Journal of Inflammation Research

Dovepress
Taylor & Francis Group

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ORIGINAL RESEARCH

CpG ODN Combined with Gold Nanorods Enhances Immune Activation and Its Potential Mechanism

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Background: Immune escape of tumor cells is a common problem with tumor photothermal therapy utilizing gold nanorods (Au NRs). Whether CpG ODN, an immune adjuvant, can synergize with Au NRs to activate the immune response and its potential mechanism is not clear.

Methods: The effect of Au NRs combined with CpG ODN (Au NRs-C) on the activity of various immune-related cells, such as double-positive T cells, macrophages, NK cells, Th17, and Treg. The expression levels of various immune and inflammation-related factors, such as IL-1R1, IL-6, IL-17, and TNF-α were characterized. Transcriptome sequencing analysis was used to explore the potential immunomodulatory mechanisms of Au NRs-C. Whether immune activation was enhanced by antibody-functionalized Au NR upon binding to CpG ODN was assessed.

Results: Flow cytometry and ELISA analyses indicate that both Au NRs and CpG ODN increase pro-inflammatory cytokine levels and immune activation. However, Au NRs-C demonstrated superior immune activation potential. Furthermore, Au NRs stimulate the expression of Treg, while Au NRs-C significantly inhibit this effect. This suggests that the conjugation of CpG ODN with Au NRs not only greatly enhances the immune activation but also compensates for some of their deficiencies in eliciting immune responses. Transcriptome sequencing uncovered DEGs mainly localized to immune and pro-inflammatory cytokine pathways. PPI analysis identified six hub genes: *FOXM1, HMOX1, UBE2C, E2F1, PECAM1*, and *FCGR3A*. Moreover, CpG conjugation with antibody functionalization- Au NRs enhances immune stimulation.

Conclusion: Au NRs-C promotes immune activation by eliciting changes in the activity of immune-associated cells and expression of inflammatory factors through multiple pathways, such as MHC antigen presentation and Toll-like receptor-mediated immune processes.

Keywords: gold nanorods, functionalized modification, immunity, immunotherapy, transcriptome sequencing

Introduction

Nanorods (NRs) are one-dimensional, rod-shaped nanoparticles with diverse geometries. As synthesis methods continue to improve, researchers have selected various materials to coat around gold nanorods (Au NRs) to enhance their plasmonic properties. Au NRs are particularly favored in theranostics due to their small size, ability to be functionalized, and adjustable plasmon frequency. Applications of Au NRs include thermotherapy for cancer treatment, photothermal conversion, and infrared neurostimulation.[1] They also facilitate Raman imaging,[2] light-assisted tissue regeneration, drug delivery, and gene therapy.[3] Functional modifications are crucial in Au NR applications, enabling targeted therapies. Typically, cancer biomarkers are conjugated with Au NRs to create specific multifunctional nanoprobes, effectively targeting and eliminating tumor cells via photothermal therapy.[4] Our previous study demonstrated that EGFR antibody-

