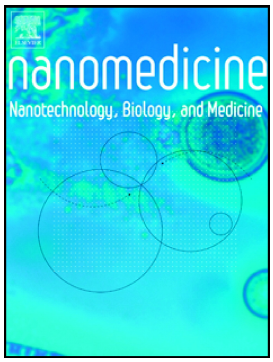

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Effects of topical methotrexate loaded gold nanoparticle in cutaneous inflammatory mouse model

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GP designed research, IF, IV provided the chemical synthesis of the AuNPs and bioconjugates; SAM developed the topical formulation; LB, CV, PA, CM, did the biological tests; EB, AC, LB, VC, MF, carried out the in vivo experiments; IF, LB EB, HB analyzed data; GP, IF, LB and AC critically revesed the paper. All authors contributed to the manuscript.

Abstract

Gold nanoparticles functionalized with 3-mercapto-1-propansulfonate (AuNPs-3MPS) have been prepared and loaded with Methotrexate (MTX), an immunesuppressive agent used in the systemic treatment of moderate-severe inflammatory deseases. The effects of the AuNPs-3MPS@MTX topically administered *in vitro* on skin model and *in vivo* on imiquimod-induced psoriasis-like mice model, have been studied. Clinical response, epidermal thickness, cell proliferation rate and inflammation were tested. AuNPs-3MPS@MTX treated mice showed a decreasing of scaling and erythema score, reduction of epidermal thickness, parakeratosis and hyperkeratosis, compared to AuNPs-3MPS treated mice. Immunohistochemistry analysis staining displayed that Ki67, K6 CD3 and CD8 stainings were reduced in AuNPs-3MPS@MTX treated mice. Blood evaluation showed no differences in blood count and in ALT and AST levels before and after AuNPs-3MPS or AuNPs-3MPS@MTX treatment. Topical AuNPs-3MPS@MTX treatment is able to induce a reduction of keratinocytes hyperproliferation, epidermal thickness and also inflammatory infiltrate *in vivo* on imiquimod-induced psoriasis like mice model.

Keywords

functionalized gold nanoparticle; topical psoriasis treatment; methotrexate transepidermal delivery; percutaneous delivery.

Background

The use of functionalized metal nanoparticles (MNPs) has fascinated researchers of many different fields, for applications ranging from optics[1] to optoelectronics,[2] catalysis,[3] sensors,[4] and biotechnology.[5] Among others, gold nanoparticles (AuNPs) have been deeply investigated for medical applications, due to their unique chemical and physical properties, combined with general biocompatibility[6] and well-established strategies for surface functionalization.[7] For example, AuNPs can be used as imaging/diagnostic tools,[8] as radio-sensitizer agents[9] or in photo-thermal therapy.[10] Among biomedical applications, their use as drug delivery systems is also well established, in particular for those drugs with hydrophobic characteristics that need to be vehiculated in a biological system.[11,12] One of the objective of drug delivery systems focuses on the transdermal delivery to avoid size effects and uses in across-skin permeation.[13] However, there are some limits for the delivery of high molecular weight drugs due to low permeation. The understanding of the permeation mechanism at molecular level was recently studied for AuNPs with different sizes and surface functionalizations.[14,15] The development of an effective method for the enhancement of therapeutic agents permeation was deeply investigated, thus interesting complex platforms were proposed in the literature: for example composites between AuNPs and copolymeric micelles were used for the delivery of hydrophobic drugs.[16]

Among hydrophobic drugs, methotrexate (MTX) is currently being used for the treatment of different diseases. MTX is a synthetic folic acid analogue, an antimetabolite used as immunosuppressive agent.[17] MTX showed anti-inflammatory and immunomodulation properties, leading to its use in the treatment of different inflammatory diseases, such as psoriasis, multiple sclerosis and rheumatoid arthritis.[18] MTX also showed an epidermal proliferation inhibitory effects and now represents standard therapy in various cutaneous diseases. Although its use may be associated with undesirable adverse events such as hepatotoxicity and myelosuppression, MTX is

prescribed systemically in moderate and severe psoriasis.[19] These side effects could be overcome by the topical use of MTX, particularly in mild psoriasis patients. Psoriasis is a quite common chronic inflammatory skin disease, characterized by inflammatory infiltrate and keratinocyte hyperproliferation.[20] Different topical MTX formulations have been used in different studies with encouraging but not fully satisfactory results.[21] This was mostly due to MTX insufficient percutaneous penetration induced by high molecular weight, water solubility, poor solubility in ointment formulations, and the ionized nature at the physiological pH causing a reduction in the passive diffusion through layers of the skin, especially in psoriatic plaques which are characterized by increased epidermal thickness and hyperkeratosis. Therapeutic applications of MTX delivery by using different nanovehicles were recently reviewed by Choi et al[22] highliting the possibility to reduce adverse effects such as toxicity. Several formulations have been tried in order to improve its topical delivery. Strategies varied from using penetration enhancer, [23] adhesive tapes as occlusive covering, iontophoresis,[24] liposomes,[25,26] nanogels,[27,28] lipid carriers,[29] to the development of novel drug delivery carriers.[30] One of the main points of focus was the use of gold nanoparticles (AuNPs) due to their suitable physical and chemical properties as their surfaces are easy for chemical modification by many bioactive molecules.[31]

The present study aimed to evaluate the effects of AuNPs, functionalized with sodium 3-mercapto-1-propansulfonate (AuNPs-3MPS) loaded with MTX (AuNPs-3MPS@MTX). Preliminary results highlighted that AuNPs-3MPS are not toxic in vitro and in vivo and that are able to increase the MTX delivery in the epidermis and also in the dermis compared to MTX alone in mice skin.[32]

STEM images on normal human keratinocytes cultured *in vitro* clearly revealed the distribution of gold nanoparticles inside the cells and UV-vis spectra for *in vivo* tracing of the conjugate on bare mouse skin after 24 h of application, show increased delivery of Methotrexate in the epidermis and dermis using AuNPs-3MPS@MTX conjugate, compared to MTX alone. In particular a loading efficency up to 80% (*i.e.* 160 μg MTX/mg AuNP) was obtained and with a fast release (80% in one hour, up to 95% in 24 h).

