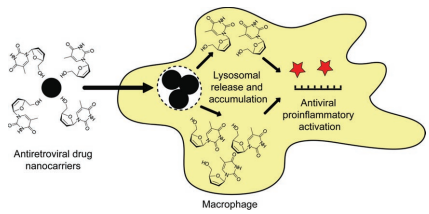
Gold Nanocarriers for Macrophage-Targeted Therapy of Human Immunodeficiency Virus

Hinojal Zazo,* Clara I. Colino, Klaudia T. Warzecha, Mareike Hoss, Uwe Gbureck, Christian Trautwein, Frank Tacke, José M. Lanao, Matthias Bartneck*

The human immunodeficiency virus (HIV) continues to be a global pandemic and there is an urgent need for innovative treatment. Immune cells represent a major target of virus infection, but are also therapeutic targets. Currently, no antiretroviral therapy targets macrophages, which function as portal of entry and as major long-term deposit of HIV. It has been shown before that human macrophages efficiently internalize gold nanoparticles, a fact which might be used to target them with drug-nanoparticle conjugates. Here, the authors use gold nanocarriers to facilitate delivery of stavudine, a widely used antiretroviral drug, to primary human macrophages. Using an ease-of-use coupling method, a striking potentiation of stavudine intake by macrophages using gold nanocarriers is shown. Further, the carriers induce a specific subtype of proinflammatory activation indicative for antiviral activity of macrophages, which suggests promising novel treatment options for HIV.



H. Zazo, Prof. C. I. Colino, Prof. J. M. Lanao
Area of Pharmacy and Pharmaceutical Technology
University of Salamanca, Spain
Av. Lcdo Méndez Nieto, 37007 Salamanca, Spain
E-mail: hinojal@usal.es
K. T. Warzecha, Prof. C. Trautwein, Prof. F. Tacke, Dr. M. Bartneck
Department of Medicine III
Medical Faculty, RWTH Aachen
Pauwelsstr. 30, 52074 Aachen, Germany
E-mail: mbartneck@ukaachen.de
Dr. M. Hoss
Electron Microscopic Facility (EMF)
Medical Faculty, RWTH Aachen
Pauwelsstr. 30, 52074 Aachen, Germany
Prof. U. Gbureck
Department and Chair of Functional Materials in Medicine and Dentistry
University Hospital Würzburg
Pleicherwall 2, 97070 Würzburg, Germany

1. Introduction

1.1. Role of Immune Cells in Antiretroviral Treatment of Human Immunodeficiency Virus

1.1.1. Key Problems in Targeting Immune Cells

The human immunodeficiency virus (HIV) continues to be a major global health challenge with almost 40 million people being infected and a global HIV prevalence of 0.8%.[1] Most patients receive antiretroviral therapies, which aim to abrogate virus replication, and represent the standard treatment of acquired immune deficiency syndrome (AIDS), and help to reduce disease severity. Stavudine is still in extensive use, specifically in the developing countries, due to low cost, despite its side effects such as lipoatrophy and weight loss.[2,3] Furthermore, an increasing number of patients are diagnosed with drug-resistant virus strains,[4] resulting in an urgent need for overcoming side effects and novel

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therapeutic options. One key issue in HIV pathogenesis is a fatal disabling of immune cells, which leads to the inability to protect the body efficiently from invading pathogens. In detail, lymphocytes (T helper cells) are killed by HIV, whereas another type of immune cell, macrophages (MΦ), serves as the portal of entry and viral deposit.

