concentration used for each experimental group (**Table S5.2**) at 4 (light colors) and 24h (darker colors) exposure times: (A) 15 nm GNPs; (B) 40 nm GNPs; and (C) GNRs. Cytotoxicity was evaluated with the LDH release assay. Each data point was recorded in triplicate. The graphs present an average of two independent repeats; error bars indicate standard deviations calculated using GraphPad Prism software. Unpaired t-test was used to calculate the significance (ns = not significant, $p \geq 0.05$; * = p within 0.01-0.05; ** = p within 0.001-0.01; *** = p within 0.0001-0.001). For the cytotoxicity profiles covering the full nanoparticle concentration range see **Figure S5.4**.

This data is consistent with published results of different GNP samples in different cell types. PEGylated GNPs and GNRs were shown to have a limited effect on LDH release (<20% after 24-hours exposure)^{13, 24} and cell death (<15% after 24-hours exposure).^{1, 16, 18, 24} According to Fytianos *et al.* polymer-coated GNPs (15 nm in diameter) did not induce cell death independent of the surface charge,¹⁸ while Bhamidipati *et al.* reported that surface chemistry contributes towards cytotoxicity, but not the shape when spherical (20 nm in diameter), rod-like (53 by 23 nm), and star-like GNPs (63 nm) were compared.²⁴ Kang *et al.* reported that neither 10, 22, or 33 nm OVA protein-coated GNPs induced cytotoxicity.¹⁶ It has also been reported that PEGylation of GNRs abolishes cytotoxicity for CTAB-stabilized GNRs.^{24, 27} The results presented here suggest that employing thiolated peptide amphiphiles as GNP and GNR stabilizers abolishes the cytotoxicity reported

for citrate-capped GNPs and CTAB-protected GNRs. Additionally, changing the surface chemistry by addition of a bioactive peptide did not induce acute cytotoxicity.

5.3.4 Cellular uptake of OVA-decorated GNPs and GNRs by BMDCs

Cellular uptake defines the antigen fate inside the cell and is crucial for establishing a link between the evoked immune reaction and the nanoparticle type. Cellular uptake of peptide-gold nanoconjugates by DCs was quantified using ICP-MS and the subcellular localization of endocytosed GNPs and GNRs was visualized with TEM. For this study, BMDCs were differentiated from bone marrow progenitor cells and exposed to epitope-bearing GNPs and GNRs for 4 hours. Particles in exposure medium were removed and the cells were subsequently washed with PBS and examined as described in **5.5.11**.

In order to analyze subcellular localization of GNP- and GNR-treated cells were embedded in a resin, cut into thin (100 nm) sections, and stained. GNPs and GNRs were found inside subcellular vesicles but not dispersed in the cytosol, or accumulated in organelles (**Figure 5.3** and **Figure S5.5**). There was no apparent difference in nanoparticle distribution as a function of peptide amphiphile composition. However, the appearance of these vesicles and number of entrapped particles depended on the nanoparticle core shape and size. For example, **40-1** and **40-2** showed fewer particles per vesicle (<10) than **15-1**, **15-2**, **Rods-1**, or **Rods-2**. For **40-1** and **40-2** the volume of some vesicles was very low: particles appeared to be encased in the endosomal membrane. For 15 nm GNPs and GNRs, the vesicles looked

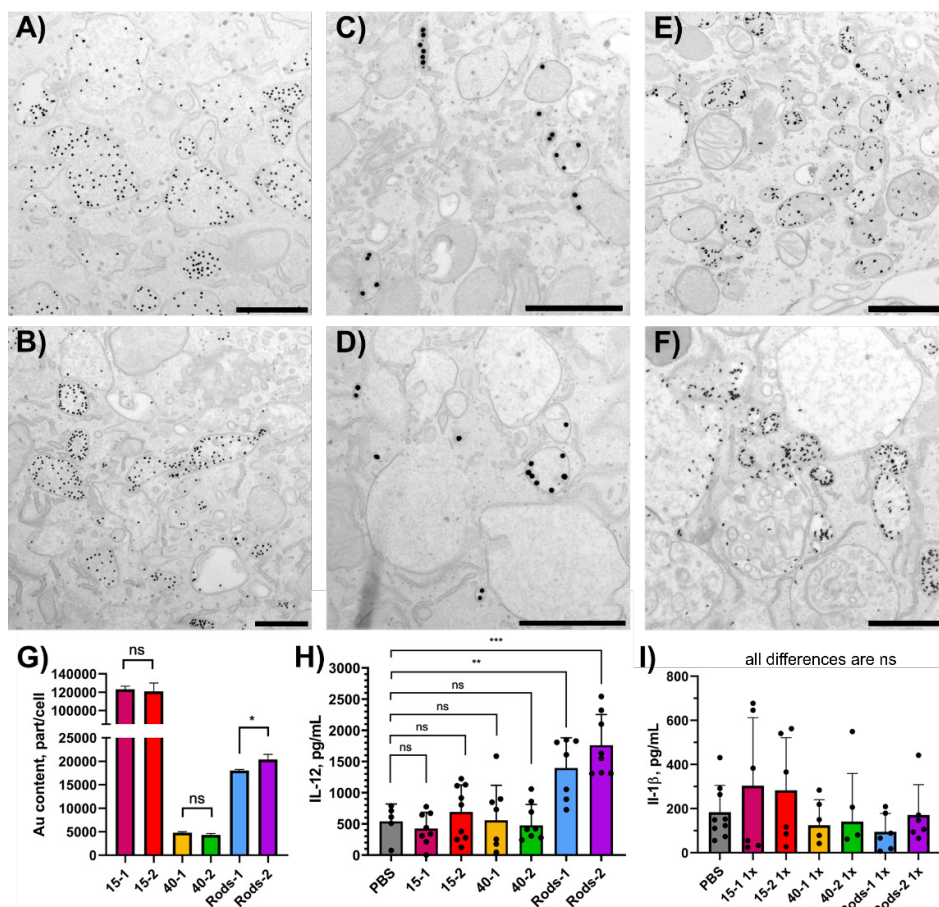


Figure 5.3. GNPs uptake was studied using TEM imaging (A-F), and quantified with ICP-MS (G), while BMDCs activation was checked via IL-12 (H) and IL-1β (I) levels in cell culture medium. Localization of GNPs and GNRs inside BMDCs sections after a 4-hour incubation was studied using TEM: (A) **15-1**; (B) **15-2**; (C) **40-1**; (D) **40-2**; (E) **Rods-1**; and (F) **Rods-2**. All studied particles were trapped in vesicles. Scale bars: 500 nm for A-B and E-F, and 1 μm for C-D. A representative image of a DC treated with PBS and low magnification images can be found in **Figure S5.5**. For more uptake quantification using ICP-MS, see **Figure S5.7** (measured in triplicate). Only IL-12 and IL-1β levels for the highest GNP concentration (1x) in exposure medium after a 24-hour incubation are shown in (H and I), for 4x and 16x concentrations see **Figure S5.9, S5.10** (for H, three independent experiments, three test wells each, were tested; for I, two independent experiments were tested; negative values are not shown; values above 3000 pg/mL of IL-12 were excluded). Error bars indicate standard deviations and were calculated in GraphPad Prism software, as well as the results significance (ns = not significant, $p \geq 0.05$; * = p within 0.01-0.05; ** = p within 0.001-0.01; *** = p within 0.0001-0.001). Unpaired t-test (G) or two-way ANOVA (H) were used to calculate the significance.

similar: larger vesicles with high particle numbers (>10) dominated, although small vesicles (< 100 nm) containing fewer particles were also observed.

The type and function of these vesicles could not be determined from TEM analysis, but these vesicles could be divided into several groups based on their size. Small vesicles with a low number of particles inside are most likely to be endocytic vesicles that are to be fused with endosomes to start the endo-lysosomal pathway.^{7, 8} Reformed endosomes undergo a maturation process and subsequently fuse with lysosomes. Lysosomes can be distinguished by their darker contents in the TEM images.⁴⁵ Reformed lysosomes perform antigen digestion, which is crucial for antigen presentation through MHC-epitope interactions. Analysis of the TEM images (**Figure 5.3**) for the vesicles' dimensions and number of entrapped particles showed differences between the different gold nanoparticles (**Figure S5.6,S5.7**). It should be noted that this analysis was based not on a full volume of a vesicle, but on its partial volume restricted to a thin section with a thickness of 100 nm. The analysis was carried out for nanoparticles decorated with **1** (OVA₂₅₇₋₂₆₄). Based on the analysis of 177 vesicles inside the thin sections of BMDCs exposed to **40-1**, there were no vesicles with more than eight particles inside and 65.5% of the vesicles contained only one particle (**Figure S5.6B,C**). Interestingly, it was possible to divide vesicles into groups based on their size and particle number. Small vesicles (40-120 nm) with low particle numbers (<6) are likely to be endocytic vesicles.

Vesicles with an average size up to 500 nm could be both endosomes and lysosomes, and large vesicles (up to 1200 nm) are either lysosomes or multivesicular bodies (MVB). It was also possible to detect fusion of two vesicles: presumably a small vesicle released its content into a larger vesicle (**Figure S5.7D**). On the other hand, **15-1** and **R-1** showed a different

behavior. Only 15.3% of vesicles ($n = 137$) contained one **15-1** particle, while for **R-1** this value was 13.0% ($n = 200$, **Figure S5.6A,B,E,F**). At the same time, vesicles with ≤ 10 entrapped particles were moderately present in BMDCs exposed to **15-1** (48.9%), but formed the majority in the case of **R-1**-exposed cells (74.0%). In some images it was possible to see formation of endocytic vesicles: **15-1** was endocytosed in vesicles with one particle inside or by interactions between the plasma membrane and multiple particles (**Figure S5.7A-C**). This suggests that multiple endocytosis pathways are involved for **15-1** uptake. Interestingly, **R-1** exposed cells showed the presence of endosomes with two kinds of membrane: rough and smooth, although no significant deviation in vesicle size was observed (**Figure S5.7E,F**). Also, nanoparticles were seen in the cytosol, but these events were rare ($<0.5\%$ for all samples).

These findings indicate that different endocytosis pathways might be involved in GNP/GNR uptake. Published data suggests the following pathways for coated GNPs and GNRs: receptor-mediated^{20, 41} and macropinocytosis²¹ for GNPs within the 15 - 40 nm size range; and receptor-independent²¹ and a combination of clathrin/caveolae- and lipid raft-mediated pathways for GNRs.¹⁷ Ding *et al.* also reported that endocytosis pathways for GNPs can be influenced through their surface chemistry.²¹ Solely based on the TEM images it was not possible to distinguish what specific endocytosis pathways peptide amphiphile-coated GNPs and GNRs employ. To address this, more uptake studies focusing on the use of endocytosis inhibitors should be conducted.^{17, 21, 46}

In order to study the immunological impact of nanoparticles of different size and shape, it was important to determine the gold content per cell. Quantification of cellular uptake based on the TEM images would not be

reflective of the entire cell population since the images provide information on only one section of a cell. For that reason, inductively coupled plasma mass spectrometry (ICP-MS) was used. BMDCs were subjected to GNPs in the same manner as described above: after a 4-hour incubation, the cells were thoroughly washed with PBS, and digested in *aqua regia*.

The gold content per cell was calculated by dividing the determined gold mass derived from gold mass concentration by the number of cells digested. Based on the dimensions of a nanoparticle, the mass of a single particle could be determined and thus the average number of particles per cell could be calculated. To make a fair comparison, the number of added nanoparticles was fixed to the gold core size, meaning the same number of **15-1** or **15-2** was added. After the 4-hour exposure time **15-1** and **15-2** were taken up to the same extent (123×10^3 versus 121×10^3 particle/cell), and 40 nm GNPs and GNRs showed the same trend (**Figure 5.3G**). This suggests that altering the active peptide in the shell composition did not have an effect on GNPs or GNRs uptake by BMDCs. It should be noted that 15 nm and 40 nm GNPs, although taken up in the same mass (in the range of 3.6-4.0 pg/cell), significantly differed in number (~25x times more 15 nm GNPs) and delivered total surface area (~3x times more for 15 nm GNPs, see **Figure S5.8**). The surface area available for contact that was delivered into the cells could be correlated to the number of delivered antigens and it decreases in the following order: 15 nm GNPs > GNRs > 40 nm GNPs. In our study epitope-decorated 15 nm GNPs could deliver ~3x times more antigens than 40 nm GNPs and ~2x times more than GNRs.

As part of the uptake studies, cytokine release by BMDCs was studied. Interleukin 1 beta (IL-1 β) and interleukin 12 (IL-12) cytokines provide information on activation of DCs by particulate formulations.^{2, 3, 18, 47, 48} For

this, BMDCs were exposed to epitope-decorated GNPs and GNRs at three concentrations (1x, 4x, and 16x) for 24 hours and the cytokine levels were determined with an enzyme-linked immunosorbent assay (ELISA) (**Figure 5.3H,I** and **Figure S5.9,S5.10**).

IL-12 is a cytokine secreted by DCs in response to Pathogen Recognition receptor (PRR) stimulation and promotes differentiation of naïve T cells into Th1 cells and stimulates production of IFN- γ from T cells.^{36, 47} IL-12 also mediates enhancement of CD8+ cytotoxic T lymphocytes activity. Compared to the PBS negative control, only GNRs showed significant levels of IL-12 secretion when comparing the highest concentration of nanoparticles in the exposure medium (**Figure 5.3H**). Interestingly, all tested concentrations of **15-1** and **15-2** failed to induce IL-12 secretion, which indicates that 15 nm GNPs do not activate DCs, although they showed potential in delivering high antigen loads (**Figure S5.9A**). On the other hand, 40 nm GNPs showed a peculiar behavior: high number concentrations had less impact than lower number concentrations. This was attributed to possible overstimulation of BMDCs, since it was determined that at these doses 40 nm GNPs do not cause cytotoxicity. It is remarkable that such low number concentrations of 40 nm GNPs were as effective as high number concentrations of GNRs (**Figure S5.9B,C**). GNRs showed a concentration-dependent effect on IL-12 secretion. Previously, protein-coated GNPs (40 nm in diameter) and gold nanocubes (40 by 40 nm) were reported to induce IL-12 secretion, but not smaller GNPs (20 nm in diameter) or GNRs.² This data therefore supports this previous assertion that small GNPs do not induce IL-12 secretion, but contradicts the previous report as GNRs are capable of inducing IL-12 secretion in this study.

Additionally, the capacity of epitope-decorated GNPs and GNRs to induce inflammation was studied through IL-1 β secretion (**Figure 5.3I** and **Figure S5.10**). None of the tested particles induced significantly elevated levels of IL-1 β compared to the negative control (PBS) indicating that the observed activation of BMDCs did not follow an inflammasome-mediated pathway. These findings are in agreement with previous studies using spherical GNPs: irrespective of the GNP size or coating, they did not induce inflammasomes formation.^{2, 18} Interestingly, there is no consensus between the results presented here and published data on GNRs. Depending on the coating, GNRs were reported to cause significant levels of IL-1 β secretion. For example, this was observed for GNRs coated with the West Nile virus envelope protein, while spherical and cubical gold nanoparticles did not induce IL-1 β secretion.² GNRs coated with recombinant Sm29 proteins derived from *Schistosoma mansoni* also induced high levels of IL-1 β , however CTAB-stabilized or cysteamine-stabilized GNRs did not.⁴⁸ Furthermore, PEGylated GNRs also did not induce IL-1 β .²⁷

In summary, both GNPs and GNRs are likely taken up *via* an endo-lysosomal pathway. Cellular uptake was governed by the core size but not by the displayed epitope. Moreover, despite the potential of 15 nm GNPs to deliver antigens, they failed to activate BMDCs, while 40 nm GNPs possibly caused cell overstimulation. GNRs were capable of delivering significant antigen loads and caused concentration-dependent activation of BMDCs. None of the tested particles caused inflammation. These results are in agreement with previously published studies for GNPs, but contradict published data for GNRs.