In this work, the AuNPs-3MPS@MTX was topically administered *in vitro* on skin model and *in vivo* on imiquimod-induced psoriasis like mice model,[33] testing the clinical response, epidermal thickness, cell proliferation rate and inflammation comparing AuNPs-3MPS alone and AuNPs-3MPS@MTX treatment for 24 and 48 hours. Non cytotoxic AuNPs-3MPS were used for the first time in this work on psoriatic murine models as therapeutic agents. It is noteworthy that topical MTX formulation drugs for psoriasis are at present not commercially available and in this work MTX is associated to a gold nanoparticle and topically administred. The clinical evidences on epidermal thickness, cell proliferation rate and inflammation have been studied in topical application. In vivo and histological results demonstrated reproducibility and efficacy.

Methods

Bioconjugation of AuNPs-3MPS@MTX

Gold nanoparticles functionalized with sodium 3-mercaptopropane sulphonate (AuNPs-3MPS) were prepared and characterized according to literature procedure.[34,35] Briefly, AuNPs-3MPS were prepared with Au/thiol (Au/S) molar ratio. Starting from 200 mg of $HAuCl_4 \cdot 3H_2O$ in 20 mL of deionized water, 20 mL of a solution of the thiol 3MPS (0.25 M) in deionized water was added. Under vigorous stirring a reducing solution (*i.e.* 190 mg of $NaBH_4$ in 20 mL of deionized water) has been added. The mixture was allowed to react at room temperature for 3 hs and at the end the brown solid was recollected and purified by centrifugation (13000 rpm, 20 min, 5 times with deionized water). Bioconjugation of AuNPs-3MPS@MTX was carried out as previously described on analogue systems.[36] Briefly, AuNPs-3MPS nanoparticles (10 mg) were dissolved in water (4 mL pH 5.5) and vigorously stirred for 4 hours at room temperature in the presence of 2 mg of MTX (Sigma Aldrich, M 8407) in order to have a weight ratio AuNPs/drug = 5/1. The reaction mixture was maintained in the dark to avoid MTX degradation with light. After 4 hours, the mixture was purified by centrifugation (at room temperature, 13000 rpm, 90 min) in order to separate the

AuNPs-3MPS@MTX bioconjugate from the free MTX rug in solution. The loading efficency was 80% (*i.e.* 160 µg MTX/mg AuNP). Field Emission Scanning Electron Microscopy (FE-SEM) images were acquired with the Auriga Zeiss 405 instrument (resolution 1 nm, applied voltage 6–12 kV) on freshly prepared drop casted films and UV-vis with a Varian Cary 100 in H_2O with quartz cells.

Preparation of AuNPs-3MPS@MTX and AuNPs-3MPS cream formulation

Both AuNPs-3MPS and AuNPs-3MPS@MTX were mixed in Locobase, a frequently used cream for galenic formulation, applied to obtain an emollient effect with no therapeutical role regarding inflammation and keratinocytes proliferation. AuNPs-3MPS@MTX and AuNPs-3MPS Locobase cream formulation were done to facilitate topical application. AuNPs-3MPS@MTX and AuNPs-3MPS cream formulations were prepared mixing freshly prepared AuNPs-3MPS@MTX bioconjugate or AuNPs-3MPS with Locobase cream (Astellas Pharma Europe B.V), following this procedure: 100 µg of the bioconjugate or AuNPs-3MPS were mixed with 1 mg of Locobase cream under constant manual stirring at room temperature avoiding light irradiation for 15 minutes till appearance of homogenous mixture. This formulation was then preserved to -20°C in a sterile syringe. Locobase is an emollient and hydrating cream contening petrolatum, water, paraffinum liquidum, cethearyl Alcohol, ceteareth 25, methylparaben, citric acid, sodium citrate.

Toxicity tests on skin equivalent

MTT test was performed on human skin equivalents (HSEs) in order to exclude acute toxicity[37]. After the authorization of the local Ethical Committee, adult human keratinocytes were seeded on a dermal substitute consisting of a collagen type I matrix and fibroblasts. A highly differentiated and stratified epidermis model was obtained after 21-days culture period. Following the ECVAM (European Center for the Validation of Alternative Methods) guidelines[37] for the testing of Chemicals, 100 µg AuNPs-3MPS@MTX bioconjugate or AuNPs-3MPS alone were topically

applied to the skin model. Positive and negative controls were performed with SDS solution 5% in water and PBS alone respectively; the materials were applied topically on the epidermal model for 15 and 30 minutes. Exposure to the tested chemicals was terminated by rinsing with PBS. After, the skin equivalents were incubated at 37°C with culture medium for 42 additional hours (post treatment incubation). For each test material, three independent tests were used. After this time the viability was assessed by incubating the tissues with MTT solution and quantified spectrophotometrically at 570 nm (Siemens BEP – III, Marburg, Germany). For each treated tissue the viability is expressed as the % of the negative control tissues. Values under 50 % will qualify the product as irritant.

Imiquimod-induced psoriasis like mouse model

Experiments were approved by the ethic committee, authorization number 379, protocol DE15B.61. The ethic committee approval was conferred for a pilot study only for a low number of mice, considering that this is a pivotal study with MTX loaded AuNPs, used in mouse psoriatic model for topical application. Eight-week-old wild type mice (Charles River), all on C57BL/6 background were used for the in vivo experiments. Mice were maintained in standard animal cages under specific phatogen-free condition in the animal facility. The experiments were carried out in accordance with The EU Directive 2010/63/EU. The mice received a dose of 50 ± 3 mg of 5% imiquimod (IMQ) cream (Aldara; MEDA AS) applied on their shaved backs daily for 6 days. On day 7, mice were randomly distributed into 4 groups (four mice per groups): 1) AuNPs-3MPS@MTX cream topically treated daily for 24 (day 8) at the dosage of 200 µg of bioconjugate (i.e. 40 µg of MTX) for a total amount of 2 mL; 2) AuNPs-3MPS@MTX cream topically treated daily for 48 hour (day 9) at the dosage of 200 µg for a total amount of 2 mL; 3) AuNPs-3MPS cream topically treated daily for 24 (day 8) at the dosage of 200 µg total amount of 2 mL of cream preparation; 4) AuNPs-3MPS cream topically treated daily for 48 hours (day 9) at the dosage of 200 µg, total amount of 2 mL of cream preparation. The animals were subsequently euthanized and skin

biopsy was immediately taken from treated areas fixed in formalin and paraffin embedded for histological and immunohistochemical analysis.

Mice blood samples

Retro-orbital blood sampling was performed on all mice on day 2 after IMQ treatment, and on day 8 or 9, in order to perform complete blood count and dosage of Aspartate aminotransferase (AST) and alanine aminotransferase (ALT).