Received: 14 March 2025
Accepted: 9 August 2025
Published: 3 September 2025

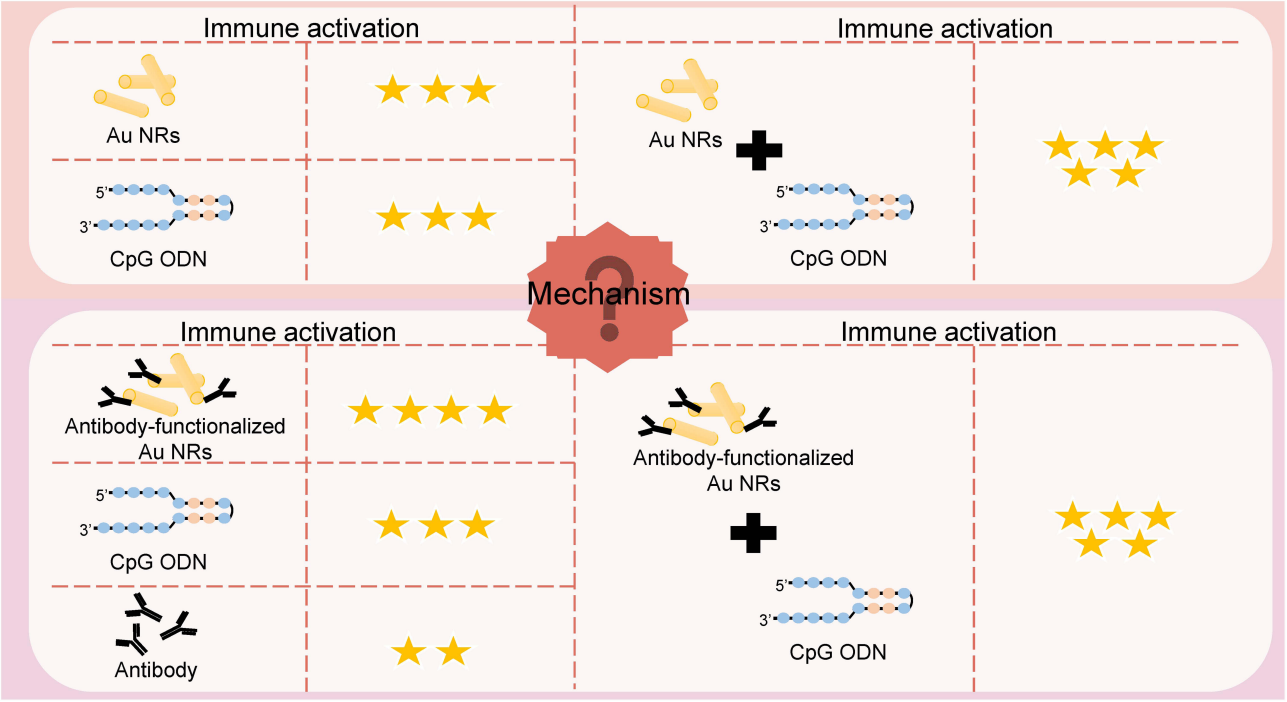

Figure 1 Hypothesis diagram of this study. The pentagram represents the intensity of immune activation.

modified Au NRs can enhance squamous cell carcinoma treatment by promoting apoptosis,[5] although the involvement of the immune response remains uncertain.

The immune system consists of innate and adaptive immunity. Immune defense against infection relies on the collaboration of T and B lymphocytes, natural killer cells, granulocytes, and various immune factors.[6,7] Research highlights that cytokines IL-1,[8] IL-6,[9] IL-17,[10,11] and TNF-α[12] significantly influence immune cell recruitment, activation, proliferation, and the inhibition of excessive activation. Activating the immune system for therapeutic benefit in cancer has long been a goal in immunology and oncology.[13] The utilization of Au NRs in immunotherapy has consistently drawn the interest of researchers. Studies indicated that synergistic immunotherapy using Au NRs effectively inhibits tumor recurrence post-surgery[14] while enhancing populations of CD3+/CD4+ and CD3+/CD8+ T lymphocytes.[15,16] Functionally modified Au NRs represent a promising approach for cancer treatment as they can selectively deliver therapeutics to cancer cells or induce direct tumor cell death.[17] Moreover, CpG oligodeoxynucleotide (CpG ODN,) serves as an established immune adjuvant, stimulating TLR9-expressing cells and activating an immunoregulatory cascade.[18,19] In addition, it has been found that the dual Toll-like receptor agonist delivery system CPG@Au NRs/m-R848 enhances photothermal immunotherapy for melanoma.[20] However, the potential of only combining CpG ODNs with Au NRs (Au NRs-C) to enhance the immunotherapeutic effects of these nanorods, along with the underlying mechanisms, remains unexplored, which has attracted our attention.