5.3.5 Activation of CD25 and induced proliferation of CD4+ and CD8+ cells in OT-I and OT-II mouse models

Studying T-cell immune response is a challenging task as there is no direct *in vitro* method for detection. Classical approaches require a significant number of lab animals for intensive *in vivo* or *ex vivo* experiments. OT-I and OT-II mouse lines are transgenic lines that have T cells specific to the OVA-derived epitopes.³⁴ CD8+ T cells in the OT-I mice only express the TCR specific towards the cytotoxic epitope OVA₂₅₇₋₂₆₄, while CD4+ T cells of OT-II mice are specific to the T-helper epitope OVA₃₂₃₋₃₃₉. This model is of particular use for vaccine platform evaluation since immunization of animals is not required and challenging of the T cells with the epitope-decorated formulations can be done directly in an *ex vivo* assay. The OT-I/OT-II technique saves time and allows for faster and more thorough screening of potential vaccine platforms with the minimal number of animals required.

The strategy used in this study included differentiation of bone marrow progenitor cells into BMDCs with exposure of the latter to the epitope-decorated GNPs and GNRs. BMDCs will endocytose the nanoparticles and antigens will be cleaved from the gold surface and presented back to the surface of the DCs, waiting for interaction with specific T cells. DCs differentiation time was kept constant (10 days) for all experiments. After 4h exposure to the BMDCs, the GNPs were removed and isolated CD8+ (OT-I) or CD4+ T (OT-II) cells were added to the activated BMDCs and the cells proliferated for 72 hours. To track proliferation, CD8+ and CD4+ cells were stained with 5(6)-carboxyfluorescein diacetate N-succinimidyl ester (CFSE) after isolation. The degree of T-cell activation was determined with FACS using CD25 gated *versus* CFSE stained signals (CD25⁺CFSE^{low} T-cell subset, for gating strategy see **Figure S5.11**). CD25 is the high-affinity IL-2

receptor present in T cells and it is upregulated and moved to the cell surface upon activation by cytokines.^{39, 49} This cell population positive for CD25+ and showing CFSE dilution (a decrease in fluorescence with respect to the negative control related to cell division) represent proliferated T cells. Next, proliferation rates as a function of epitope dosage were transformed into dose-response curves shown in **Figure 5.4**.

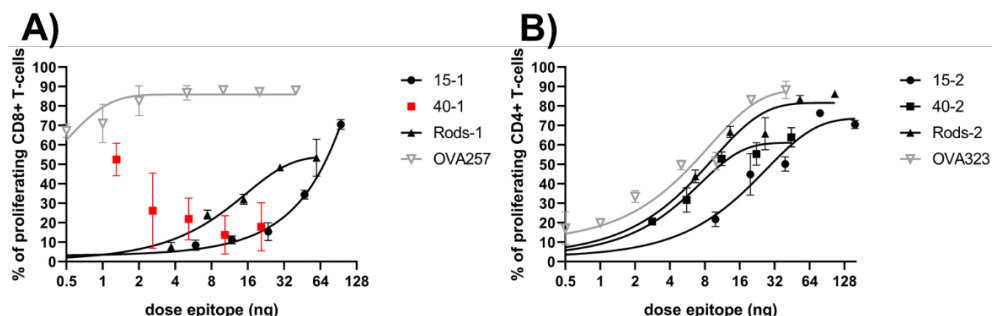


Figure 5.4. Dose-response curves reflecting proliferation rates of: (A) OT-I mice derived CD8+ cells and (B) OT-II mice derived CD4+ cells in response to OVA epitope-carrier GNPs (15 and 40 nm in diameter) and GNRs. % of proliferating cells was calculated from FACS data as CD25+/CFSE dilution (arising from the specific T-cells; for gating strategy, see **Figure S5.11**). The gray line shows the positive controls free epitopes. Three independent experiments were conducted for each GNP preparation, and a representative example is shown here. Data points represent a mean result of three individual wells. Error bars indicate standard deviations. Data sets were fitted into a dose-response curve using GraphPad Prism software.

The assays revealed a striking difference in GNPs and GNRs ability to induce different T-cell mediated responses. As expected, 15 nm GNPs were the least effective carriers in both cases. It was attributed to their inability to induce IL-12 secretion, hence there was no activation of BMDCs. 40 nm GNPs were more effective than 15 nm GNPs for induction of CD4+ cells (T-helper cells), but the CD8+ response was not trivial to interpret. **40-1** was the only formulation that did not show a typical S-shaped dose-response curve: proliferation of CD8+ cells was suppressed for all **40-1** concentrations except

the lowest. To test the overstimulation hypothesis drawn from the IL-12 secretion and CD8+ proliferation profiles, the number of BMDCs was doubled, while the ratio between BMDCs and CD8+ T cells was preserved. This alteration resulted in lower proliferation rates for all three formulations: almost negligible CD8+ proliferation in case of **15-1** and **Rods-1** formulations (**Figure 5.5**). On the other hand, **40-1** did cause significant levels of CD8+ proliferation in a concentration-dependent manner. This observation indicates that, indeed, **40-1** are capable of causing BMDCs overstimulation. The difference between the effect caused by **40-1** and **40-2** can be explained by different pathways that different MHC molecules employ to present processed antigens back to the plasma membrane. MHC-I (involved in CD8+ activation) is supplied with antigens present in the cytosol, while MHC-II (involved in CD4+ activation) loads antigens directly inside lysosomes.³⁸ Egress of antigens from lysosomes into the cytosol is a complex process that is not well understood.^{50, 51} The data presented here suggests that 40 nm GNPs lead to significantly more cleaved epitopes escaping into the cytosol than GNRs, possibly defining a size-dependent trend. Following the idea of MHC-II loading inside lysosomes, **Rods-2** were expected to outperform **40-2**, since the former deliver more antigens in cells (**Figure 5.3G**), and this trend was proven experimentally (**Figure 5.4B**). Importantly, neither the **base-coated** GNPs or GNRs evoked any CD25 upregulation in both OT-I and OT-II experiments (**Figure S5.12**).

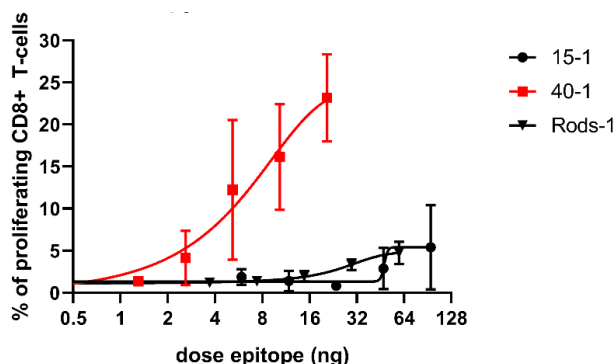


Figure 5.5. Dose-response curves showing proliferation rates of OT-I mice derived CD8+ cells exposed to BMDCs activated with GNPs and GNRs after the ratio between BMDCs and nanoparticles was doubled relative to **Figure 5.4**. % of proliferating cells was calculated from FACS data as CD25+/CFSE dilution (arising from the specific T-cells; for gating strategy, see **Figure S5.11**). Data points represent a mean of three individual wells. Error bars indicate standard deviation. Data sets were fitted into a dose-response curve using GraphPad Prism software.

‘Splitting’ the T-cell immune response into two components (cytotoxic and T-helper) highlighted several aspects. Firstly, there is potentially a size threshold below which DCs and hence T cells only become activated when a very high antigen loading is applied (**Figure 5.4B**). Secondly, CD4+ activation is less sensitive to the size or shape of a carrier particle, but is governed by cellular uptake and the number of delivered antigens. Thirdly, CD8+ activation does not seem to be directly linked to uptake, but is influenced by the size and/or shape of carrier nanoparticles. These aspects should be taken into account when a new vaccine platform is being developed and one type of immune response is preferred over the other.

In order to obtain additional details on the performance of GNPs and GNRs decorated with OVA₂₅₇₋₂₆₄, and to clarify the effect **40-1** exhibits on BMDCs, the cell activation through upregulation of cellular receptors was studied.

5.3.6 DCs activation monitored through cellular receptors upregulation

Mature BMDCs were exposed to **15-1**, **40-1** and **Rods-1** for 4 hours. The cell number per well was increased to 50×10^3 cells instead of the previously used 20×10^3 cells, but the particle concentrations added to the wells were not changed. Nanoparticles were subsequently removed and the cells were analyzed with FACS directly after NP removal and after 24 hours. CD80, CD86, and the full complex of MHC-I/OVA₂₅₇₋₂₆₄ were chosen to monitor BMDCs activation (**Figure S5.13-S5.16**).

Both CD80 and CD86 are necessary for T-cell activation and survival through co-stimulation with CD28 expressed on T cells.^{36, 39} Both receptors are already upregulated on BMDCs due to their handling during the culture procedure, which is reflected in their staining in the negative control sample (DMEM). With respect to the negative control, geometric mean fluorescent intensity (GMFI) of the CD80 channel did not significantly differ in the positive control (free OVA₂₅₇₋₂₆₄), **15-1**, or **Rods-1** at both time points, with one notable exception: **40-1**, which raised the CD80 level to a statistically significant level (**Figure 5.6A** and **Figure S5.14**). Interestingly, CD86 was more readily activated by **40-1** and less by **Rods-1**, but not by **15-1** or free OVA₂₅₇₋₂₆₄ (**Figure 5.6B** and **Figure S5.15**). Enhancement provided by **40-1** was almost double compared to the effect of **Rods-1**, and showed a dose dependence. This assay helped clarify the effect of 40 nm GNPs on BMDCs activation: most likely, suppressed secretion of IL-12 (**Figure 5.3H**) and CD8⁺ T-cell proliferation (**Figure 5.4A**) observed earlier at higher particle concentrations could be attributed to BMDCs overstimulation, when lower cell numbers are exposed to the particles (50×10^3 cells *versus* 20×10^3 cells).

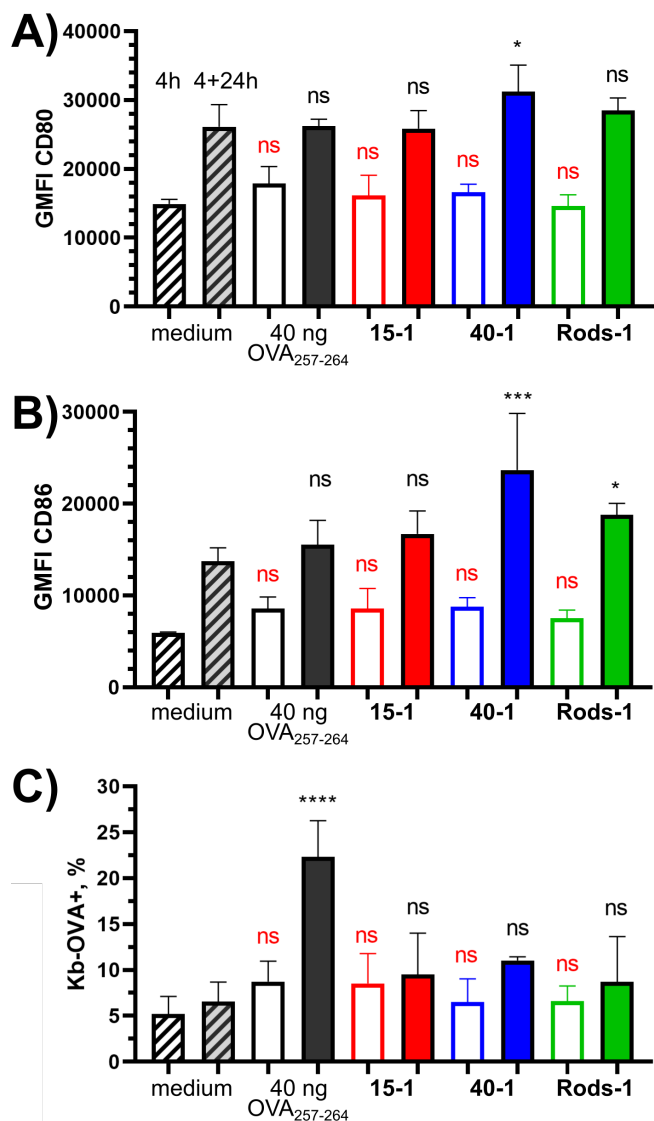


Figure 5.6. Activation of BMDCs upon exposure to GNP and GNR decorated with the OVA₂₅₇₋₂₆₄ epitope through cellular receptors upregulation: (A) CD80; (B) CD86; and (C) Kb-OVA₂₅₇₋₂₆₄. CD80 and CD86 datasets were calculated using the geometric mean fluorescent intensity (GMFI) of corresponding channels (Figure S5.13-S5.15), and for Kb-OVA₂₅₇₋₂₆₄ the positive cell subset is shown (Figure S5.16). Significance of the datasets mean values was determined through comparison to the negative control (supplemented DMEM). Empty columns correspond to 4-hour exposure time, and solid columns correspond to 24 hours post exposure. Only highest particle concentrations are shown here, for more detail, see Figure S5.13-S5.16.

The results of CD80 and CD86 activation of BMDCs are consistent with previously reported data: 33 nm GNPs coated with full OVA were shown to induce greater upregulation in both CD80 and CD86 expression than 10 or 22 nm GNPs coated with OVA.¹⁶ Additionally, antigen-coated GNRs were able to induce simultaneous upregulation of CD86, MHC-I, and MHC-II.⁴⁸

To investigate antigen processing and presentation mediated by GNPs of different shape and size, the ability of BMDCs to present MHC-I loaded with OVA₂₅₇₋₂₆₄ was assessed by means of FACS using an antibody specific towards the whole complex (K_b-OVA₂₅₇₋₂₆₄, **Figure 5.6C**). Consistent with the T-cell proliferation data, 15 nm GNPs did not induce significant epitope presentation, while GNRs induced very low presentation at 24 hours post exposure. However, 40 nm GNPs showed slight epitope presentation directly after exposure, but after 24 hours there was no significant MHC-I/OVA₂₅₇₋₂₆₄ upregulation detected. Taking into account suppressed IL-12 production and CD8+ T-cell proliferation, the K_b-OVA data shows that with decreasing concentration of **40-1**, an increase in antigen presentation is detected. Clearly, when the BMDCs are overloaded with 40 nm GNPs, this affects cross presentation. Other possible reasons for such seemingly poor presentation can be: (i) insufficient time after exposure to process most of the antigen and present it back to the cell membrane; (ii) the K_b-OVA₂₅₇₋₂₆₄ antibody was suitable for detection of direct MHC-I loading with free SIINFEKL, but it is not sensitive enough for detection of processed SIINFEKL; (iii) the epitope is being cleaved off the gold surface into a set of fragments that, when loaded onto MHC-I, cannot be recognized by the K_b-OVA₂₅₇₋₂₆₄ antibody with the same specificity as OVA₂₅₇₋₂₆₄ itself.

The results of upregulation of cellular receptors of DCs upon activation with GNPs of different size and shape were consistent with the other results.

Despite high loading and intense cellular uptake, 15 nm GNPs did not stimulate BMDCs on multiple levels (IL-12 secretion, CD80 and CD86 upregulation) and, hence, failed to induce T-cell proliferation. 40 nm GNPs showed great potential in delivering epitopes into cells and inducing BMDCs activation, even causing overstimulation in some cases. Also, 40 nm GNPs seem to be more effective in directing processing epitopes into the cell cytosol. GNRs took the middling position: they are capable of inducing activation of BMDCs and CD8⁺ cells, but to a lesser extent in comparison with 40 nm GNPs.