1.1.2. Macrophage-Directed Therapy of HIV

Due to the proliferative activities of lymphocytes, HIV leads to an accelerated cell death of T cells[5] whereas MΦ are much less sensitive to toxic effects of the virus. In terms of intracellular copy number, macrophages can store about 1000 copies of virus particles whereas T helper cells are killed by 20–50 virus particles per cell only.[6] Accordingly, MΦ can store the virus for longer times and at larger copy numbers, thus infecting large numbers of other immune cells during their large lifespan.[7] Despite numerous innovative attempts targeting T helper cells, few were made to target MΦ, a heterogeneous cell population that is known for extensive internalization of pathogens, but also of gold nanoparticles (AuNPs).[8] MΦ are composed of two basic heterogeneous subtypes: proinflammatory M1-MΦ which express proinflammatory cytokines such as interleukin 1 β (IL1β) and are also of therapeutic interest in HIV therapy since they exhibit antiviral activities.[9] On the contrary, M2-MΦ express cytokines like IL10, which deactivate many immune cells.[10] Targeting MΦ as a novel strategy for treatment of HIV can make use of their phagocytic activities for drug delivery, since they are efficient in the uptake of various types of nanoparticles.[8,11] Experts envision targeting viral reservoirs of HIV as a major step toward improved therapies.[6,12,13] Recently, we have shown that AuNPs distribute in diverse organs upon intravenous injection in mice, specifically liver and spleen, where large portions of them are internalized by local MΦ,[14] which means that tissue macrophages can be targeted in vivo by bloodstream injections. Targeting of bone marrow, an important HIV reservoir, however, might require equipping nanocarriers with additional specific targeting moities.[15]

1.1.3. Development of Macrophage-Targeting Nanocarriers

Therefore, the aim of this study is to generate AuNP-based nanocarriers for the targeted delivery of antiretroviral drugs to human primary macrophages as a prototypic novel nanotechnology-supported HIV therapy. We have prepared a nanoparticle library comprising different sizes (10, 40, and 70 nm) and surface stabilizers such as citrate, polyethylene glycol (PEG), and polyethylene imine (PEI). Stavudine was coupled to the nanoparticles, which was verified using UV–vis spectroscopy and zeta potential measurements. We used a state-of-the-art in vitro screening system based on human primary macrophages, which provides the highest possible approximation to the human in vivo situation. Molecular studies on the intracellular distribution of the nanocarriers were assessed using transmission electron microscopy (TEM) and inductively coupled plasma mass spectrometry (ICP-MS). The influence of the different conjugates on macrophage activation was studied using quantitative real-time polymerase chain reaction (PCR).

2. Experimental Section

2.1. Gold Nanocarriers

2.1.1. Generation of Nanocarriers

AuNPs with sizes 10, 40, or 70 nm, either functionalized with citrate, PEI, or PEG were obtained from nanoComposix (San Diego, CA, USA). Stavudine was obtained from ArQuifar (Barcelona, Spain). To generate stavudine-AuNP conjugates, AuNPs were mixed with a solution of stavudine. 1 mL of AuNP dilution (0.05 mg Au mL^{-1}) was incubated with mild agitation with 0.5 mL of stavudine (3 mg mL^{-1}) for 24 h at room temperature. The dispersion was centrifuged at 18 000 *g* for 15 min and the conjugates were resuspended in 10 μL of RPMI 1640 for usage in cell culture experiments.

2.1.2. Characterization of Nanocarriers

A zetasizer Nano Z (Malvern Instruments, Worcestershire, UK) was used to determine the nanoparticle charge; further, the carriers were characterized by UV–vis spectroscopy using a UV160A (Shimadzu, Tokyo, Japan). Drug release was determined in phosphate-buffered saline (PBS) solution at pH 7.4 and 5.5. To this end, 100 μL of the conjugates were suspended in 1 mL of PBS buffer and kept in an orbital shaking water bath Unitronic-Orbital (JP Selecta, Barcelona, Spain) at 37 °C under shaking conditions for up to 72 h. At each time, samples were centrifuged at 18 000 *g* for 15 min, and the drug concentration in supernatant was analyzed using ultra high-performance liquid chromatography (UHPLC). The quantification of stavudine in cell culture supernatant was done using UHPLC using a Shimadzu UHPLC (Tokyo, Japan) with a PDA SPD-M20A detector (Shimadzu, Tokyo, Japan) at 265 nm. The chromatographic separation was performed by a Kinetex C18 column (50 mm × 2.10 mm, particle size 1.7 μm, Phenomenex) thermostated at 45 °C. The mobile phase was water:acetonitrile (94:6 v/v) at a flow rate of 0.5 mL min^{-1}, as described earlier in detail.[16] The inter-day and intra-day variability of the instrument was below 5%.