Histological analyses

The paraffin embedded punch biopsies were sectioned and haematoxylin and eosin (HE) stained for histological evaluation. Epidermal thickness was measured as an average of 15 random measurements of the distance from the stratum corneum to the deepest part of the epidermis, employing LEICA IM50 software, version 4.0.

Immunohistochemistry

For the immunohistochemical staining, a polyclonal antibody directed against the Ki-67 was used in order to investigate epidermal proliferation (Abcam,Cambridge,UK) and monoclonal antibody against cytokeratin 6 (Genetex , International corporation, Irvine, CA, USA). Staining of the T – cell subsets was performed with CD3 polyclonal antibody (Abcam,Cambridge,UK) and CD8 monoclonal antibody (Epitomics, Burlingame, CA U.S.A.). For Ki67 staining, the cell count was performed from the basement membrane up to the stratum corneum and this was expressed in positive cells per millimeter skin length. In order to analyse Ki67 positive cells, a line, with known length 1 mm, following the basal layer was drawn after choosing a representative 'region of interest' (ROI).[38,39] All positive cells above this line were counted. CD3 positive and CD8 positive cells were counted across the whole section. Results were given in percent, the percentage of

decrease was calculated comparing AuNPs-3MPS- and AuNPs-3MPS@MTX cream treated mice for 24h, and AuNPs-3MPS- and AuNPs-3MPS@MTX cream treated mice for 48h.

Statistical analysis

The data were summarized with the mean as a measure of central tendency and standard deviation as a measure of dispersion. The difference between two means was tested by unpaired Student's t-test. Multiple groups was evaluated via one-way ANOVA. A p value of 0.05 or less was chosen as the limit of significance.

Results

The hydrophilic AuNPs-3MPS nanoparticles were prepared by the well assessed wet reduction method and loaded with MTX drug, as schematically described in figure 1, together with a scanning electron microscopy image representing the well dispersed and uniformly sized AuNPs-3MPS of about 5 nm. In order to apply AuNPs-3MPS@MTX on mice skin, we first excluded the toxicity in vitro on HSE. MTT test on HSEs was performed using 100 µg AuNPs-3MPS@MTX bioconjugate or AuNPs-3MPS alone, topically applied to the epidermal model for 15 and 30 minutes, the same concentration employed for *in vivo* treatments. Results showed that AuNPs-3MPS@MTX are not toxic in vitro skin model (Figure 2).

Erythema, scaling and epidermal thickness

Imiquimod-induced psoriasis like mice was topically treated for 24 or 48 hour with AuNPs-3MPS or AuNPS-3MPS@MTX cream. All mice were assessed for the severity of skin inflammation on days 7 after IMQ treatment and subsequently on day 8 for the groups treated for 24 hours, and on day 9 for the groups treated for 48 hours, using 2 elements of the Psoriasis Area Severity Index, assigning a score of 0–4 (0, none; 1, mild; 2, moderate; 3, severe; 4, very severe) for each of the

parameters erythema and scaling[40] After IMQ treatment on day 7 (baseline), mice showed a mean score of 2.75 on erythema, and 3 on scaling. During treatment, control group (AuNPs-3MPS cream treated mice) showed no difference in the erythema, and mild improvement in the scaling after 24 and 48 hours of treatment (not statistically significant). AuNPs-3MPS@MTX cream treated mice showed a decreasing of scaling score from an average of 3 at the baseline, after IMQ treatment, to 1 after 24 and 48 hours of treatment (Fig.3A and 3B). Similarly, erythema score modified from an average of 2.75 at the baseline to 1 after 24 and 48 hours of treatment, respectively (Fig 3A, C)**.** Histological evaluation of mice skin on day 8 and 9 showed increased thickness of the epidermis (acanthosis), and of the keratin layer (hyperkeratosis) and the presence of parakeratosis in the AuNPs-3MPS-cream control group. By contrast, we observed a reduction of epidermal thickness, parakeratosis, and hyperkeratosis in AuNPs-3MPS@MTX cream treated mice; Epidermal thickness decreased from an average of 81 to 44 micrometers, in AuNPs-3MPS and AuNPs-3MPS@MTX 24 hours treated mice, and from 82 to 41 micrometers, in AuNPs-3MPS and AuNPs-3MPS@MTX 48 hours treated mice, respectively. (Fig. 3D). Blood evaluation showed no differences in blood count, ALT and AST level before and after AuNPs-3MPS or AuNPs-3MPS@MTX treatment. Moreover, no differences were observed between the group treated with AuNPs-3MPS or AuNPs-3MPS@MTX (data not shown).

Proliferation rate

In accordance with epidermal thickness clinically observed, nuclear immunoreactivity for Ki67 showed a strong Ki67 staining at the basal and suprabasal layers of the epidermis in AuNPs-3MPS cream treated mice. On the contrary, Ki67 positive cells were reduced in the number and staining intensity and were confined to the basal layer in AuNPs-3MPS@MTX cream mice especially after 48 hours (Fig 4 A, B, C, and D). The percentage of decrease in Ki67 positive cell number was 31% and 47% at 24h and 48h of AuNPs-3MPS@MTX treatment, respectively (Fig 4 E).

Expression of K6

To better characterize the effect of AuNPs-3MPS@MTX bioconjugate on epidermal hyperplasia keratin 6 (K6), a well-known hyperproliferation associated marker in activated keratinocytes was analyzed. Strong and homogeneous cytokeratin immunoreactivity was detected in the whole epidermis at all layers in AuNPs-3MPS treated mice, while, AuNPs-3MPS@MTX treated mice demonstrated a slight decrease of K6 staining intensity (Figure 5 A and B).

Inflammatory infiltrate

We observed a decreasing of CD3 positive cells in AuNPs-3MPS@MTX treated mice (Fig 6 A, B, C, and D) in the dermis after 24 hours and markedly after 48 hours ($p = 0.01$, $p = 0.05$, respectively) when compared to AuNPs-3MPS treated mice. The percentage of decrease in dermal CD3 positive cells was 47% and 44% after 24h and 48h of AuNPs-3MPS@MTX treatment, respectively (Fig 6E). Highly significant time-related effects were observed also at the epidermis level. The percentage of decrease in epidermal CD3 positive cell number was 32% and 60% after 24h and 48h of AuNPs-3MPS@MTX treatment, respectively (Fig. 6F). The reduction in cell counts was much more statistically significant in comparison to the dermis.