5.4 Conclusions

The data reported here on peptide-gold nanoconjugates comprising a gold core of differing shape and size (15 and 40 nm in diameter, and rods of 44 by 15 nm) and a peptide shell, showed that these nanoconjugates are safe in terms of their stability under physiological conditions and do not cause cytotoxicity. Cellular uptake of GNPs and GNRs by BMDCs was governed by the gold core parameters, irrespective of the displayed antigen. All three types of nanoparticles were trapped inside subcellular vesicles, which were analyzed for their size and number of entrapped particles. Observed differences might indicate that every type of GNPs and GNRs utilizes a different endocytosis pathway or mixed pathways. Despite potential in delivery of high antigen loads, 15 nm GNPs failed to activate BMDCs while both 40 nm GNPs and GNRs caused significant BMDCs activation through IL-12 secretion and upregulation of CD80 and CD86. Using the OT-I/OT-II mouse model, it was possible to decouple the cytotoxic and helper T-cell immune responses and show that larger spherical conjugates (40 nm in diameter) caused better CD8⁺ induction (cytotoxic response), while rod-shaped conjugates (44 by 15 nm) caused better CD4⁺ activation (T-helper response). In terms of CD4⁺ T-cell proliferation, GNRs consistently showed higher rates than GNPs of 40 nm in diameter. However, it cannot be concluded that GNRs are superior to the GNPs, as the latter also induced significant CD4⁺ proliferation. 15 nm GNPs induced both responses but only at very high doses. The observed difference in CD8⁺ T-cell proliferation can possibly be attributed to more effective egress of processed epitopes from lysosomes with 40 nm carrier GNPs in comparison to GNRs. This way epitopes are delivered into the cytosol more efficiently and have a higher chance of being loaded onto MHC-I and inducing a cytotoxic response. The

observed difference in CD4⁺ T-cell proliferation was attributed to the difference in epitope loading: GNRs due to their higher relative surface area carry more antigen than 40 nm GNPs, thus GNRs are more likely to load antigens onto MHC-II inside the lysosomes. It should be noted that the difference between different NPs in CD8⁺ T-cell activation had a more dramatic character than the difference observed in CD4⁺ T-cell activation. These findings allow for rational design of a carrier nanoparticle in vaccine development.

5.5 Experimental Section

5.5.1 Materials

All chemicals were purchased from Sigma-Aldrich unless stated otherwise. For GNR and GNP synthesis, the purity of CTAB and the composition of the gold salt are critical for reliable particle formation. For synthesis of the particles described in this chapter CTAB ($\geq 99\%$, H6269) and $\text{HAuCl}_4 \times 3 \text{H}_2\text{O}$ (49% Au basis, G4022) were purchased from Sigma-Aldrich. Silver nitrate (99.999%) and Oxyma Pure were purchased from Carl Roth GmbH. TFA, piperidine, DMF, DCM, methanol, and acetonitrile were purchased from Biosolve. TEM grids (Formvar/Carbon, 200 mesh, on copper support) were purchased from Electron Microscopy Sciences. FCS, penicillin/streptomycin mixture, L-glutamate, DMEM and RPMI 1640 cell culture media were purchased from Lonza. Mouse granulocyte-macrophage colony-stimulating factor (GM-CSF) was purchased from PeproTech. CD4⁺ and CD8⁺ isolation kits (*via* MACS magnetic separation) were purchased from Miltenyi Biotec. LDH leakage assay (LDH-Cytox™ Assay Kit) and the specific antibody against the whole MHC-I/OVA₂₅₇₋₂₆₄ complex (PE anti-mouse H-2Kb bound to OVA₂₅₇₋₂₆₄ antibody, K_b-OVA₂₅₇₋₂₆₄) were purchased from Biolegend. ELISA standards (IL-12 and IL-1 β) and antibodies, TMB substrate, as well as FACS immunostaining antibodies were purchased from eBioscience.

5.5.2 Gold Nanoparticles Synthesis

Spherical particles (GNPs) of 15 and 40 nm in diameter were synthesized *via* citrate reduction and overgrowth as described in **Chapter 3** of this thesis.²⁹ The average size of the GNPs in suspension was analyzed using dynamic light scattering (DLS), and the results are shown in **Table 5.1**. Size

parameters of the obtained GNPs were also determined by transmission electron microscopy (TEM): mean diameters were 15.0 nm and 43.5 nm. The mass of one nanoparticle was calculated to be 34.1 and 832.2 ag (for 15 and 40 nm GNPs, respectively) and the surface areas were determined to be 707 and 5945 nm².^{19, 52}

Gold nanorods (GNRs) were synthesized *via* the two-step seed-mediated approach as described in **Chapter 4** of this thesis.^{53, 54} The average dimensions were 44.0 ($\pm$ 4.0) by 14.8 ($\pm$ 1.9) nm. The mass of one nanorod was calculated to be 129.8 ag, and the surface area – 2046 nm².

5.5.3 Peptide and peptide amphiphile synthesis

Peptides and peptide amphiphiles were synthesized by solid-phase peptide synthesis using standard Fmoc-chemistry protocols. The synthesis was performed on an automated microwave peptide synthesizer, Liberty Blue (CEM), using standard settings. All peptides and amphiphiles were synthesized on a Wang resin preloaded with the corresponding C-terminal residue. 20% piperidine in DMF was employed as the deprotection solution, (deprotection was performed at 90 °C, for 60 secs), and N,N'-diisoprorylcarbodiimide (DIC)/OxymaPure (activator/activator base) were used to activate Fmoc-protected α -amino acids (5 equivalents relative to the resin loading were used, coupling was facilitated at 95 °C, 4 mins). 11-mercaptoundecanoic acid was coupled to the peptide N-terminus using the same protocol as for amino acid coupling but for a longer time (5 equivalents relative to the resin loading, 95 °C, 10 mins). The terminal thiol of the acyl chains was blocked with 2,2'-Dithiobis(5-nitropyridine) (DTNP, 1 eq. in DMF, 3 hours). The molecules were cleaved from the resin using a cleavage cocktail comprising 2.5% triisopropylsilane (TIS), 1.5% deionized water,

2.5% phenol in trifluoroacetic acid (TFA, RT, 2 hours). The crude peptide compounds were precipitated into cold diethyl ether, pelleted by centrifugation, redissolved in water, and lyophilized prior to purification. HPLC purification was performed on a Shimadzu system equipped with two LC-20AR pumps, an SPD-20A UV-Vis detector and a Phenomenex Kinetex EVO C18 column (21.2 by 150 mm) with water and acetonitrile as mobile phases. 0.1% TFA was added to the mobile phases, except for purification of the **base** sequence where 0.1% NH₃ was added. Purity of the compounds was confirmed with LC-MS (**Figure S5.17-S5.22**). The purified molecules were lyophilized and stored at -20 °C.

5.5.4 Preparation of Peptide Amphiphile-Capped GNPs

Coating of the GNPs was performed *via* the ligand exchange procedure as described in detail elsewhere.²⁹ Briefly, the desired coating molecules were dissolved in DMSO, mixed at 9:1 molar ratio (**base:1** or **base:2**), and added to a stirred GNP suspension. The peptide amphiphile molar excess over the GNPs was at least 200 000. The final concentration of DMSO in the mixture was 50% (v/v). After a 1-hour incubation the samples were centrifuged to pellet the modified GNPs and the supernatants were discarded and replaced with 25% DMSO (v/v). Samples were incubated overnight and after a final centrifugation cycle, the GNPs were dispersed in 5% DMSO. Size exclusion chromatography (SEC, NAP-25 columns, GE Healthcare) was performed with PBS as an eluent to remove free ligands and DMSO according to manufacturer's manual.

Particle size distributions, determined by dynamic light scattering (DLS), and Zeta-potential before and after modification were obtained using a Zetasizer Nano-7 S (Malvern Instruments). The average of three separate

measurements was reported. Transmission electron microscopy (TEM) measurements were performed on a JEM 1400 Plus (JEOL) microscope, with an accelerating voltage of 80 kV and with a CDD camera. Sample preparation was as follows: a 10 μ L droplet of the sample was deposited on a carbon-coated copper grid for 20 mins. The excess sample was removed by blotting with a fibreless tissue. The grid was washed with MilliQ water once and blotted again. 1% (w/v) uranyl acetate staining was applied, followed by immediate blotting and drying.

UV-Vis spectra were recorded in the 900-350 nm range using a Cary 300 UV-Vis spectrometer (Agilent). Molar concentrations of GNPs and GNRs were determined by UV-Vis using extinction coefficients published by Haiss et al.⁵⁵ for spherical particles, and by Orendorff et al.⁵⁴ for rod-like particles. To calculate molar concentrations of nanoparticles, The Lambert-Beer law was used (equation 1):

$$A = \varepsilon \cdot l \cdot c \cdot N, \quad (1),$$

where A is absorption, ε is extinction coefficient (in $M^{-1}cm^{-1}$), l is the optical path length ($l = 1$ mm), c is molar concentration (in M), and N is the dilution factor.

5.5.5 Circular dichroism

Circular dichroism (CD) spectra of the amphiphiles were recorded using a JASCO J-815 spectropolarimeter, fitted with a Peltier temperature controller. All measurements were performed at 20 °C. Lyophilized powders of peptide amphiphiles were dissolved in 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP), mixed at the molar ratio of 1:9 = active peptide amphiphile (**1** or **2**):base peptide amphiphile, and dried under a nitrogen flow. The peptide amphiphile

thin film was rehydrated with PBS (pH 7.2) to achieve the following concentrations: 20 μ M of active peptide amphiphile (**1** or **2**) and 180 μ M of **base** peptide amphiphile. As a reference sample, **base** was dissolved in PBS (pH 7.2) to a final concentration of 200 μ M. Samples were left in the dark in sealed vials for 14 days. Samples were measured in quartz cuvettes with a 2 mm path length, and spectra were recorded from 260 to 190 nm at 2 nm intervals with a 1 nm bandwidth. All spectra were converted to mean residue ellipticity ($\text{deg cm}^2 \text{dmol res}^{-1}$) using equation 2:

$$[\theta] = \frac{100 \times [\theta]_{obs}}{((C_{base} \times n_{base}) + (C_X \times n_X)) \times l} \quad (2),$$

where $[\theta]_{obs}$ is the observed ellipticity in mdeg; C_{base} is the concentration of **base** in mM; n_{base} is the number of amino acids in **base** ($n_{base} = 11$); C_X is the concentration of **1** or **2** in mM; n_X is the number of amino acids in **1** or **2** ($n_1 = 17$ and $n_2 = 26$); and l is the path length of the cuvette in cm.

5.5.6 Number concentrations of GNPs and GNRs

Gold (Au) mass quantification using inductively coupled plasma mass spectrometry (ICP-MS) was conducted as follows: the concentration of Au in the samples was measured using a PerkinElmer NexION 2000 equipped with a quartz nebulizer and a glass cyclonic spray chamber operating in standard mode (1600 W radio frequency power; 18 mL/min plasma gas flow; 1.2 L/min nebulizer gas flow; and 1.12 L/min auxiliary flow). The peptide amphiphile-coated GNPs or GNRs (in suspension, OD $\sim$ 0.3-0.5) were mixed 1:1 (v/v) with *aqua regia* ($\text{HNO}_3\text{:HCl} = 1\text{:}3$) and were subjected to digestion at 70 $^\circ\text{C}$ for 1 hour. Mixtures were diluted 25x times with MilliQ water and directly measured for ^{197}Au isotope content. Knowing the optical densities of the suspensions before digestion with *aqua regia*, the mass of digested gold, and

the calculated mass of one nanoparticle, it was possible to link optical density (OD) of GNP or GNR suspensions with their number concentration. Small deviations in the determined gold concentration between the samples with the same core were not significant (**Figure S5.3A**), and could be attributed to incomplete nanoparticle digestion and analysis error, which can lead to deviations of up to 10%.¹⁹

5.5.7 Antigen loading displayed onto GNPs and GNRs

Antigen loading was expressed as shown in equation 3:

$$AL = d_W \times S_{NP} \times N_{NP} \quad (3),$$

where AL is the antigen loading that 1 mL of a GNP or GNR suspension with an optical density of 1.0 at the plasmon band (OD_{LSPR}) contains; d_W is coverage density of a model molecule **W** (see **Scheme S5.1**) displayed on the gold surface; S_{NP} is the square area of one nanoparticle; and N_{NP} is the number concentration of nanoparticles in a suspension with $OD_{LSPR} = 1.0$ (determined using ICP-MS as described in **5.5.6**).

Coverage density d_W was determined as described in **Chapter 4** of this thesis (protocol #3 in **4.5.10**). Briefly, **base** and **W** peptide amphiphiles were dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP, 1.0 mg/mL) and mixed at a molar ratio of 9:1. This mixture was diluted with HFIP to obtain a final concentration of **base** of 0.50 mg/mL. Next, it was added to the GNPs and GNRs as follows: for every 0.3 mL of the peptide amphiphile mixture, 0.5 mL of a citrate- or CTAB-protected nanoparticle suspension was taken ($OD_{519} = 4.5$ for 15 nm GNPs, $OD_{530} = 2.5$ for 40 nm GNPs, and $OD_{756} = 3.2$ for GNRs). After a 1-hour incubation under stirring, the nanoparticles were pelleted *via* centrifugation (Eppendorf Centrifuge 5425, 14 000 rpm, 40 mins

for 15 nm GNPs, 10 mins for 40 nm GNPs, and 20 mins for GNRs). The pellets were rehydrated and lyophilized. The dry residue was redissolved in HFIP and nanoparticles were pelleted again. Both supernatants were analyzed using UV-Vis spectroscopy and concentrations were determined using the Lambert-Beer law (equation 1, tryptophan absorbance was analyzed using $\epsilon_{280}(W)=5600 \text{ M}^{-1}\text{cm}^{-1}$). Knowing the molar concentrations of GNPs and GNRs and their surface areas, as well as added concentrations of **W**, and the amount that did not bind to the gold surface, the following d_w values were calculated: 0.58 peptide/nm² for 15 nm GNPs; 0.35 peptide/nm² for 40 nm GNPs; and 0.46 peptide/nm² for GNRs. Using a simple proportion based on the molecular weight of OVA₂₅₇₋₂₆₄, and OVA₃₂₃₋₃₃₉, and particle number concentrations obtained using ICP-MS, the coverage densities were recalculated for epitope loading (**Table S5.1**).

5.5.8 Animals

C57BL/6, OT-I, and OT-II transgenic mice were purchased from Jackson Laboratory (CA, USA). The animals were bred in-house in agreement with standard laboratory conditions, and provided with water and food *ad libitum*. The animals were treated in compliance with the Dutch government guidelines and the Directive 2010/63/EU of the European Parliament. Experiments were approved by the Ethics Committee for Animal Experiments of Leiden University.

5.5.9 Isolation and maturation of bone marrow-derived dendritic cells (BMDCs)

Bone marrow was extracted from tibias, femurs, and thigh bones of C57BL/6 or OT-I/OT-II mice. A single-cell suspension of bone marrow cells was

prepared using a 70- μ m cell trainer (Greiner Bio-One B.V.). The cell culture medium used for the maturation was DMEM supplemented with 2 mM L-glutamine, 5% (v/v) FCS, 100 U/mL penicillin/streptomycin. Bone marrow cells were seeded in 95 mm Petri dishes at a density of $8-10 \times 10^6$ cells per dish (Greiner Bio-One B.V.) and incubated at 37 °C and 5% CO₂ for 10 days. Medium was refreshed four times during the 10-day period, 20 ng/mL GM-CSF was added to the medium to induce and maintain the maturation of progenitor cells into BMDCs.