2.2. Cells

2.2.1. Cell Isolation, Culture, and Analyses

Human peripheral blood mononuclear cells (PBMC) were isolated from buffy coats obtained from transfusion medicine

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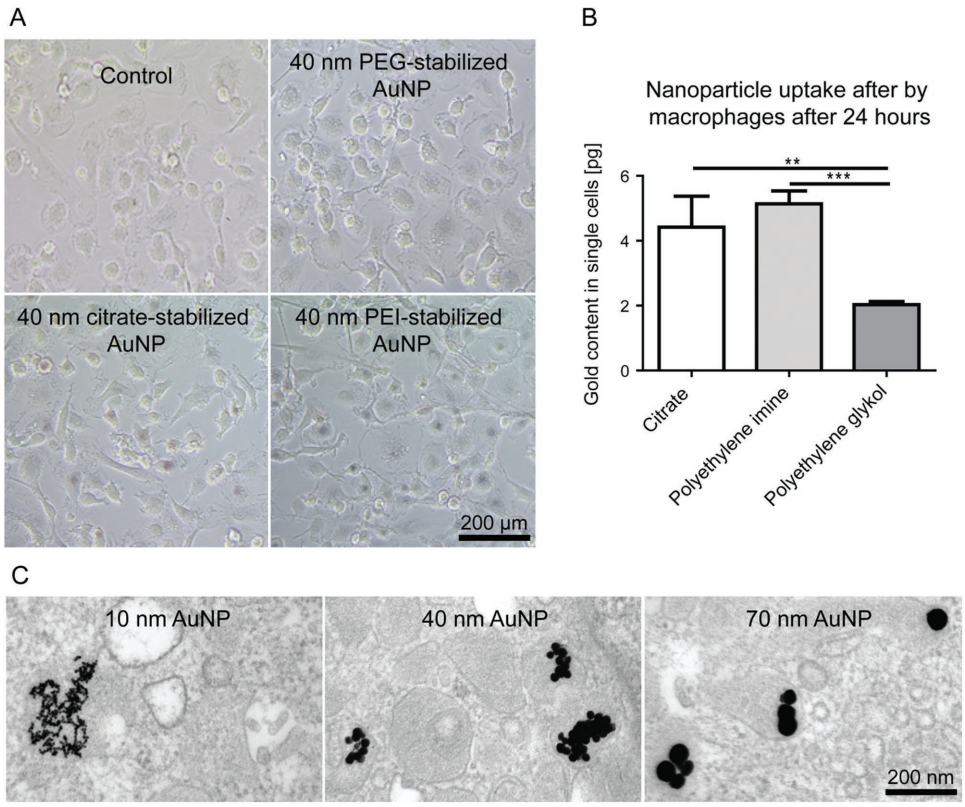

Figure 1. Impact of nanocarrier surface stabilizer on intracellular localization and accumulation in macrophages. A) Human primary macrophages cell cultures after 24 h of incubation. B) Quantifications of the amount of gold in single macrophages determined using inductively coupled plasma mass spectrometry. C) Transmission electron microscopy of ultrathin sections of macrophages after incubation for 24 h with sizing 10, 40, or 70 nm. Data are expressed as mean ± SD; $^*P < 0.05$ (unpaired Student's *t*-test).

of RWTH Aachen University Hospital using Ficoll-based density gradient centrifugation as described earlier.[11] Briefly, PBMC were incubated at 37 °C on uncoated, bacterial grade Petri dishes (2 million cells mL^{-1}) in RPMI1640, containing 5% human autologous serum, for 35 min in a humidified incubator with 5% CO_2. During this period, monocytes adhere to the dish and lymphocytes are removed with the supernatant. To obtain human primary macrophages, monocytes were cultured for 7 d in RPMI1640 medium supplemented with 5% autologous human serum. Macrophages were incubated with 10 µL of the adjusted solutions and samples were taken after up to 72 h of incubation as reported earlier.