Moreover, we observed a decreasing of CD8 positive cells in AuNPs-3MPS@MTX treated mice both in the dermis and in the epidermis (Fig 7 A, B, C, and D). The percentage of decrease in dermal CD8 positive cell number was 20% and 59% after 24h and 48h of AuNPs-3MPS@MTX treatment, respectively (Fig. 7 E). The percentage of decrease in epidermal CD8 positive cell number was 29% and 55% after 24h and 48h of AuNPs-3MPS@MTX treatment, respectively (Fig. 7 F).

Discussion

AuNPs have been proposed for a number of applications in many different fields and are particularly attractive since their valuable size and shape-dependent properties are easy to control and modify. In our previous work, we demonstrated that AuNPs-3MPS@MTX was not toxic for keratinocytes in monolayer cultures. Moreover, TEM images allowed us to see clearly the internalization of gold nanoparticles inside the keratinocytes.[32] These findings and the fact that MTX, even if serving as standard systemically therapy in psoriasis,[19] is not yet available for topical formulation due to its solubility, insufficient percutaneous penetration,[41,42] led us to test whether or not topical AuNPs-3MPS@MTX formulation in vivo would have an effect on psoriasis-like skin inflammation in a mouse model, by using the IMQ model described by van der Fits et al. Imiquimod treatment induced a psoriasis-like skin inflammation and keratinocyte hyperproliferation leading to epidermal thickness.[33] In order to apply AuNPs-3MPS@MTX on mice skin, we first excluded the toxicity in vitro on the skin equivalent at the same concentration employed for *in vivo* treatment. In a previous paper we have confirmed the contribution of the AuNPs-MPS to the translocation of MTX into the skin performing in vivo mice skin permeation experiments using AuNPs-3MPS@MTX conjugate mixed in locobase and locobase cream containing MTX alone. UV–vis spectrophotometer analysis of epidermis and dermis showed that MTX drug can be observed both in the epidermis and also in the dermis in AuNPs-3MPS@MTX conjugate treated mice skin. On the contrary, samples treated with the formulation based on MTX alone, showed a MTX peak in the epidermis while in dermis it was absent, suggesting deeper penetration of the drug into the skin using the gold nanoparticles as drug carrier. [32]

IMQ-induced psoriasis like mice skin was topically treated daily for 24 or 48 hours with AuNPs-3MPS or AuNPs-3MPS@MTX cream. All mice were assessed for the severity of skin inflammation using 2 elements of the Psoriasis Area Severity Index, erythema and scaling.[40]

Interestingly, AuNPs-3MPS@MTX could decrease the severity of erythema and scaling of skin lesions compared to AuNPs-3MPS treated mice. Control group (AuNPs-3MPS treated mice) showed no differences in the erythema, and mild improvement in the scaling compared to baseline,

probably due to hydration effect of Locobase cream. On the contrary, AuNPs-3MPS@MTX treated mice showed a decrease especially in scaling as well as erythema score compared to baseline and to AuNPs-3MPS treated mice. These findings were confirmed by measurements of epidermal thickness showing about a 50% of epidermal thickness in the 24 hours and 48 hours AuNPs-3MPS@MTX treated mice compared to control groups. If the natural recovery from imiquimod challenge occurs we should have observed in the mice group treated with AuNPs-3MPS a reduction of the psoriatic phenotype between 24 and 48 hs. In our experiments this effect was not observed and the psoriatic phenotype was maintained.

Finally, the histopathological evaluation of psoriasis-like mouse skin verified the finding that in the AuNPs-3MPS@MTX group we observed a reduction of epidermal parakeratosis and hyperkeratosis compared to AuNPs-3MPS group. To better characterize the effect of AuNPs-3MPS@MTX on epidermal hyperplasia K6 and Ki67, well-known hyperproliferation markers associated in activated keratinocytes were analyzed. In accordance with the previous data, we observed that AuNPs-3MPS@MTX treated mice demonstrated a slight decrease of K6 staining intensity in the epidermis compared to AuNPs-3MPS group. The number of Ki67 positive nuclei increased with positive cells also in the suprabasal layer, which is a common feature in the psoriatic epidermis, in AuNPs-3MPS group. On the contrary, in AuNPs-3MPS@MTX groups, we found that Ki67 positive cells were reduced in the number and staining intensity and confined to the basal layer after 24 hours of treatment, and this seems even more evident after 48 hours of treatment. Cycling epidermal cells seem to be the primary target for topical AuNPs-3MPS@MTX cream in psoriatic-like mouse skin, in comparison to the pretreatment lesions. Ki-67 reduction also has been previously reported in oral MTX treatment.[43] Swinkels et al. analyzed the effect of a single dithranol application (2% cream) on the proliferation, keratinization, and inflammation of the psoriatic lesions. They confirmed the mechanism of reduction of Ki-67 index by the inhibition of keratinocyte proliferation. Four days only after single application, the index was reduced to 54% and to 66% after 12 days.[44] Similar findings were observed when topical 0.05% clobetasol propionate was used for only three days in

patients with moderate psoriasis with the number of Ki-67 positive cells dropped to 47%.[45] Moreover, we analyzed the effects of AuNPs-3MPS@MTX treatment on the inflammatory infiltrate. We observed lower numbers of CD8 and CD3 positive inflammatory cells both in the dermis and epidermis of AuNPs-3MPS@MTX treated mice. The percentage of decrease in dermal CD3 positive cell number was 47% and 44% after 24h and 48h of AuNPs-3MPS@MTX treatment, respectively compared to AuNPs-3MPS group. Interestingly, the percentage of decrease in epidermal CD8 positive cell number was 29% and 55% after 24h and 48h of AuNPs-3MPS@MTX treatment, respectively compared to AuNPs-3MPS group.

Finally, to test the safety of the topical treatment we performed blood evaluation that showed no differences in blood count and ALT and AST level before and after AuNPs-3MPS or AuNPs-3MPS@MTX treatment. Furthermore, no differences were observed between groups treated with AuNPs-3MPS or AuNPs-3MPS@MTX. These results as a whole provide evidence that topical AuNPs-3MPS@MTX treatment was able to induce a reduction of keratinocytes hyperproliferation, epidermal thickness and also inflammatory infiltrate in Imiquimod-induced psoriasis-like mice model. It is noteworthy that the mice number waas limited and also the treatment period was very short, only 48 hours once per day. ~~and~~ In light of this, it might conceivable to suppose that a more prolonged treatment or a raised starting dose could be more effective. Moreover, it is to be highlighted that short AuNPs-3MPS@MTX treatment exerts its pharmacological action at both epidermal and dermal compartments. Our data suggest that topical AuNPs-3MPS@MTX treatment may be therapeutically effective in treating psoriatic plaques, and it may represent an alternative topical treatment for mild psoriasis, which is the most frequent form of the disease, that required lifelong, often rotational or combination therapy. Moreover, MTX topical formulation may avoid adverse events associated with MTX systemic administration and may work synergistically with other psoriasis topical treatments. After a systematic literature review, this is the first study assessing those parameters in psoriatic model under the cutaneous effect of bioconjugate of both MTX and gold nanoparticles. In addition, the data may contribute to clarify the possibility of

enhancing the topical delivery of methotrexate by the aid of gold nanoparticle delivery. This study demonstrated a preliminary proof of percutaneous gold nanoparticle enhanced drug delivery.