5.5.10 LDH leakage assay

LDH leakage assay was performed to determine cytotoxicity of the peptide-gold conjugates in BMDCs. For the concentration range employed see **Table S5.2** (dilutions of 1x, 4x, and 16x were used for this assay). For this assay, BMDCs were cultured as described above. After 10 days of culturing, DCs (20×10^3 per well) were plated in 96-well plates (Greiner Bio-One B.V.) and were exposed to GNPs and GNRs for 4 and 24 hours at 37 °C (5% CO₂). A negative control (50 μ L of PBS) and a positive control (50 μ L of the assay lysis buffer) were added to every plate. The added volumes of peptide-gold conjugate samples were kept the same for all experiments – 50 μ L per well (200 μ L total well volume). After the exposure, the plates were centrifuged (1500 rpm, 5 mins) and 30 μ L of the supernatants were taken for analysis which was performed according to the manufacturer's manual (non-homogenous assay protocol). Cytotoxicity was expressed as the difference between the negative control and fully lysed cells (equation 4) and the results are shown in **Figure 5.2** and **Figure S5.4**.

$$Cytotoxicity (\%) = \frac{(A-C)}{(B-C)} \cdot 100 \quad (4),$$

where *A* is the signal arising from the GNP- and GNR-treated wells, *B* - the signal from the cell treated with assay lysis buffer, and *C* - is the signal coming from the negative control wells (PBS-treated). Signals were derived from absorbance at 490 nm measured using a microplate reader (Infinite 200 PRO Tecan). Every sample was tested in three individual wells. The data shown is an average of two independent experiments.

5.5.11 GNPs and GNRs uptake by BMDCs

This step was duplicated for two independent experiments: measuring cellular uptake of GNPs and GNRs *via* direct detection of the gold content per cell (ICP-MS assisted) and visualization of subcellular localization with TEM. Samples analyzed were: **15-1**, **15-2**, **40-1**, **40-2**, **Rods-1**, and **Rods-2**.

Mature DCs were seeded at a density of 1×10^6 cell per well in 6-well plates (Greiner Bio-One B.V.). For the cell visualization experiment, a coverslip (Thermanox, 24 mm in diameter, Nunc) was added in each well. 250 μ L of sample (top concentration in **Table S5.1**) were diluted 2x times with DMEM and added to the wells (3 mL total volume). The cells and the samples were incubated for 4 hours at 37 °C (5% CO₂). Next, the cells were washed with PBS (pH 7.2) to remove the non-associated nanoparticles.

For the visualization experiment, the cells were fixed with 2% (v/v) glutaraldehyde in sodium cacodylate buffer (0.1 M, pH 7.2, 2 hours at room temperature). After fixation, 1% (w/v) OsO₄ was applied for 1 hour at room temperature. Next, the cells were dehydrated through ethanol graded series and embedded into Agar 100 resin (Agar Scientific, AGR1043). Thin sections (100 nm) were cut from the resin using a Leica Ultramicrotome equipped with a diamond knife. The images were acquired on a JEM 1400 Plus microscope, fitted with a CCD camera, at an accelerating voltage of 80 kV. The vesicles

dimensions were determined using Fiji ImageJ software. Since vesicles are not completely spherical, each vesicle was measured across its longest and shortest axes, and a mean value was reported in **Figure S5.6**.

ICP-MS was also used to determine the average gold content per cell. After 4 hours of incubation time, the BMDCs were washed with PBS (pH 7.2, 3x times), detached with a cell scraper (Sarstedt) and counted (1 mL total volume). The cells were lysed with aqua regia (1:1 dilution, 1 hour at 70 °C). Lysates were diluted 25x times with MilliQ water and directly measured in triplicate using the method described in **5.5.6**. Since both the cell numbers and total gold content were known, the average gold content per cell could be calculated (**Figure 5.3G** and **Figure S5.8**).

5.5.12 Activation of BMDCs

Activation of BMDCs was monitored through cytokine release and upregulation in expression of cellular receptors.

Mature BMDCs (20×10^5 cell per well in 96-well plates) were treated with GNPs and GNRs for 24 hours in the same manner as described in **5.5.8**. The cell culture supernatants were checked for IL-12 and IL-1 β levels using ELISA. A 'sandwich' ELISA was performed according to a standard protocol at room temperature and involved the following steps: (1) coating the 96-well plates (half area, Costar) with capture antibodies (eBioscience, overnight at 4 °C); (2) blocking the wells from non-specific interactions by incubation with BSA (2.5% w/v in PBS, 1 hour); (3) incubation (2 hours) with the supernatant of GNP-exposed cells (diluted with block buffer), PBS-treated samples, IL-12 standard at different concentrations, and blank wells (blocking buffer); (4) addition of detection antibodies and complexation with SAV-HRP (1 hour). The read out was based on the reaction of the HRP conjugate with an TMB

substrate (BD Bioscience). The reaction was stopped with 1 N H₂SO₄ when the blue color in the well became intense. Absorbance at 570 nm was subtracted from absorbance at 450 nm (recorded using a microplate reader Infinite 200 PRO Tecan). Based on the signals obtained for the cytokine standards, calibration curves were built. Samples from three independent experiments were analyzed. For results see **Figure 5.3H** and **Figure S5.9,S5.10**.

Next, mature BMDCs (50×10⁵ cell per well in 96-well plates) were exposed to GNPs and GNRs for 4 hours. Cell culture supernatants were removed and half of the cells were analyzed directly, and the other half was supplied with fresh medium and analyzed after 24 hours. The procedure included immunostaining for CD80, CD86, and K_b-OVA₂₅₇₋₂₆₄, as well as for viability. Flowcytometry was performed on a CytoFLEX S (Beckman Coulter). FACS data analysis was done using FlowJo software (Treestar). The gating strategy can be found in **Figure S5.13** and the results are shown in **Figure S5.14-S5.16**.

5.5.13 Isolation of CD4⁺ and CD8⁺ T cells from OT-II and OT-I mice

BMDCs were cultured as described above for 10 days, and pulsed for 4 hours with GNPs and GNRs or controls (PBS or free epitopes) followed by complete removal of the particles from the cell culture. Spleens were removed from OT-I or OT-II mice and strained through a 70-μm cell strainer to yield a single-cell suspension. Red blood cells (erythrocytes) were lysed with Ammonium-Chloride-Potassium (ACK) lysis buffer (0.15 M NH₄Cl, 1 mM KHCO₃, 0.1 mM Na₂EDTA; pH 7.3). CD8⁺ or CD4⁺ T cells were isolated *via* magnetic separation using a CD8⁺ or CD4⁺ T cell isolation kit according to

the supplier's protocol. Enriched T-cell fractions were stained with CFSE-FITC and then added to the plates with BMDCs in the ratio of 3:1 = specific T cells:BMDCs in complete RPMI 1640 medium (supplemented with 2 mM glutamine, 5% FCS, 100 U/mL penicillin/streptomycin, and 50 μ M β -mercaptoethanol). T cells were left to proliferate for 72 hours in the same plates (37 °C, 5% CO₂). Cells were immunostained for Thy1.2, CD4 or CD8, CD25, as well as for viability, and analyzed by flow cytometry. FACS data analysis was done using FlowJo software. Each experiment was repeated three times, and a representative example is shown in **Figure 5.4**. The gating strategy can be found in **Figure S5.11**. The proliferation rate was expressed through a CD25+ cell subset showing lowered CFSE-FITC fluorescence of daughter (divided) cells. GraphPad Prism version 7.00 for Windows (GraphPad Software) was used to analyze the data and to plot the dose-response curves.

5.6 References

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54. Orendorff, C. J.; Murphy, C. J., Quantitation of metal content in the silver-assisted growth of gold nanorods. *J Phys Chem B* **2006**, 110 (9), 3990-3994.
55. Haiss, W.; Thanh, N. T. K.; Aveyard, J.; Fernig, D. G., Determination of size and concentration of gold nanoparticles from UV-Vis spectra. *Anal Chem* **2007**, 79 (11), 4215-4221.

5.7 Supporting Information

Co-assemblies of **base** and OVA epitope-decorated peptide amphiphiles **1** and **2** were β -structured in nature:

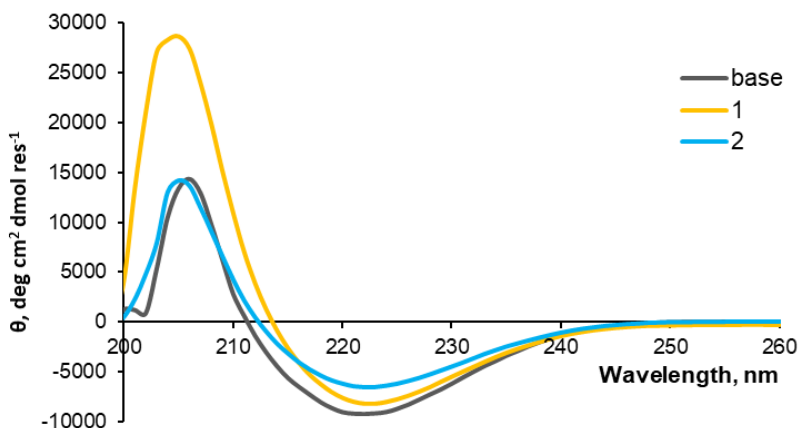


Figure S5.1. CD spectra showing that incorporation of 1 molar part of the active peptide amphiphile **1** or **2** does not affect the self-assembly behavior of the **base** peptide amphiphile: yellow line – 180 μM **base** + 20 μM **1**; blue line – 180 μM **base** + 20 μM **2**; gray line – 200 μM **base**. Samples were prepared in PBS (pH 7.2).

Size and surface charge of peptide amphiphile-coated GNPs and GNRs compared to citrate-stabilized GNPs and CTAB-stabilized GNRs:

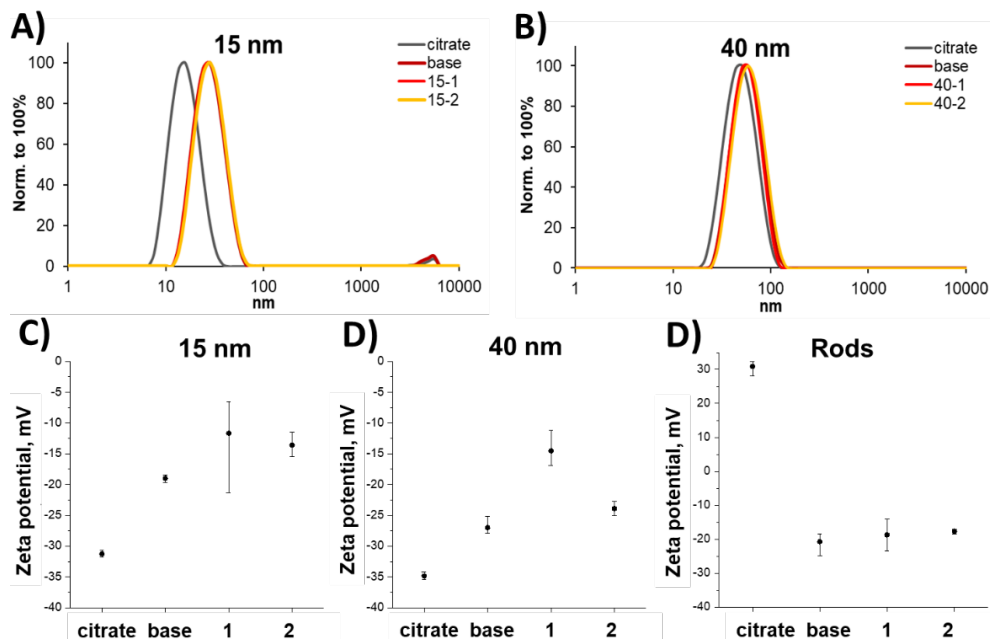


Figure S5.2. Sample characterization before and after modification obtained with DLS: (A) size distribution for 15 nm GNPs; (B) size distribution for 40 nm GNPs; (C) zeta potential values for 15 nm GNPs; (D) zeta potential values for 40 nm GNPs; (E) zeta potential values for GNRs. (A, B) samples were prepared in PBS (pH 7.2); (C-E) samples were prepared in PBS (pH 7.2) and diluted 10 times with MilliQ prior the measurement. Zeta potential is shown as a mean of three measurements with error bars indicating max and min values. Due to their shape, GNRs could not be analyzed for their hydrodynamic diameter.

GNP and GNR number concentrations were determined with ICP-MS:

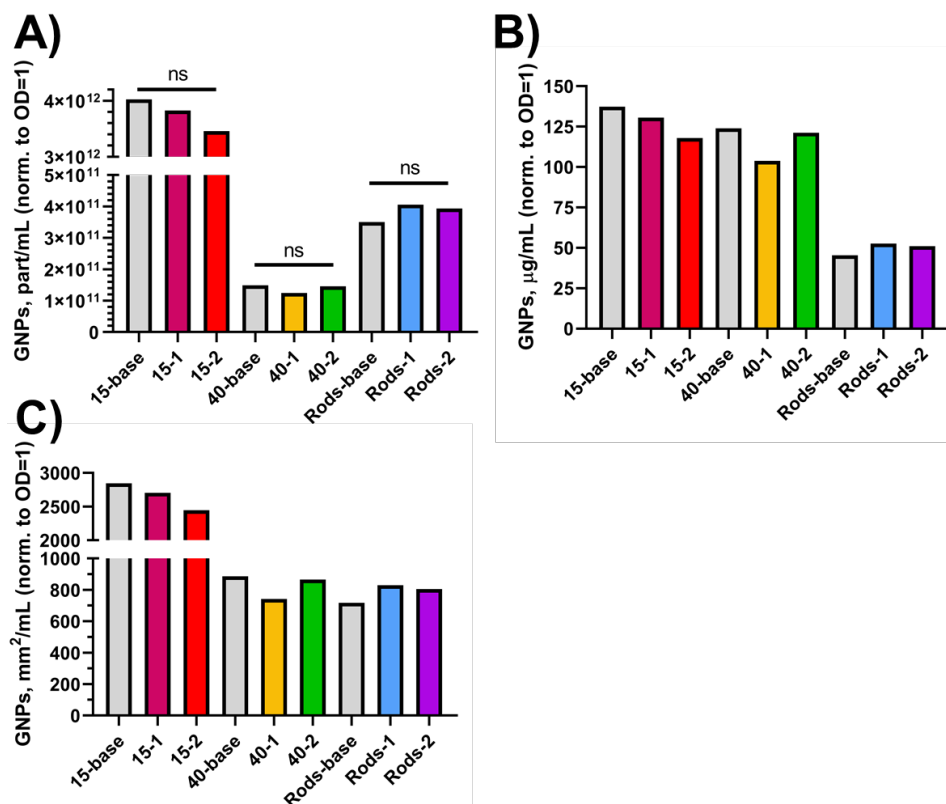
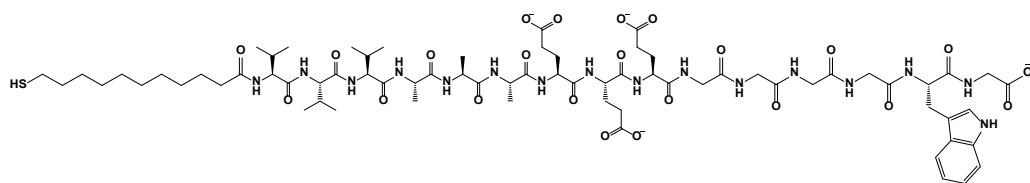


Figure S5.3. GNP and GNR characteristics determined with ICP-MS and normalized to a suspension with an optical density of 1 ($OD_{LSPR} = 1$): (A) number concentration; (B) mass concentration; and (C) total gold surface available for contact as a function of volume. The mass concentration was measured once. To determine the significance of the difference between the concentrations of particles with the same gold core but different coating, a 10% deviation was introduced. According to a two-way ANOVA test performed in GraphPad Prism software, differences in metrics of nanoparticles comprising the same gold core, were found to be not significant (ns). Mass of one nanoparticle in ag: 15 nm GNPs = 34.1; 40 nm GNPs = 832.2; GNRs = 129.8. Surface area of one nanoparticle in nm²: 15 nm GNPs = 706.86; 40 nm GNPs = 5944.7; GNRs = 2045.8.