2.2.2. Quantification of Nanocarriers in Cells and Media

The intracellular distribution and uptake of AuNP were studied by TEM and ICP-MS. To prepare cells for TEM, fixation was done in 3% glutaraldehyde for 24 h, cells were then embedded in 2% agarose, incubated for 1 h of incubation in 1% osmium tetroxide, rinsed with distilled water, and dehydrated with ethanol and propylenoxide. Preparations were cut into 80 nm thin sections and analyzed with an EM 400 T Philips at 60 kV. Microscopy images were taken using a CCD-Camera MORADA Olympus. The intracellular concentration of AuNP was measured using an ICP-MS detector (Varian, Barmstadt, Germany) with a commercial gold standard solution (Merck, Darmstadt, Germany). To prepare cells for TEM, they were dissolved in 1 mL of 37% hydrochloric acid (HCl). After 10 min of centrifugation at 3000 *g*, 0.5 mL of each supernatant was diluted with 4.5 mL double distilled water. After further centrifugation for 10 min, the solution was diluted to an equal part with 3.7% HCl and gold was quantified.

2.2.3. Gene Expression Analysis

The expression of inflammatory mediators was analyzed using real-time PCR for IL1β and IL10. RNA was purified using the peqGold kit (PEQLAB Biotechnologie GmbH, Erlangen, Germany), and complementary DNA was generated from RNA using the First Strand cDNA synthesis kit (Roche, Penzberg, Germany). Quantitative real-time polymerase chain reaction was done based on SYBR Green Reagent (Roche, Penzberg, Germany). Reactions were done as triplicates, and β-actin was used to normalize gene expression. Primer sequences are available upon request.

2.2.4. Statistical Analysis

Graph Pad Prism 5.0 was used to calculate statistical significance using the appropriate statistical tests.

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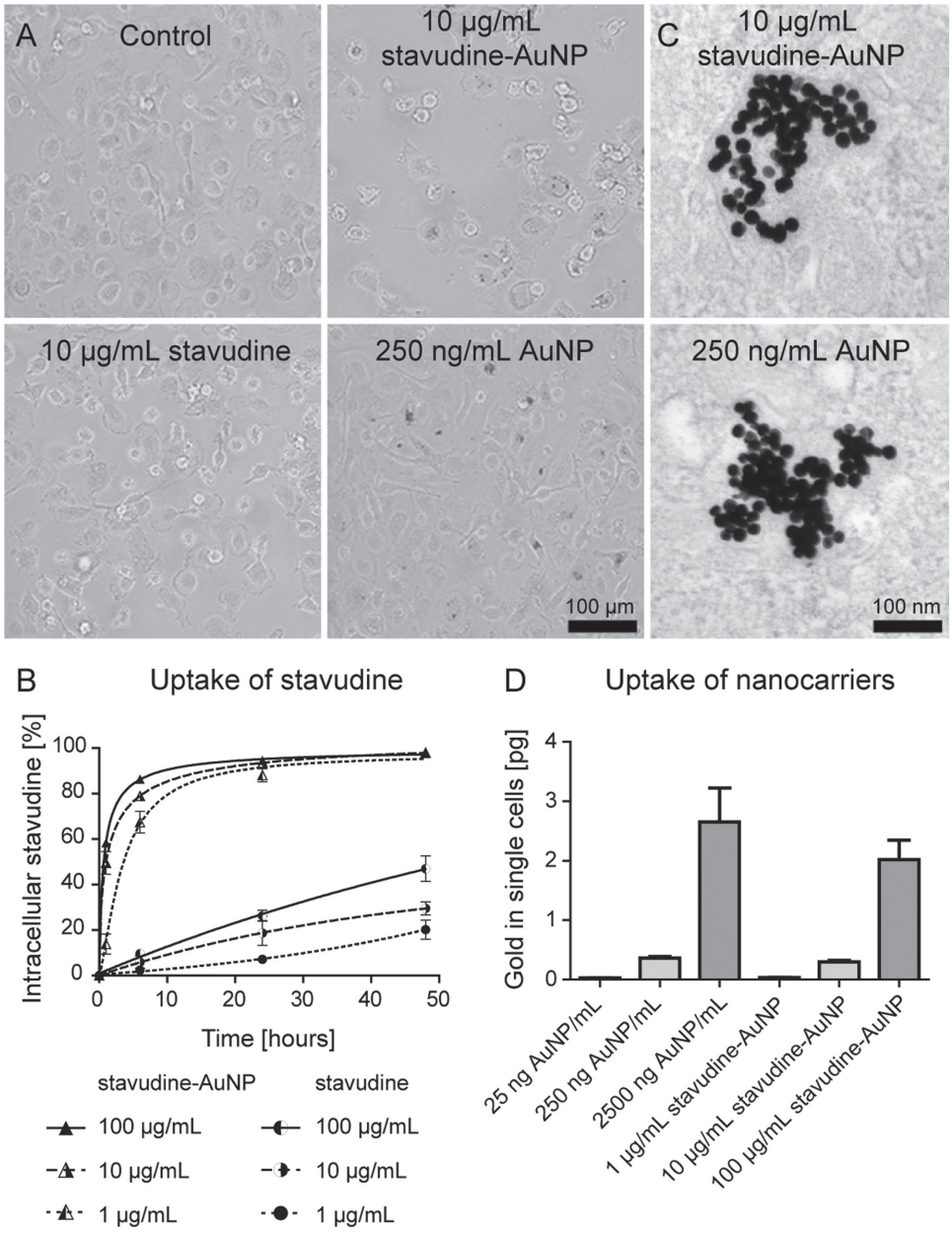