Up to day topical psoriasis treatments include: corticosteroids, vitamin D analogues, topical retinoids, calcineurin inhibitors, salicylic acid, coal tar, which act mostly reducing inflammation and/or on cellular proliferation. Topical MTX formulations have not showed complete satisfactory efficacy mostly due to insufficient percutaneous penetration. This new MTX topical formulation exhibited biological effects on the epidermis and also in the dermis in psoriatic mouse model. On this basis, it may represent a useful therapeutical option, especially in patients with mild psoriasis or in patients with contraindication to systemic therapy.

This work deals with the use of non-cytotoxic MNPs and for the first time the AuNPs used in this work have been used on psoriatic murine models as a therapeutic agent. MTX topical drugs for psoriasis are at present not commercially available and in this work, for the first time, AuNPs are associated to a nanovehicle and topically administered. The clinical evidences on epidermal thickness, cell proliferation rate, and inflammation have been studied in topical application. In vivo and histologyical results demonstrated reproducibility and efficacy**.**

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Text for the graphical abstract

Topical treatment with functionalized gold nanoparticles loaded with methotrexate was used *in vivo* on imiquimod-induced psoriasis like mice model. The AuNPs-3MPS@MTX system allowed to reduce keratinocytes hyperproliferation, epidermal thickness and inflammatory infiltrate.

Figure legends

Figure 1. MTX drug immobilization onto AuNPs-3MPS: (A) Schematic description of the MTX drug immobilization onto AuNPs-3MPS, B) FESEM images of AuNPs-3MPS and AuNPs-3MPS@MTX bioconjugate.

Figure 2. Skin Irritation. Skin Irritation test on HSEs after 15 and 30 minutes treatment with AuNPs-3MPS@MTX bioconjugate at 100 µg concentration.

Figure 3. Scaling, Erythema and Epidermal thickness. (A) Representative photograph of phenotypical presentation of mouse skin after IMQ treatment, (days 7) and after AuNPs-3MPS or AuNPs-3MPS@MTX 48 hours of treatment (B) Scaling was scored after IMQ treatment (baseline) and after AuNPs-3MPS or AuNPs-3MPS@MTX 24 and 48 hours of treatment. Bars represent scores. (C) Erythema was scored after IMQ treatment (baseline) and after AuNPs-3MPS or AuNPs-3MPS@MTX 24 and 48 hours of treatment. Bars represent scores (0-4). (D**)** Epidermal hyperplasia was scored after IMQ treatment (baseline) and after AuNPs-3MPS or AuNPs-3MPS@MTX 24 and 48 hours of treatment. Bars represent epidermal thickness in µm. On the right panels, representative H&E-stained skin sections on after AuNPs-3MPS or AuNPs-3MPS@MTX 48 hours of treatment. Data indicate Mean ±SD of all experiments.***p = 0.001,**p = 0.01, *p = 0.05. Scale bar corresponds to 20 µm.

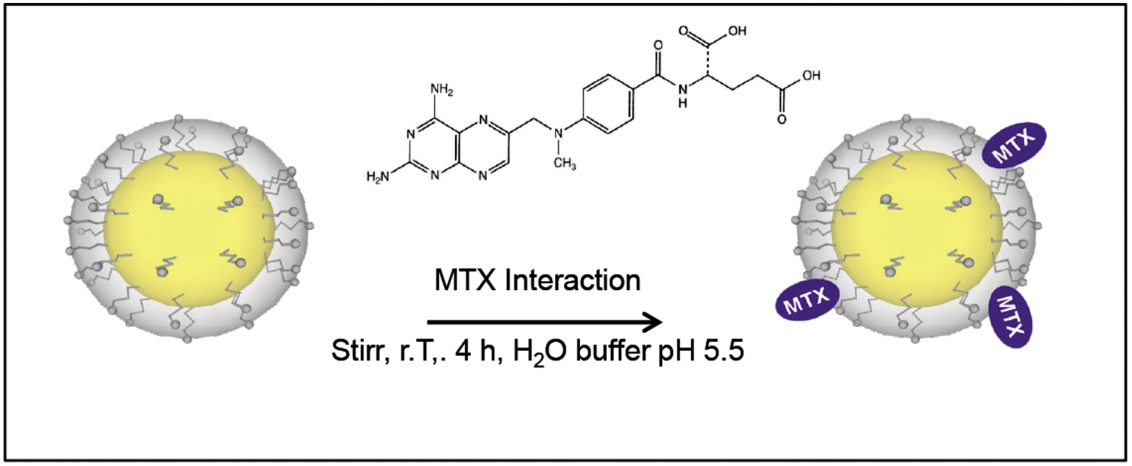
Figure 4. Proliferation rate. (A) Ki67 Immunohistochemical staining in AuNPs-3MPS treated mice after 24 or (B) 48 hours. (C) AuNPs-3MPS@MTX treated mice after 24 or (D) 48 hours. (E) Quantitative Ki67 expression. The cell counts were expressed in positive cells per millimeter skin length. Data indicate Mean ± SD of all experiments (n = 10) . ***p = 0.001,**p = 0.01, *p = 0.05. Scale bar corresponds to 20 μm.

Figure 5. Expression of K6. (A) K6 Immunohistochemical staining of AuNPs-3MPS and (B) AuNPs-3MPS@MTX treated mice after 48 hour of treatment. Scale bar corresponds to 20 μm.