Antigen loading was determined by UV-Vis with the help of a model peptide amphiphile **W**:



Scheme S5.1. Chemical structure of **W** used to determine coverage density and antigen loading onto different GNPs and GNRs.

Table S5.1. The ratio between optical density (OD) and GNP number concentration and epitope loading.

Gold core	Parameter	OD*	base	1	2
15 nm	Epitope $\mu\text{g/mL}$ (OD=1)		n/a	2.52	4.2
	Epitope $\mu\text{g/mL}$ (top conc.)	0.75	n/a	1.89	3.15
	Epitope ng in 50 μL ** (top conc.)		n/a	94.5	157.5
	Number part/mL (OD=1)		4.03E+12	3.83E+12	3.46E+12
	Number part/mL (top conc.)	0.75	3.02E+12	2.87E+12	2.59E+12
	Particles in 50 μL (top conc.)		1.51E+11	1.44E+11	1.3E+11
40 nm	Epitope $\mu\text{g/mL}$ (OD=1)		n/a	0.414	0.891
	Epitope $\mu\text{g/mL}$ (top conc.)	1.00	n/a	0.414	0.891
	Epitope ng in 50 μL (top conc.)		n/a	20.7	44.5
	Number part/mL (OD=1)		1.49E+11	1.25E+11	1.46E+11
	Number part/mL (top conc.)	1.00	1.49E+11	1.25E+11	1.46E+11
	Particles in 50 μL (top conc.)		7.45E+09	6.24E+09	7.28E+09
GNRs	Epitope $\mu\text{g/mL}$ (OD=1)		n/a	0.611	1.09
	Epitope $\mu\text{g/mL}$ (top conc.)	1.94	n/a	1.19	2.12
	Epitope ng in 50 μL (top conc.)		n/a	59.3	105.8
	Number part/mL (OD=1)		3.50E+11	4.06E+11	3.93E+11
	Number part/mL (top conc.)	1.94	7.01E+11	7.87E+11	7.63E+11
	Particles in 50 μL (top conc.)		3.50E+10	3.93E+10	3.82E+10

*- Top conc. = top concentration used throughout this study. In the text this is also denoted as 1x dilution. *- 50 μL was the volume of a sample dose per well added to BMDCs during cell experiments. Total well volume was 200 μL . Cell number varied from 10×10^3 to 50×10^3 per well depending on the assay. For full details, see **5.5** Experimental section.

The GNP and GNR concentration range used in this study was as follows:

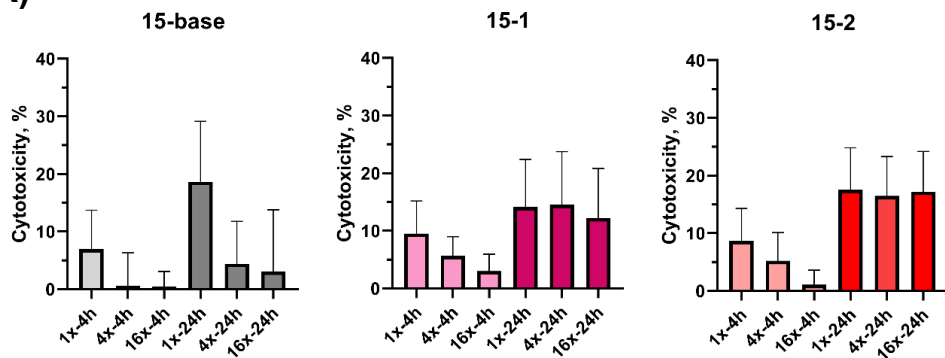
Table S5.2. GNP and GNR concentration range used for cell experiments.

Dilution	15-1	15-2	40-1	40-2	Rods-1	Rods-2
Epitope dosage, ng in 50 μ L*						
1x **	94.5	157.5	20.7	44.5	59.3	105.8
2x	47.3	78.7	10.3	22.3	29.6	52.9
4x	23.6	39.4	5.2	11.1	14.8	26.5
8x	11.8	19.7	2.6	5.6	7.4	13.2
16x	5.9	9.8	1.3	2.8	3.7	6.6
Particle number in 50 μ L						
1x	1.44E+11	1.30E+11	1.25E+11	1.46E+11	4.06E+11	3.93E+11
2x	7.18E+10	6.48E+10	6.24E+10	7.28E+10	2.03E+11	1.97E+11
4x	3.59E+10	3.24E+10	3.12E+10	3.64E+10	1.01E+11	9.83E+10
8x	1.79E+10	1.62E+10	1.56E+10	1.82E+10	5.07E+10	4.92E+10
16x	8.97E+09	8.11E+09	7.80E+09	9.10E+09	2.53E+10	2.46E+10

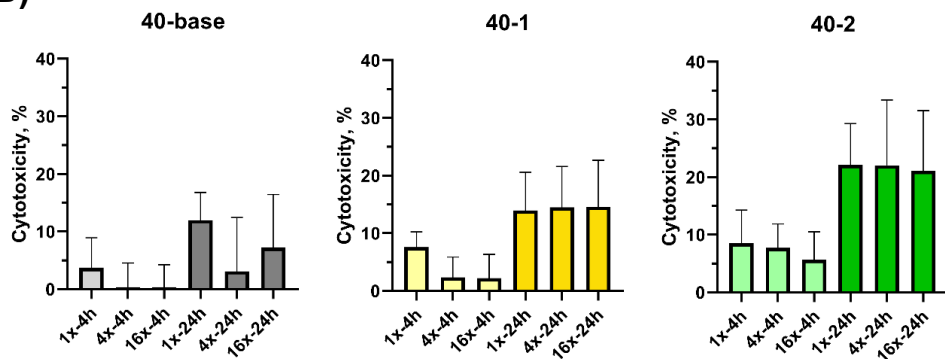
*- 50 μ L was the volume of a sample dose per well added to BMDCs during cell experiments. Total well volume was 200 μ L. Cell number varied from 10×10^3 to 50×10^3 per well depending on the assay. **- top concentration used throughout this study. In the text this is also denoted as 1x dilution. For detail, see **5.5** Experimental section.

Peptide amphiphile-coated GNPs and GNRs did not cause cytotoxicity:

A)



B)



C)

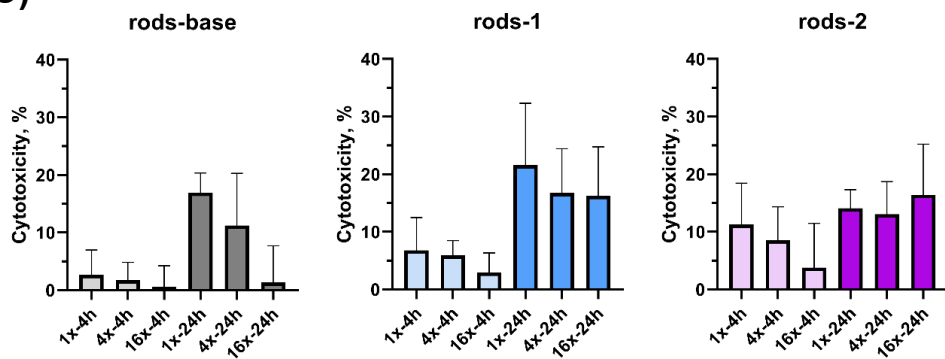
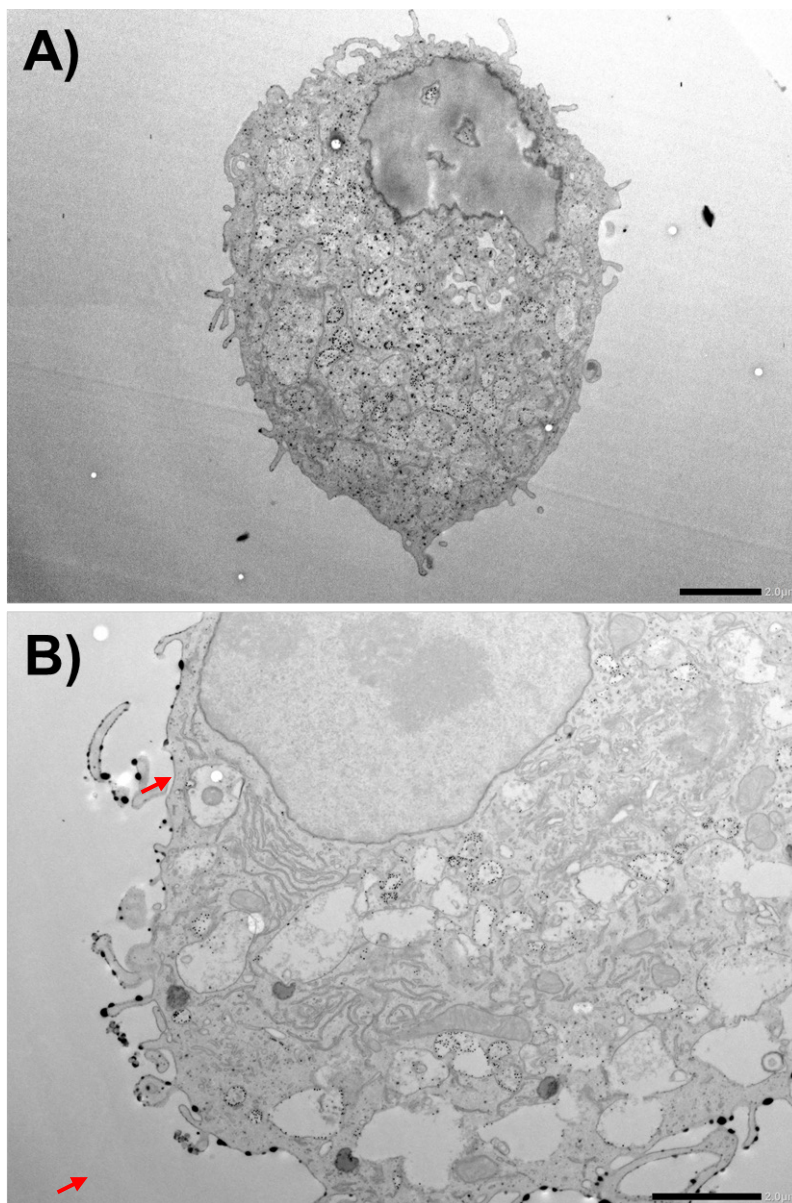


Figure S5.4. Cytotoxicity evaluated with an LDH release assay for: (panel A) 15 nm GNPs, (panel B) 40 nm GNPs, and (panel C) GNRs. GNP concentration range analyzed involved 1x, 4x, and 16x times diluted samples (**Table S5.2**). Lighter color shades indicate 4 h exposure time; darker shades – 24 h exposure time. Error bars indicate standard deviations and were calculated using GraphPad Prism software.

Subcellular localization of internalized GNPs and GNRs was assessed with TEM:



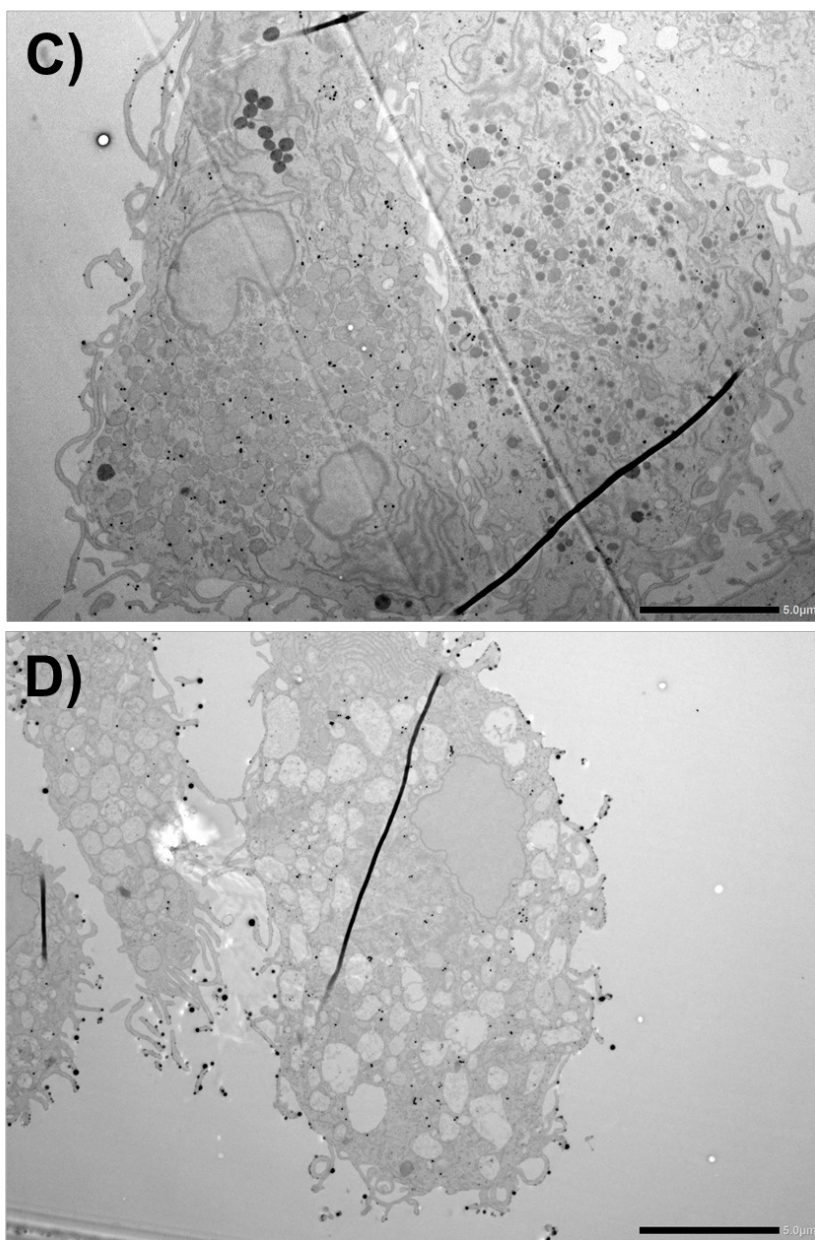
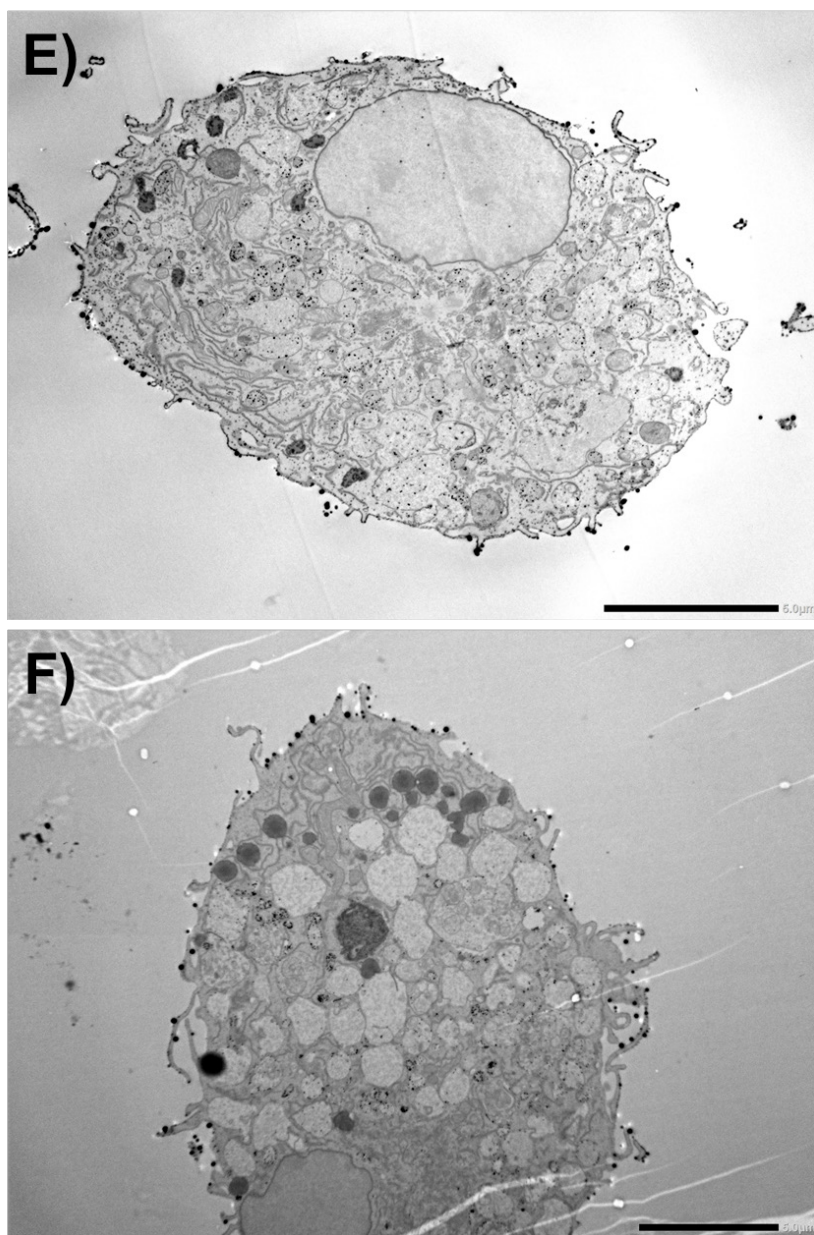


Figure S5.5C,D. TEM micrographs of cell sections: (C) BMDCs exposed to **40-1** and (D) BMDCs exposed to **40-2**. Scale bars = 5 μm. The round dark crystals on the outer side of the cell membrane are the accumulations of the OsO₄ staining.