The purpose of this study was to investigate whether combining Au NRs with the recognized immune adjuvant CpG ODN could synergistically promote immune responses and investigate its potential mechanism via transcriptomics. Our hypothesis for this work is shown in Figure 1. This study will provide a theoretical basis for the immunotherapeutic study of CpG ODN combined with Au NRs, which is of great significance for their clinical development and application.

Methods

Treatment of Au NRs

Experimental methods were referred to in our previous studies.[21] PEG Au NRs (4 mL of 0.1 mg/mL) (JK-02-006-810, Nanjing Kike Biotechnology Co., Ltd., China) were centrifugally dispersed in 10 mM MES buffer. Following this, 50 μL

of EDC (Aladdin, USA) was added and incubated at 37 °C for 30 min. Post incubation, the solution was washed by centrifugation and re-dispersed in MES, divided into two equal 2 mL tubes. EGFR (2A2H10, Thermo Fisher, USA) and VEGFR2 (1G2A8, Thermo Fisher, USA) were added separately and incubated overnight in darkness on a shaking platform (SHZ-82, Changzhou Guohua Electric Co., China). Subsequent centrifugation and redispersion in deionized water produced antibody-functionalized Au NRs. The synthesized Au NRs were examined using a transmission electron microscope (H7800, Hitachi High-Tech, Japan) to obtain average measurements of both long and transverse diameters. Zeta potential analysis confirmed the successful synthesis. CpG OND binding to Au NRs was performed according to the previous method.[22] Briefly, the concentration of Au NRs was first adjusted to 10 nm/mL, and the mixture was shaken with CpG ODN at room temperature overnight. The mixture was again incubated overnight with shaking after adjustment using 1 M PBS (pH=7.4). PBS was used to rinse the mixture several times to remove unbound DNA. The CpG ODN used in this study was ODN 2336 sodium, which belongs to class A CpG ODN.

Isolation and Culture of Peripheral Blood Mononuclear Cells

All procedures involving human subjects were approved by the Yunnan Cancer Hospital Ethics Committee (acceptance No. SLKYCS2024-046) and comply with the Declaration of Helsinki. Written informed consent was obtained from all subjects. Peripheral blood mononuclear cells (PBMCs) were extracted from 100 mL of anticoagulated blood from a mixture of all subjects through Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ) density gradient centrifugation under sterile conditions.[23] It was emphasized that samples were collected from the same batch, and blood was collected from populations with similar clinical characteristics to minimize bias. Subsequently, PBMCs were seeded into 6-well plates at a density of 2×10^5 cells per well. PBMCs were treated with PBS, antibody only (VEGFR mAb or EGFR mAb), Au NRs only, Au NRs functionalized with antibody or CpG ODN, and Au NRs functionalized with both CpG ODN and VEGFR/EGFR mAb. CpG ODN is mixed with Au NRs, while the antibody is coupled to the Au NRs. For the above materials, all were 6 μg/mL except CpG ODN, which was 0.1 mg/mL. The cells were incubated for 48 hours. Resulting in ten distinct groups named PBS, VEGFR mAb, EGFR mAb, CpG ODN, Au NRs, Au NRs-V, Au NRs-E, Au NRs-C, Au NRs-CV, and Au NRs-CE. We confirm that all methods in this experiment were performed following the relevant guidelines and regulations.