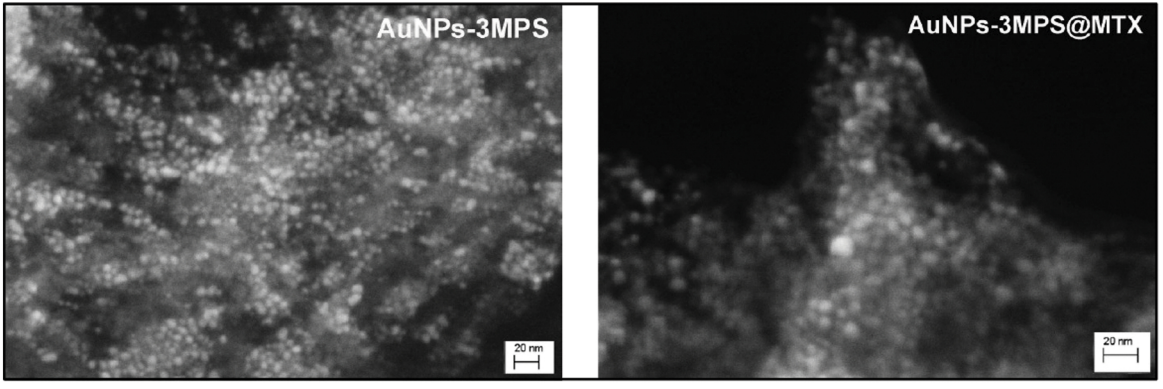
Figure 6. CD3 inflammatory infiltrate. (A) CD3 immunoistochemistry in in AuNPs-3MPS treated mice after 24 or (B) 48 hours. (C) AuNPs-3MPS@MTX treated mice after 24 or (D) 48 hours. (E) Quantitative CD3 expression in the dermis and (F) in the epidermis. The cell counts were expressed in positive cells per 1 millimiter square. Data indicate mean ± SD of all experiments (n = 10). ***p = 0.001,**p = 0.01, *p = 0.05. Scale bar corresponds to 20 μm.

Figure 7. CD8 inflammatory infiltrate. (A) CD8 immunoistochemistry in in AuNPs-3MPS treated mice after 24 or (B) 48 hours. (C) AuNPs-3MPS@MTX treated mice after 24 or (D) 48 hours. (E) Quantitative CD8 expression in the dermis and (F) epidermis. The cell counts were expressed in positive cells per 1 millimiter square. Data indicate mean ± SD of all experiments (n = 10).)
***p = 0.001,**p = 0.01, *p = 0.05. Scale bar corresponds to 20 μm.

Topical treatment with functionalized gold nanoparticles loaded with methotrexate was used *in vivo* on imiquimod-induced psoriasis like mice model. The AuNPs-3MPS@MTX system allowed to reduce keratinocytes hyperproliferation, epidermal thickness and inflammatory infiltrate.


MTX Interaction
Stirr, r.T,. 4 h, H_2O buffer pH 5.5
MTX
MTX
MTX

A


AuNPs-3MPS
20 nm
AuNPs-3MPS@MTX
20 nm

B

Figure 1

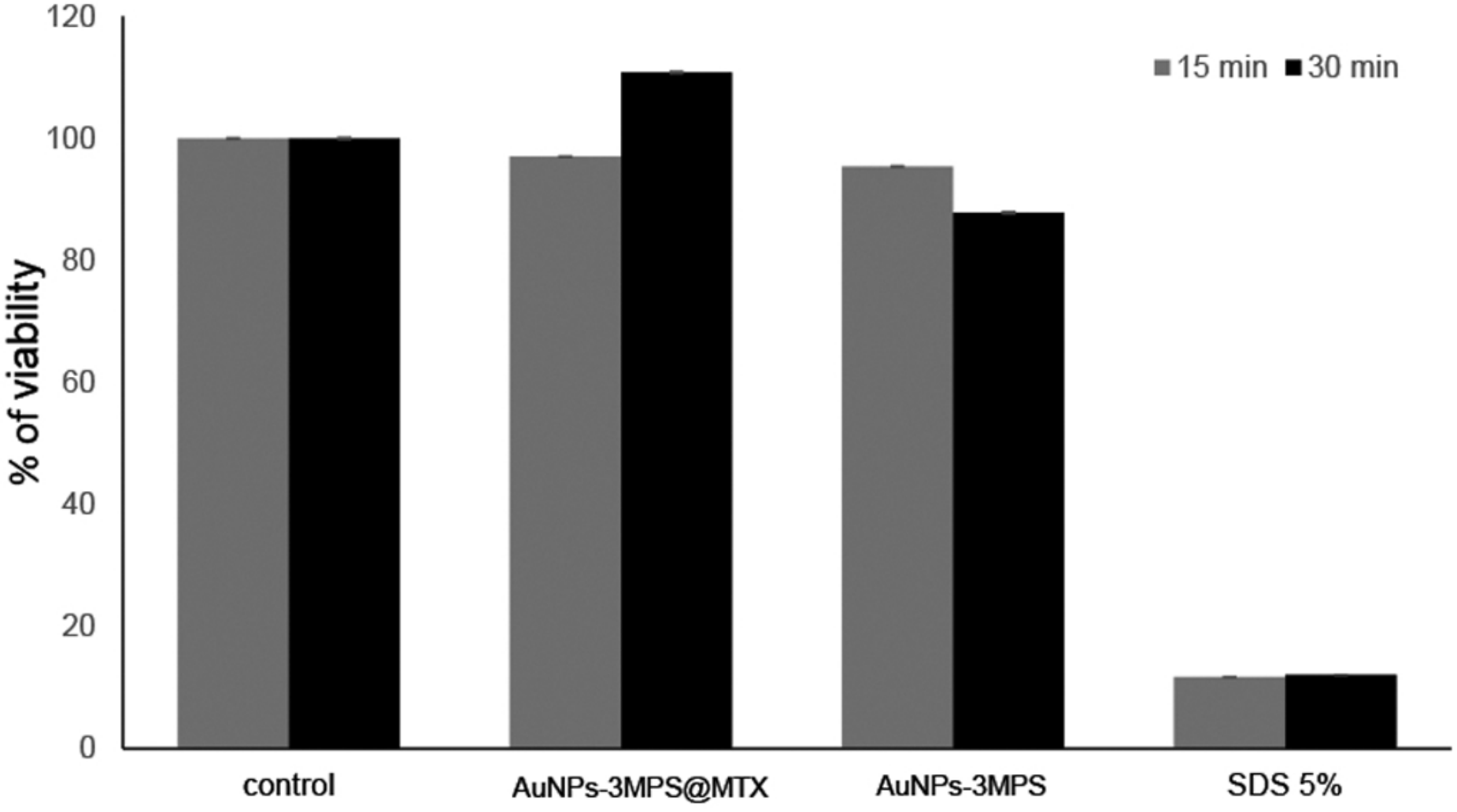
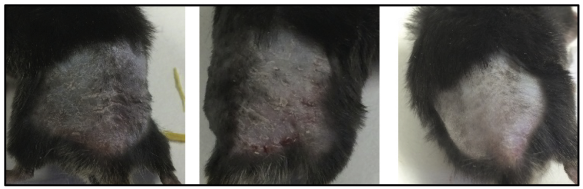