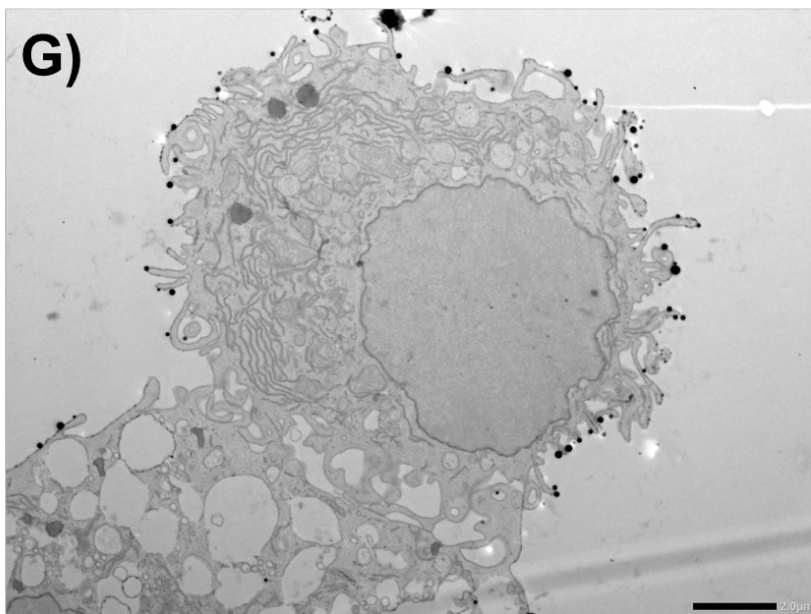


Figure S5.5G. TEM micrograph of a cell section: (G) BMDCs treated with PBS. Scale bars = 2 μm . The round dark crystals on the outer side of cell membrane are the accumulations of the OsO_4 staining.

TEM analysis of subcellular vesicles revealed differences between the uptake of GNPs of different shape and size by BMDCs:

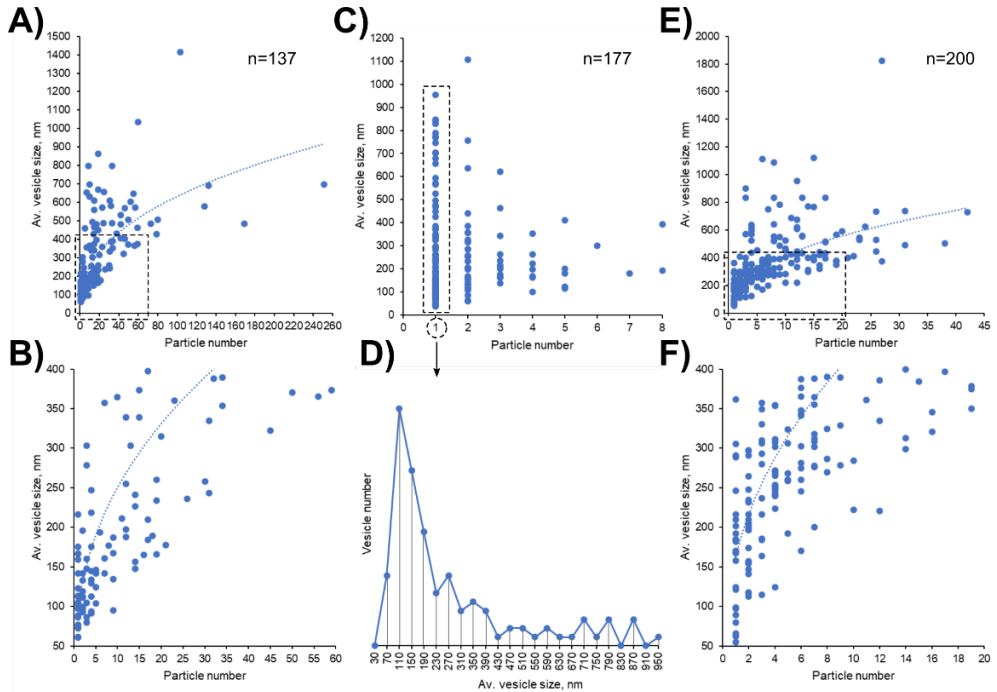


Figure S5.6. TEM analysis of BMDC sections based on average vesicle size and number of entrapped nanoparticles for: (A-B) **15-1**, (C-D) **40-1**, and (E) **Rods-1**. It was not possible to determine the vesicle type based solely on the TEM imaging. Vesicle types observed in this study were hypothesized to be different stages of endosomes, lysosomes, autophagosomes *etc.* (D) shows the size distribution of vesicles with 1 entrapped particle of **40-1**: it is suspected that endocytic vesicles have a size range of 40-120 nm.

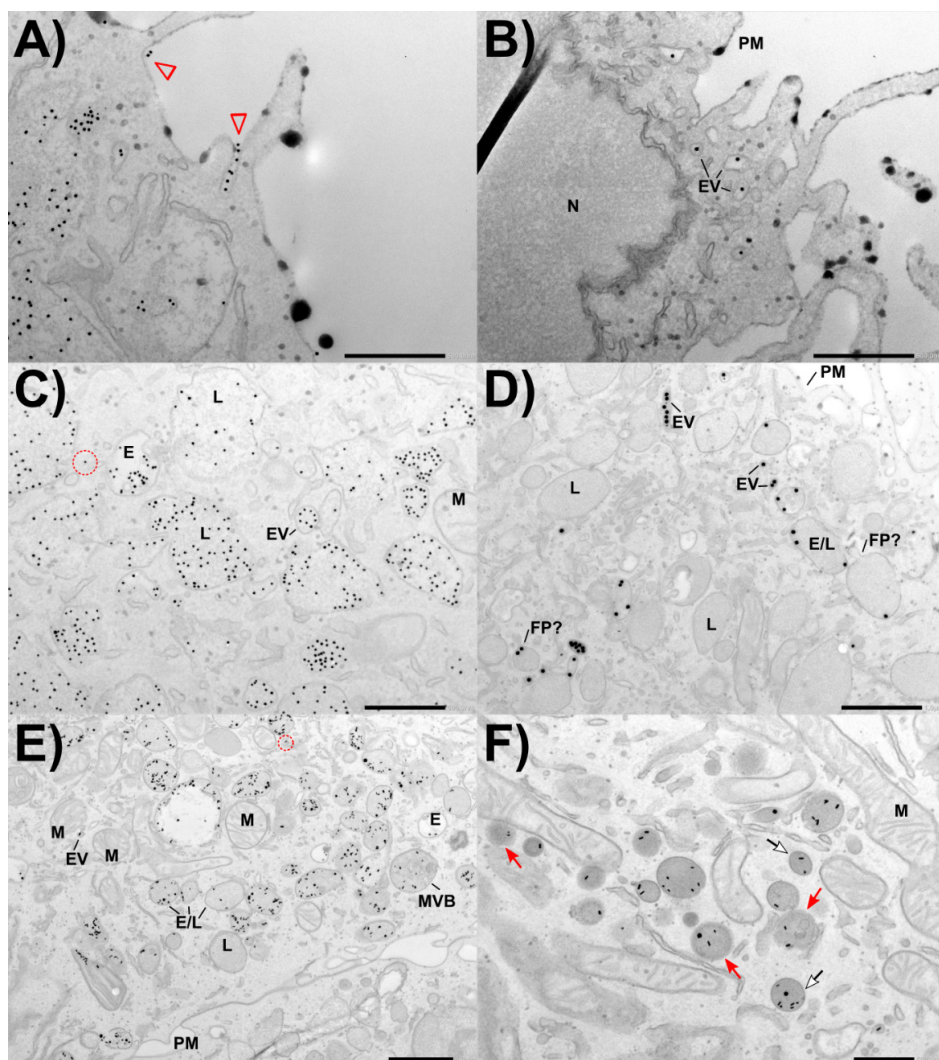


Figure S5.7. TEM images of a BMDC section: (A-C) DCs exposed to **15-1**; (D) DCs exposed to **40-1**; and (E,F) DCs exposed to **R-1**. These images show the subcellular localization of endocytosed GNPs and GNRs: EV = endocytic vesicle; E = endosome; L = lysosome; MVB = multivesicular body. Other cellular organelles present are: M = mitochondria; N = nucleus; PM = plasma membrane. It is possible that fusion pores (FP) formed by two vesicles were captured in (D). Red triangles indicate the potential entry of GNPs into the cells in panel (A). Red circles indicate GNPs and GNRs escaped into the cytosol in (C, E). Red arrows indicate vesicles with rough membranes and empty black arrows indicate vesicles with smooth membranes in panel (F). Scale bars = 1 μm (D,E) and 500 nm (A-C,F). The round dark crystals on the outer side of cell membrane are a result of the accumulation of stain (A,B).

Cellular uptake of epitope-coated GNPs and GNRs was quantified with ICP-MS:

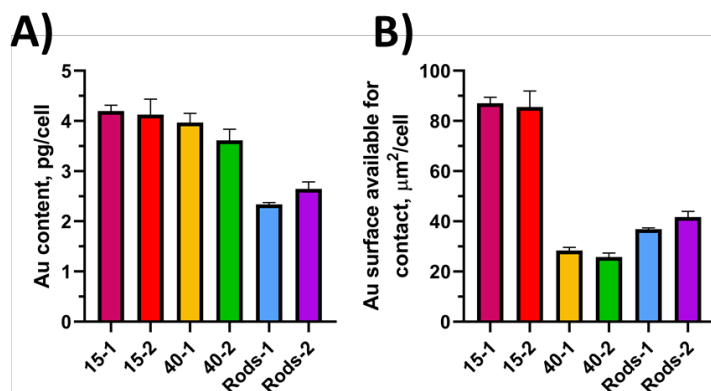


Figure S5.8. Quantification of cellular uptake visualized with TEM and shown in **Figure S5.4**. Quantification is based on ICP-MS data: (A) mean gold content in pg per cell; and (B) mean delivered gold surface available for contact in μm^2 per cell. Each column indicates a mean of three measurements; error bars indicate standard deviations and were calculated using GraphPad Prism software. More detail can be found in **Table S5.3**.

Activation of BMDCs by epitope-coated GNPs and GNRs through IL-12 and IL-1 β secretion was tested with ELISA:

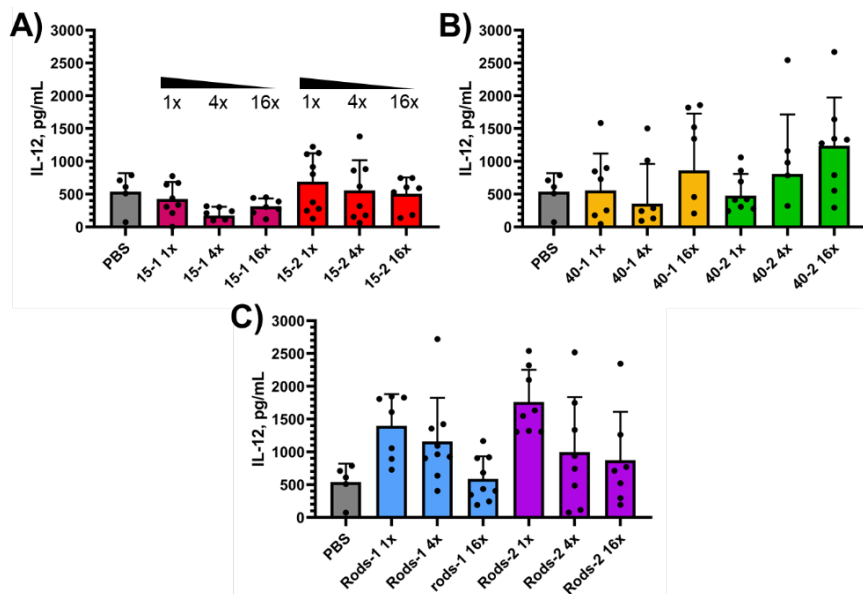


Figure S5.9. IL-12 levels in the cell culture supernatants of BMDCs after 24 h exposure to different GNPs: (A) GNPs with a 15 nm core; (B) GNPs with a 40 nm core; and (C) GNRs with a rod-like core of 45 by 15 nm in size. The BMDCs that were given PBS instead of GNPs or GNRs are present for comparison (gray color). The GNP concentration range analyzed included 1x, 4x, and 16x times diluted samples (Table S5.2).

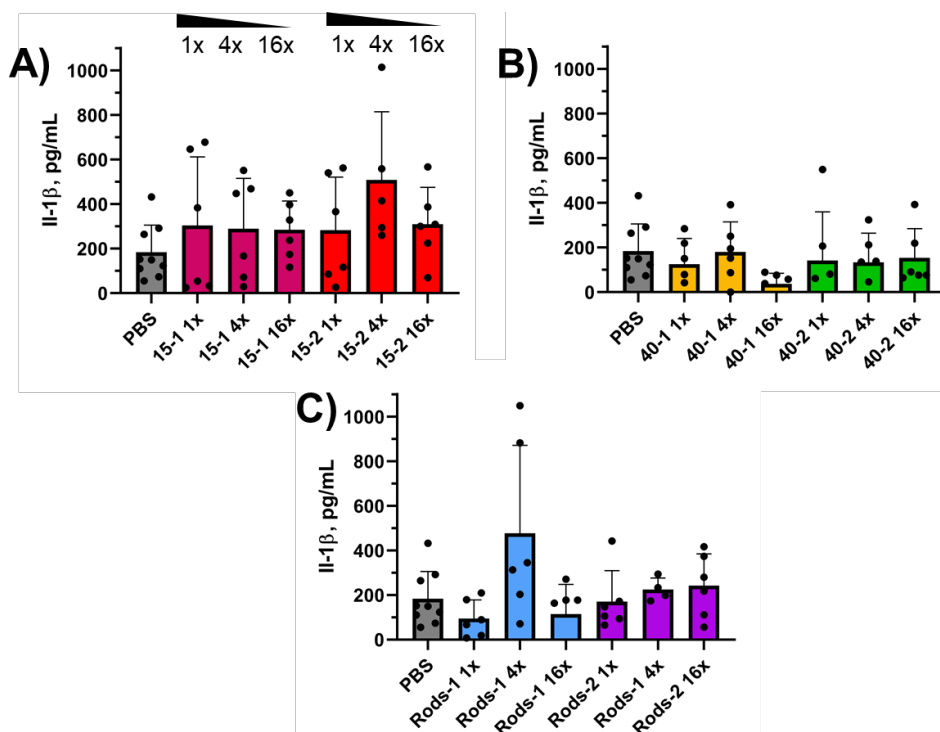


Figure S5.10. IL-1 β levels in the cell culture supernatants of BMDCs after 24 h exposure to different GNPs: (A) GNPs with a 15 nm core; (B) GNPs with a 40 nm core; and (C) GNRs with a rod-like core of 45 by 15 nm in size. The BMDCs that were given PBS instead of GNPs or GNRs are present for comparison (gray color). GNP concentration range analyzed included 1x, 4x, and 16x times diluted samples (Table S5.2).