Figure 2. Transport of stavudine into macrophages mediated by gold nanocarriers. Macrophages were incubated with stavudine nanocarriers, stavudine, or gold alone for 24 h. A) Human macrophages in cell culture after 24 h of incubation. B) Quantification of stavudine by UHPLC, C) stavudine-nanocarriers or carriers without drug inside human macrophages visualized using TEM. D) Intracellular concentrations of conjugates compared to AuNP alone.

3. Results and Discussion

3.1. Optimization of Nanocarriers

Chemical characterizations demonstrated successful generation of nanocarriers by UV–vis spectrometry (Figure S1A, Supporting Information) and zeta potential measurements (Table S1, Supporting Information). Drug release studies demonstrated that the release of stavudine was influenced by the stabilizer used (Figure S1B, Supporting Information). In order to optimize the nanocarriers functionalized with either PEG, PEI, or citric acid, we incubated macrophages for 24 h with the nanocarriers. All stabilizers evoked a similar cellular morphology (Figure 1A), but ICP-MS studies showed that citrate and PEI evoked increased uptake of nanocarriers by macrophages whereas PEG reduced it (Figure 1B). Nanocarriers sizing 70 nm compared to those sizing 10 or 40 nm further led to strongly reduced numbers

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of AuNP in macrophage lysosomes, as observed in TEM (Figure 1C). We decided to focus on the 40 nm citrate-based carriers for the following experiments because of the higher uptake by macrophages, higher loading capacity, and the generally reduced toxicity of negatively compared to positively charged nanoparticles.[17]

3.2. Uptake of Stavudine and Stavudine-Nanocarriers by Macrophages

Therefore, the next step was to compare uptake of free stavudine to carrier-coupled drug in macrophage cell cultures. We found that coupling stavudine to gold had strong effects on macrophage morphology: at a concentration of 10 μg mL^{-1} stavudine and 250 ng AuNP mL^{-1}, AuNP-stavudine nanocarriers influenced macrophage morphology with an increased number of cells exhibiting a round shape whereas neither stavudine nor gold alone influenced macrophage morphology at the identical concentration when administered separately (Figure 2A). Huge concentrations of free stavudine had a similar effect on macrophage morphology (induction of round shape), but not huge concentrations of gold nanoparticles (Figure S2, Supporting Information). This suggests that the uptake of stavudine by macrophages was strongly incremented by the nanocarriers, compared to the uptake of free drug. Apparently, the changes in cellular morphology related to an increase in intracellular stavudine by the AuNP conjugates in terms of the applied dose detected intracellularly in UHPLC (Figure 2B). In order to study the role of coupled stavudine on AuNP uptake by macrophages, TEM and ICP-MS analyses were performed for nanoparticles with and without stavudine. A similar content of AuNP in macrophage lysosomes was detected for both gold nanocarriers and gold alone (Figure 2C). ICP-MS-based quantifications confirmed very similar amounts of intracellular AuNP (Figure 2D). Our data suggest that free stavudine enters macrophages at very low amounts, which might be explained by the passive uptake of free stavudine based on diffusion,[18] which is assumed to be very low due to the hydrophilic properties of the drug.[19] In contrast, the stavudine-nanocarriers entered the cells via phagocytic uptake, as demonstrated earlier.