120
100
80
60
40
20
0
% of viability
15 min
30 min
control
AuNPs-3MPS@MTX
AuNPs-3MPS
SDS 5%

Figure 2



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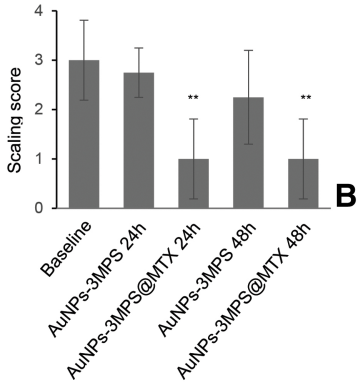

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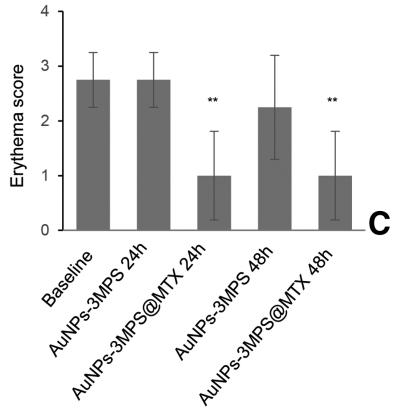

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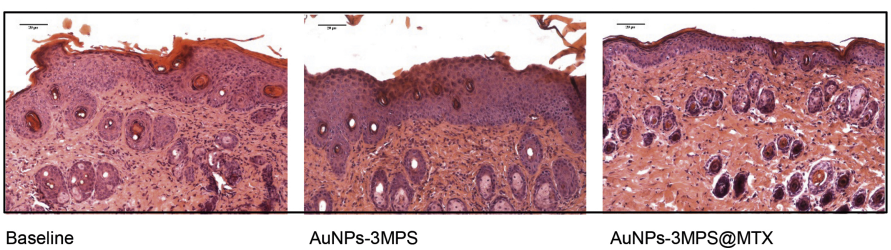

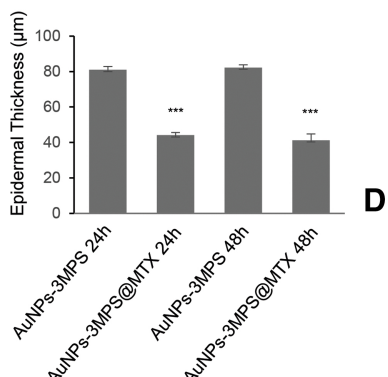

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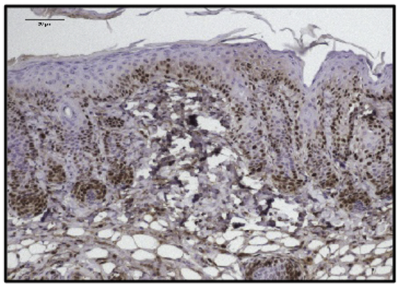

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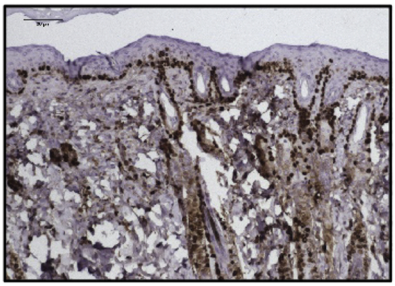

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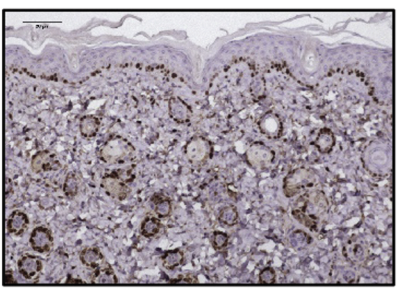

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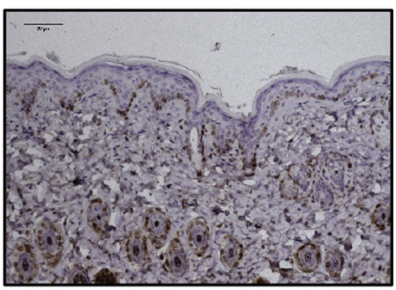

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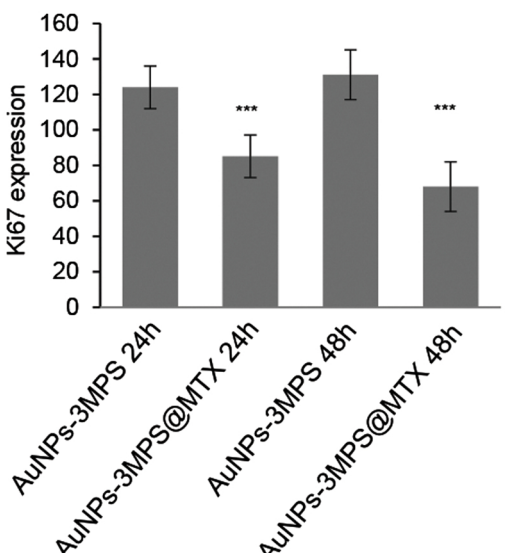

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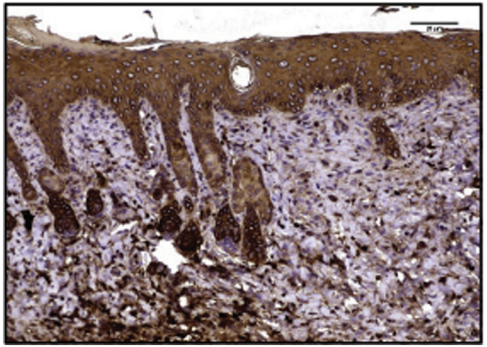
Figure 4