Activation of CD8⁺ and CD4⁺ T cells isolated from OT-I and OT-II mice by epitope-coated GNPs and GNRs was monitored with FACS according to the following gating strategy:

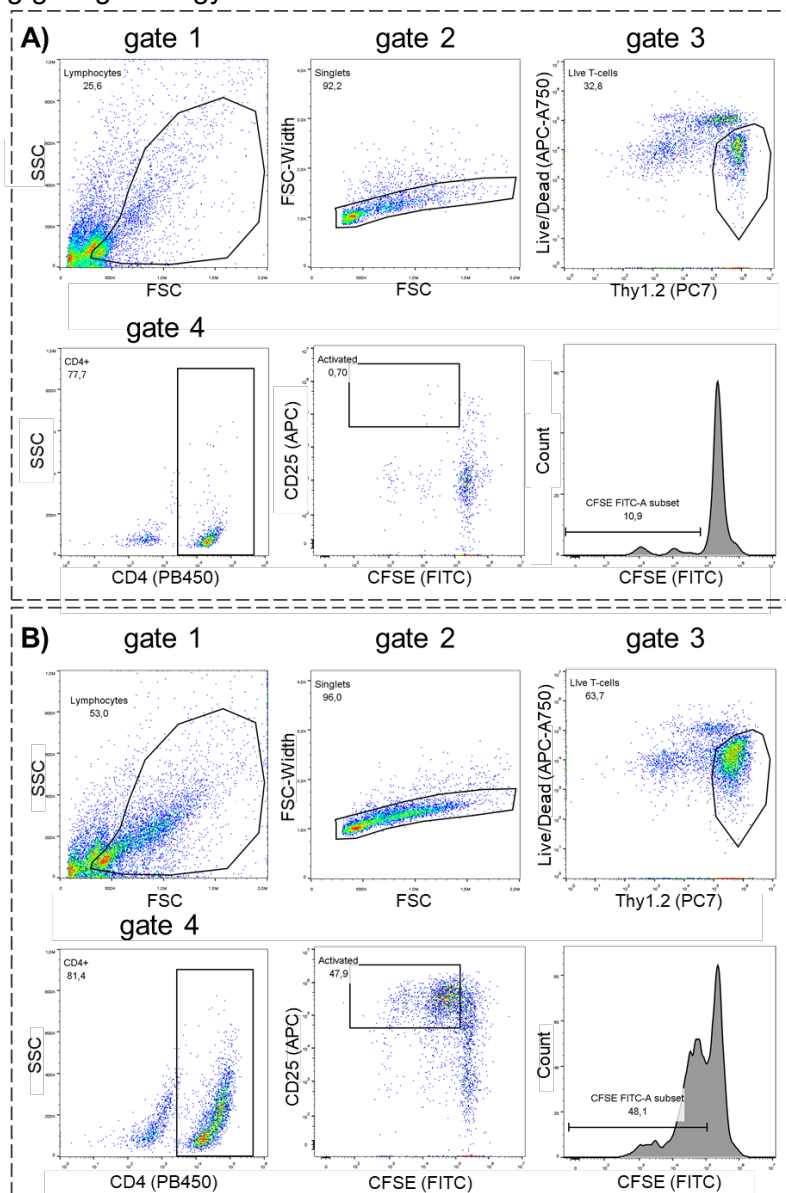


Figure S5.11. FACS gating strategy for OT-I and OT-II mice *ex vivo* experiments: (panel A) negative control – PBS and (panel B) positive control – 20 ng/well OVA₃₂₃₋₃₃₉. Data were analyzed on the area basis. Values for CD25⁺ versus CFSE^{low} are shown in **Figure 5.4, 5.5**.

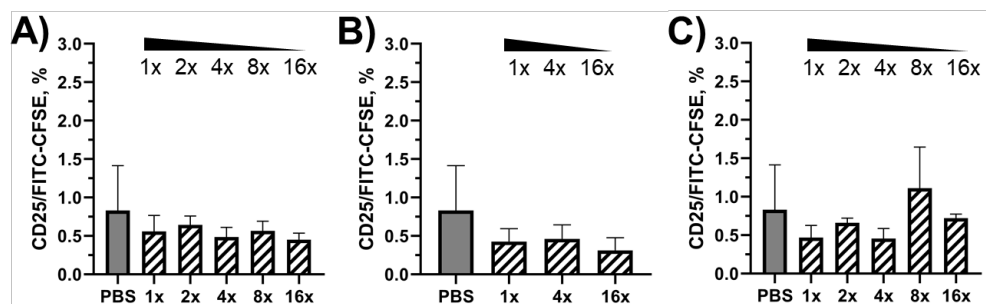


Figure S5.12. T-cell proliferation profiles of GNPs and GNRs coated with the **base** sequence (bearing no epitopes): (A) GNPs with a 15 nm core; (B) GNPs with a 40 nm core; and (C) GNRs with a rod-like core of 45 by 15 nm. Data represent a combined effect on both OT-I and OT-II derived T-cells. The BMDCs that were given PBS instead of GNPs or GNRs are present for comparison (gray color). GNP concentrations analyzed were 1x, 4x, and 16x (for 40 nm core) and 1x, 2x, 4x, 8x, and 16x (for 15 nm GNPs and GNRs) times diluted samples (**Table S5.2**).

Activation of BMDCs by epitope-decorated GNPs and GNRs through upregulation of cellular CD80, CD86, and MHC-I/OVA₂₅₇₋₂₆₄ markers:

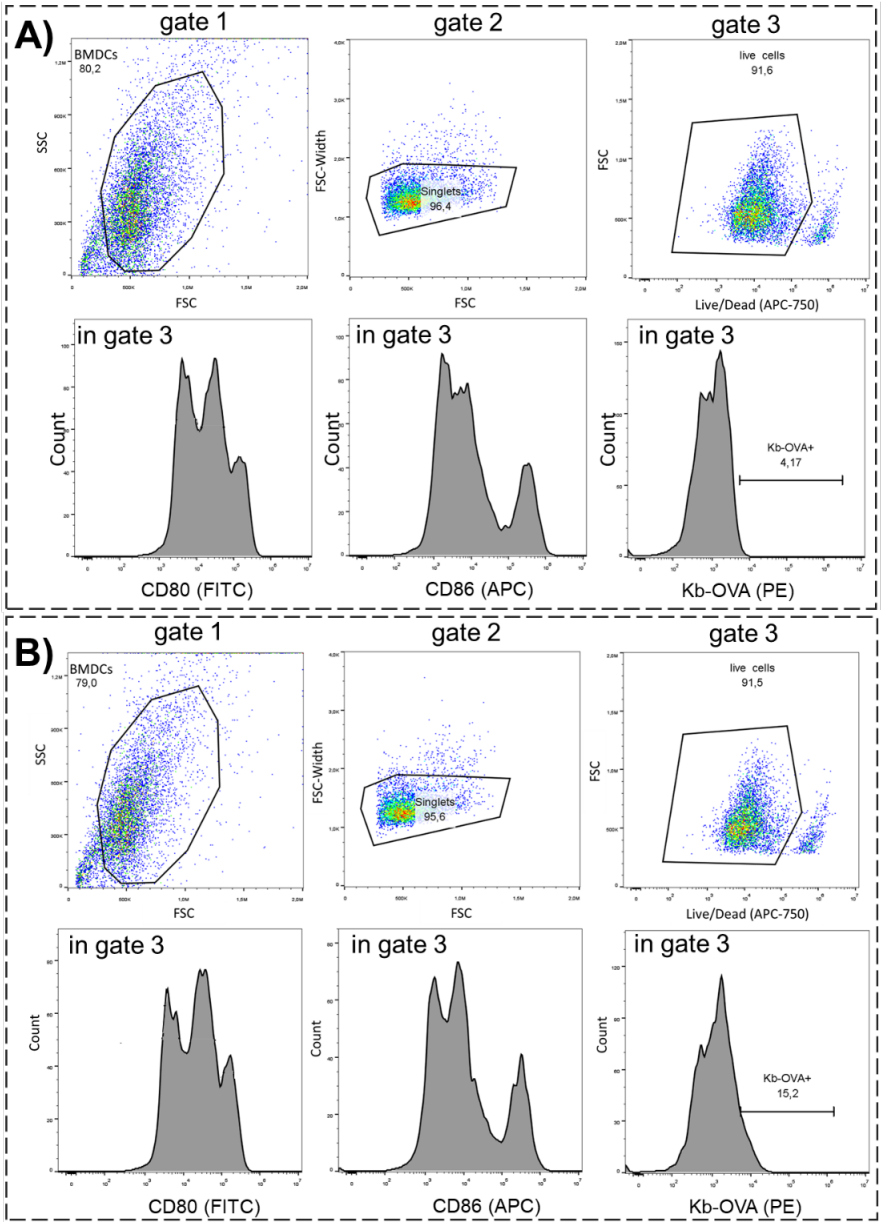


Figure S5.13. Gating strategy used in FACS analysis of BMDCs activation by epitope-decorated GNPs and GNRs. (panel A) negative control – DMEM medium and (panel B) positive control – 40 ng/well OVA₂₅₇₋₂₆₄. Data were analyzed on the basis of area.

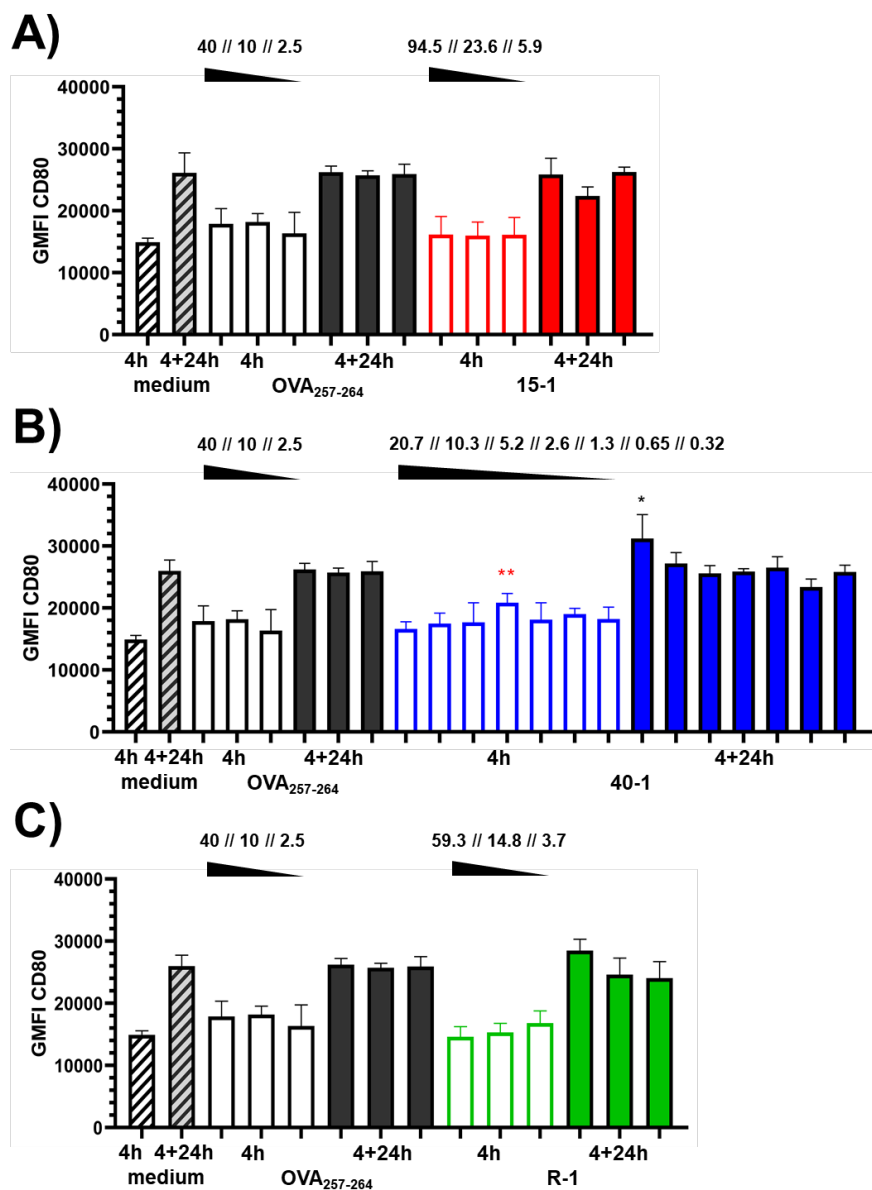


Figure S5.14. BMDCs activation was monitored through upregulation of CD80 cellular marker after a 4-hour incubation with GNPs and GNRs. Geometric mean fluorescence intensity (GMFI) gained for different samples was compared to the negative control (medium) and positive control (OVA₂₅₇₋₂₆₄): (A) **15-1**, (B) **40-1**, and (C) **R-1**. Empty columns show data right after the 4-hour incubation, and solid columns – after 24 hours after the incubation. Epitope dosage per well (in ng/well) is shown above the  indicator.