3.3. Molecular Activation of Macrophages by Stavudine-Nanocarriers

To further study molecular effects of the conjugates, we performed analysis of the expression of key inflammatory molecules of macrophages subsets after 24 h of incubation with nanocarriers or single components (Figure 3). Proinflammatory M1-MΦ express IL1β, a central molecule in inflammasome activation,[20] which is also expressed by antiviral M1-MΦ and potentially indicates therapeutic activities of MΦ.[9] Strikingly, the conjugates exhibited a strong, concentration-dependent proinflammatory polarization and thus are particularly interesting for HIV therapy because proinflammatory activation is associated with antiviral activities of macrophages.[9] The anti-inflammatory cytokine IL10, which has a deactivating function on many immune cells,[21] was not affected by the nanocarriers (except for very high carrier concentration), thereby clearly supporting their proinflammatory activation. Interestingly, the largest concentration of

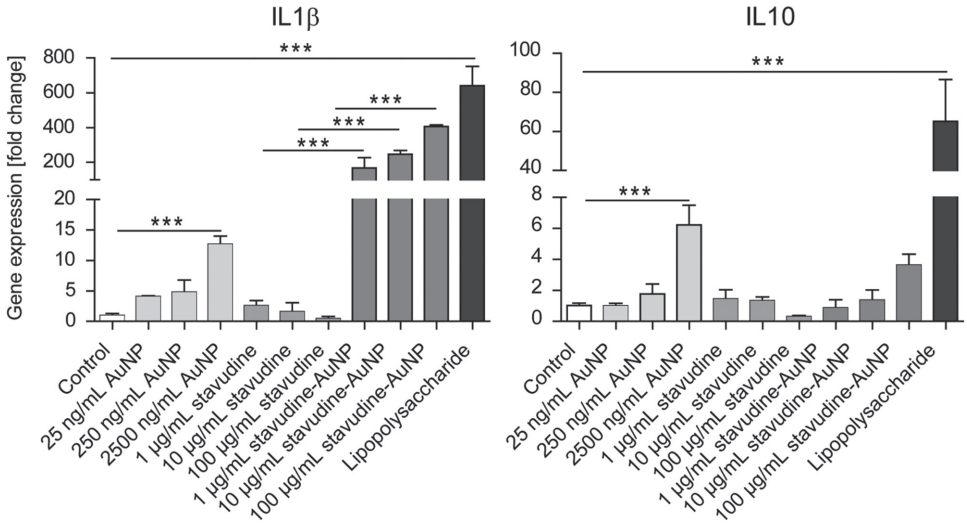

Figure 3. Expression of inflammatory genes by macrophages after incubation with nanocarriers. Human primary macrophages were incubated with either citrate-stabilized gold nanoparticles (AuNP), stavudine, or AuNP-stavudine conjugates at different concentrations for 24 h. Inflammatory gene expression of interleukin 1 β (IL1β) and IL10 was studied using real-time polymerase chain reaction. Mean data of three independent experiments. Data are expressed as mean ± SD; ***$P < 0.001$ (unpaired Student's *t*-test).

AuNP alone significantly induced IL1β and IL10 production, different from the highly specific upregulation of IL1β by the conjugates, but orders of magnitude below the nanocarriers. This demonstrates that we observed a novel state of macrophage polarization driven by stavudine-nanocarriers different from the effects of classical proinflammatory activation evoked by LPS or AuNP.

4. Conclusions

Our data indicate that our ease-of-use system could enable targeting macrophages at early stages of HIV when the virus replicates in macrophages, which in turn infect other cell types that are more sensitive to the virus.[6] Macrophages serve as a deposit for virus particles (about 1000 copies of virus particles) and distribute it to other cell types, specifically $CD4^+$ lymphocytes (20–50 virus particles per cell), which are rapidly killed by the infection.[6] The conjugation of stavudine-AuNP produces an important increment in the drug uptake by macrophages. Future trials facilitating humanized mouse models for HIV will help to validate the therapeutic potential of the nanocarriers in vivo.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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