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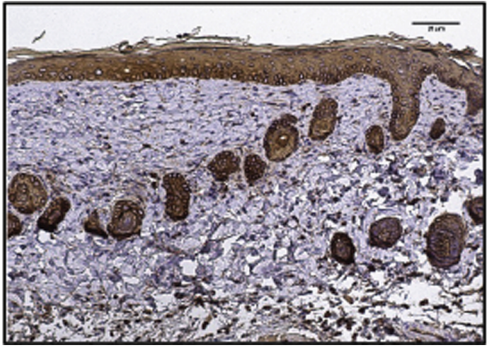

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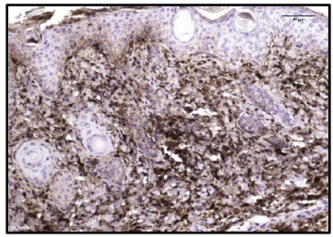

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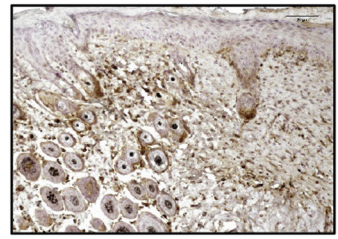

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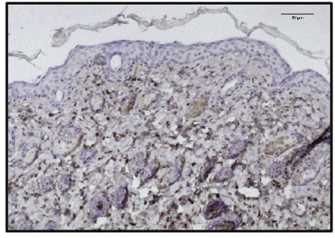

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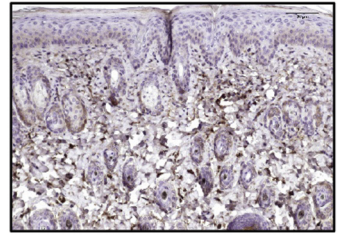

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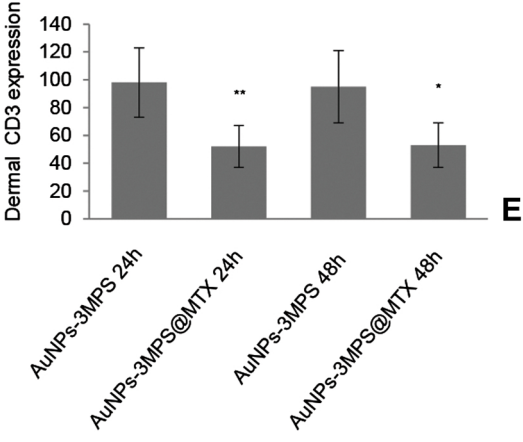

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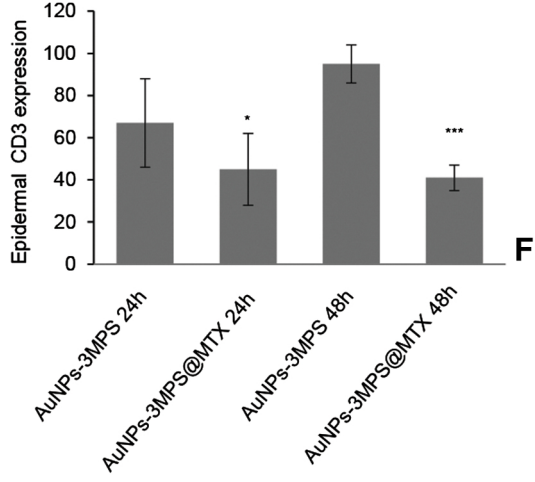

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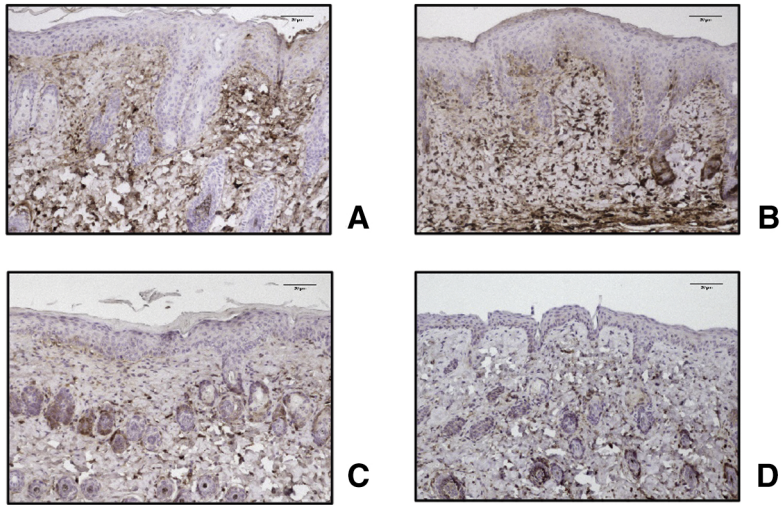
Figure 6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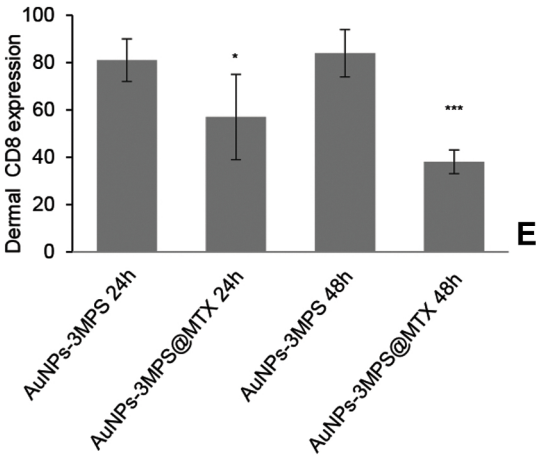
Dermal CD8 expression
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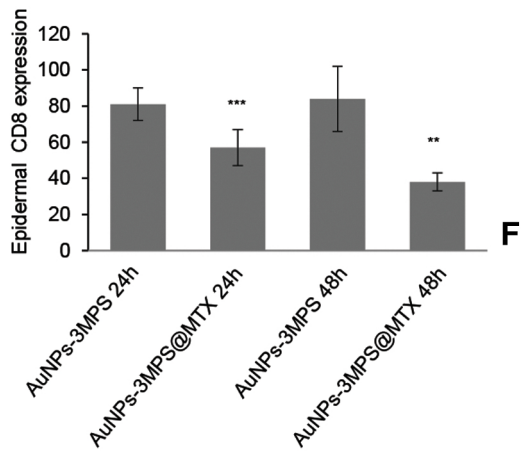
AuNPs-3MPS 48h
AuNPs-3MPS@MTX 48h


Epidermal CD8 expression
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AuNPs-3MPS 24h
AuNPs-3MPS@MTX 24h
AuNPs-3MPS 48h
AuNPs-3MPS@MTX 48h

Figure 7