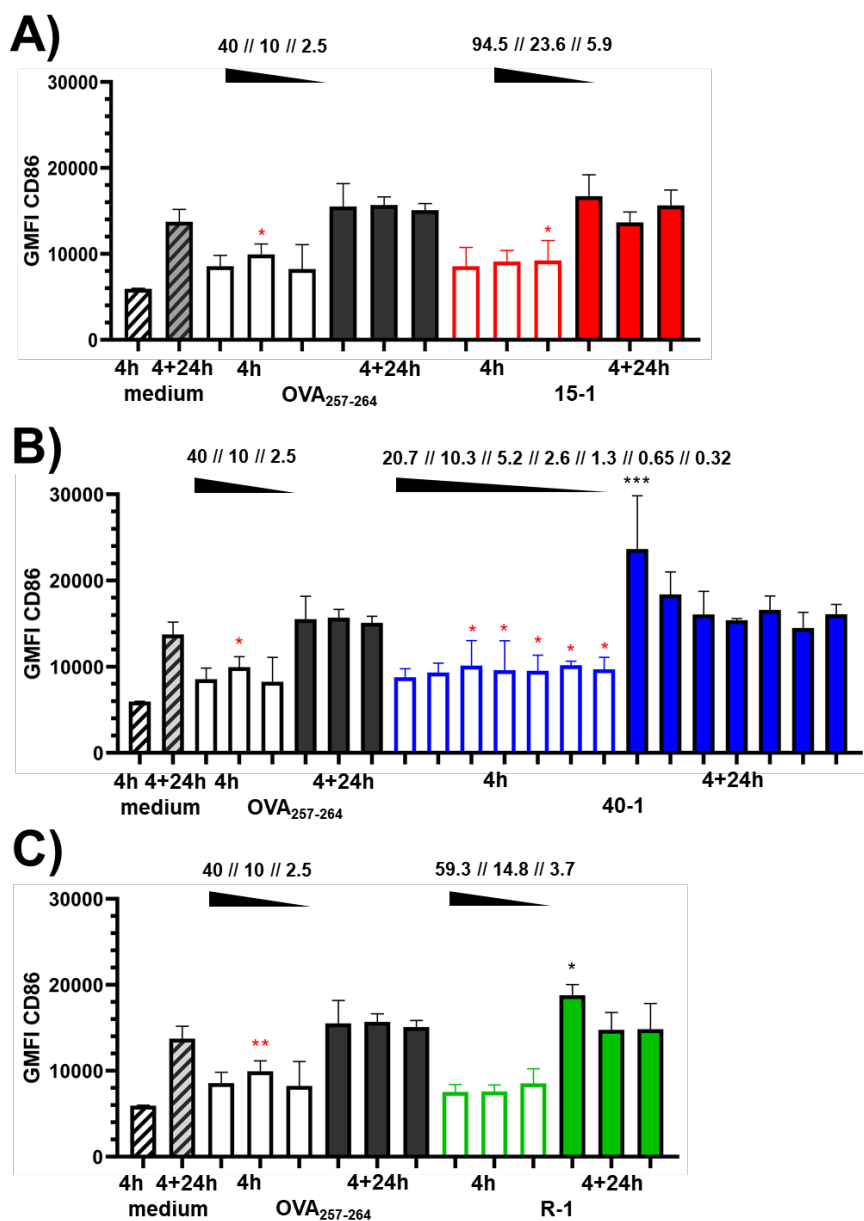


Figure S5.15. BMDCs activation was monitored through upregulation of CD86 cellular marker after a 4-hour incubation with GNPs and GNRs. Geometric mean fluorescence intensity (GMFI) gained for different samples was compared to the negative control (medium) and positive control (OVA₂₅₇₋₂₆₄): (A) **15-1**, (B) **40-1**, and (C) **R-1**. Empty columns show data right after the 4-hour incubation, and solid columns – after 24 hours after the incubation. Epitope dosage per well (in ng/well) is shown above the  indicator.

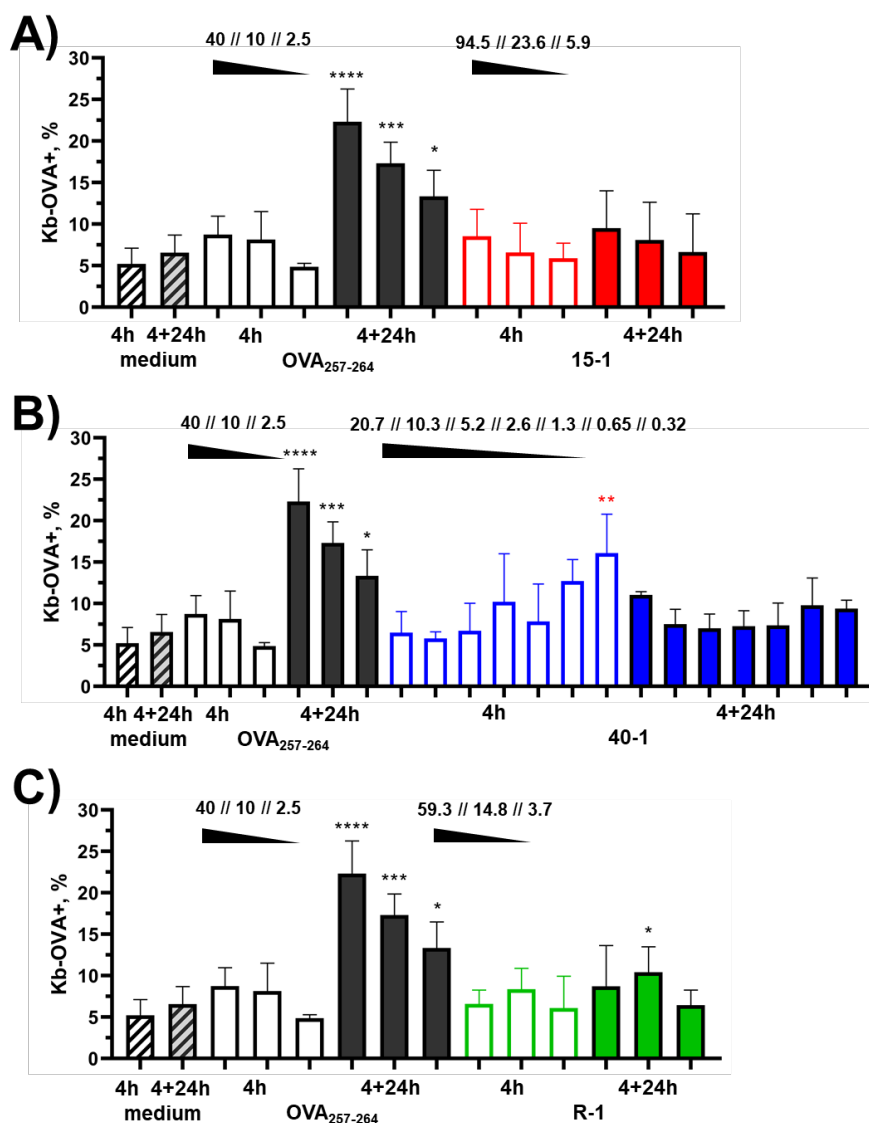


Figure S5.16. BMDCs activation was monitored through expression of MHC-I/OVA₂₅₇₋₂₆₄ complex on the cell surface after a 4-hour incubation with GNPs and GNRs. Geometric mean fluorescence intensity (GMFI) gained for different samples was compared to the negative control (medium) and positive control (OVA₂₅₇₋₂₆₄): (A) **15-1**, (B) **40-1**, and (C) **R-1**. Empty columns show data right after the 4-hour incubation, and solid columns – after 24 hours after the incubation. Epitope dosage per well (in ng/well) is shown above the  indicator.

Purity of the peptide and peptide amphiphile compounds was determined by LC-MS:

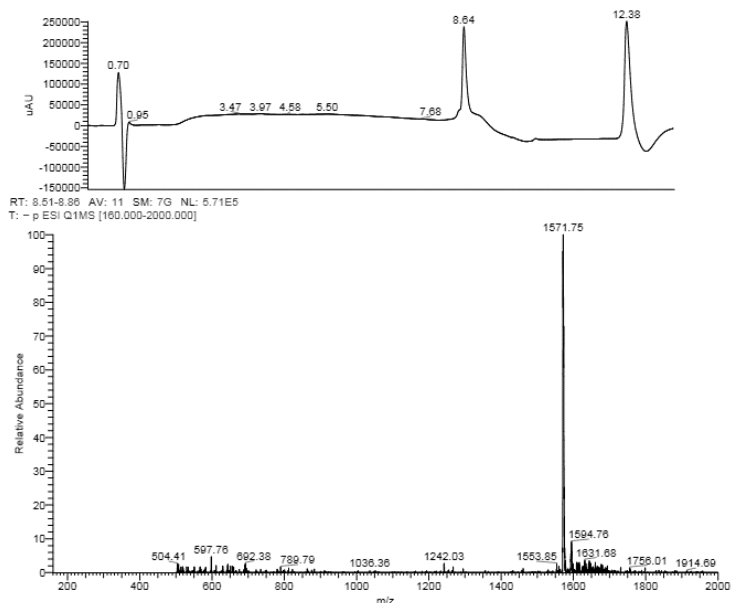


Figure S5.17. LC-MS spectrum for **base**. $[M-H]^{1-}_{\text{theor}} = 1571.83$.

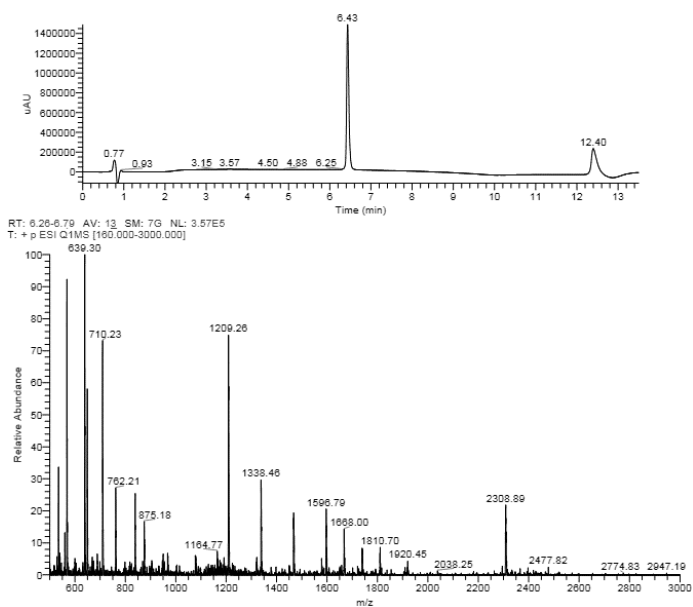


Figure S5.18. LC-MS spectrum for **1**. $[M+H]^{1+}_{\text{theor}} = 2309.26$.

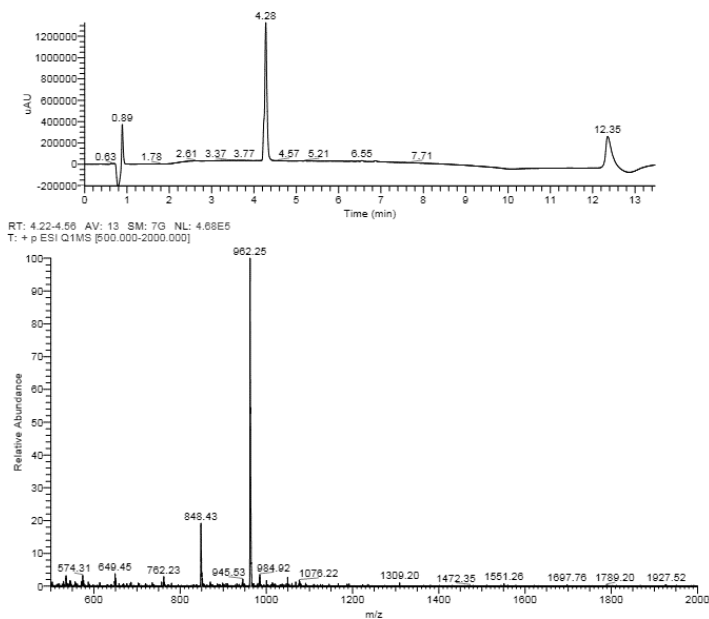


Figure S5.19. LC-MS spectrum for OVA₂₅₇₋₂₆₄. $[M+H]^+_{\text{theor}} = 962.54$. $[M+H]^+ = 848.43$ belongs to OVA₂₅₇₋₂₆₄ with a deletion of one isoleucine (I, Ile).

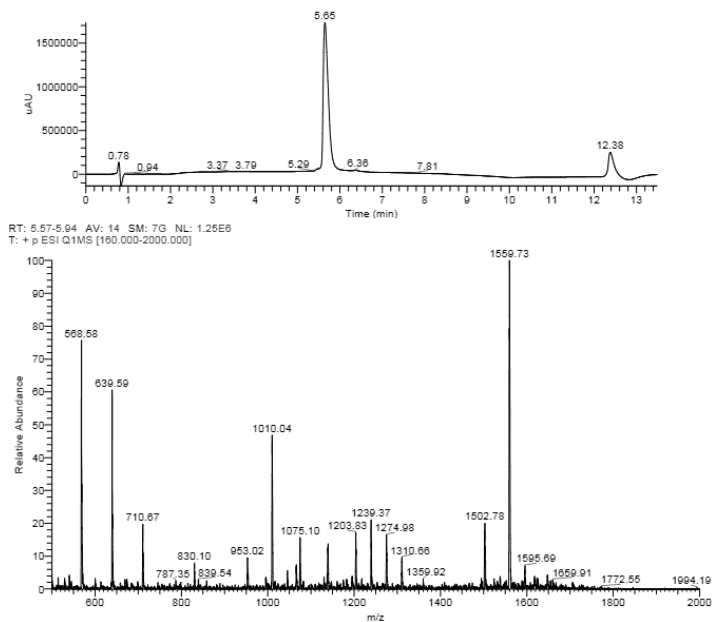


Figure S5.20. LC-MS spectrum for **2**. $[M+2H]^{2+}_{\text{theor}} = 1559.81$.

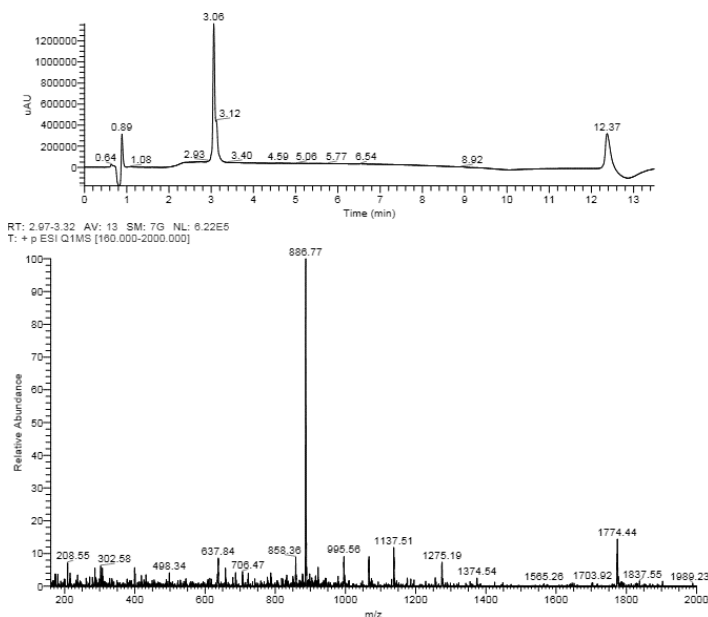


Figure S5.21. LC-MS spectrum for OVA₃₂₃₋₃₃₉. $[M+H]^+_{\text{theor}} = 1773.90$ and $[M+2H]^{2+}_{\text{theor}} = 887.45$.

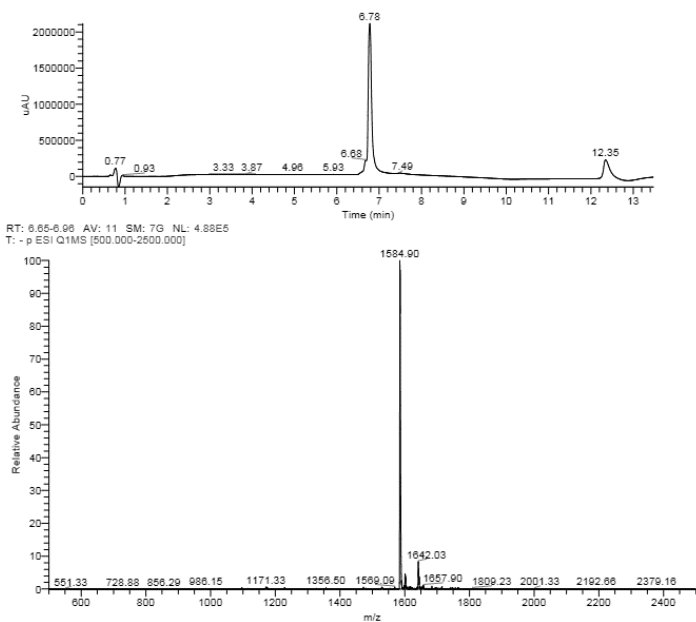


Figure S5.22. LC-MS spectrum for **W**. $[M-H]^+_{\text{theor}} = 1585.75$.