Flow Cytometry Analysis

Experimental methods were referred to in previous studies.[23,24] Before fluorescence staining of PBMCs from each experimental group, FACS lysate (349202, BD Biosciences, USA) was used to treat PBMCs after gradient centrifugation to remove erythrocyte interference. Centrifugation and washing of the cells resulted in PBMCs that could be used for fluorescence staining. Then, PBMCs were stained according to the cell surface antigen differentiation groups to identify different immune cells. These antibodies were directed against CD3+, CD4+, CD8+, RORγt, Foxp3, CD25+, CD56+, CD68+, and NKP46. Specifically, PBMC single-cell suspensions with a cell density of 1×10^6 cells/100 μL were supplemented with specific fluorescent monoclonal antibodies labeled with FITC, PE, or APC. The cells were washed, centrifuged, and resuspended after 20 min of reaction at room temperature. Cells were fixed with glutaraldehyde (5%) and analyzed by flow cytometry (Novocyte advanced VBR, Agilent, USA). Linear amplification was performed with forward-scattered light (FSC)/side-scattered light (SSC), and logarithmic amplification was performed with different bands of the spectrum. FSC-A/SSC-A and FSC-A/FSC-H were used to establish the channel to exclude dead cells and adhesions. Negative controls were performed with 100 μL of anticoagulated blood plus 20 μL of fluorescent monoclonal antibody (FITC, PE, or APC, consistent with labeled antibody). The antibody information is as follows: CD3-PE (12-0038-42), CD3-APC (17-0038-42), CD4-FITC (11-0049-42), CD8-PE (12-0081-81), CD25-APC (17-0257-42), RORγt-PE (12-6988-80), CD56-FITC (11-0566-42), CD68-FITC (11-0689-42), NKP46-PE (12-3351-82), and Foxp3-PE (12-4777-42) were all from Invitrogen (USA). Meanwhile, the Foxp3/Transcription Factor Staining Buffer Kit (00-5523-00, eBioscience, USA) was used in this experiment. The excitation and emission wavelengths of the antibodies are as follows: PE (565/495 nm, 576 nm), APC (650 nm, 660 nm), and FITC (495 nm, 519 nm).

ELISA

Cytokine analyses for IL-1R1 (E-EL-H1028), IL-6 (E-EL-H6156), IL-17 (E-EL-H6181), and TNF-α (E-EL-H0109) were conducted per manufacturer's instructions (Elabscience, China). The optical density of each well was recorded utilizing an enzyme labeling instrument (ELx800, BIO-TEK, USA) at 450 nm within 15 minutes post-reaction.

Transcriptome Sequencing Analysis

RNA sequencing was performed on Au NRs, Au NRs-C, and PBS-interacted PBMC (named Au NRs, Au NRs-C, and PBS, respectively). Each of the three groups had four independent replicates. Sample handling was unified, stored, and dispensed to minimize bias. Total RNA from the samples was extracted using the TRIzol method and evaluated for quality, purity, and concentration. Library construction and sequencing were performed by OE Biotech (Shanghai, China). The results obtained were analyzed for differential expression using the DESeq2 package[25,26] in R. After obtaining the differentially expressed genes (DEGs), the DEGs were analyzed for Gene Ontology (GO)[27] and Kyoto Encyclopedia of Genes and Genomes (KEGG)[28] enrichment using the hypergeometric distribution algorithm. Moreover, protein interactions were analyzed according to the STRING database to obtain the interrelationships of the genes.

RT-qPCR Analysis

To verify the sequencing outcomes, RT-qPCR was performed on the hub genes. GAPDH was used as an internal reference gene. The relative expression of the genes was calculated using the $2^{-\Delta\Delta Ct}$ method. Total RNA was extracted with TRIzol reagent (Thermo Fisher, USA), precipitated with ethanol, and converted to cDNA using the FastKing cDNA kit (KR116, Tiangen, China) following the manufacturer's guidelines. We amplified cDNA with Taq Pro Universal SYBR qPCR Master Mix (Vazyme, China) to quantify the target gene through real-time RT-PCR. The primers utilized for RT-PCR are detailed in Table 1.

Statistical Analysis

Data were expressed as mean ± SD of at least three independent experiments. Statistical probability was assessed using One-Way ANOVA and Tukey's post hoc tests. SPSS 23.0 was used for statistical analysis, and Origin 2021 software was used for plotting. The asterisk labeling method was used in this study. The ends of the line segments are the groups being compared, and the asterisks at the top indicate their differences.* indicate $p < 0.05$, ** indicate $p < 0.01$, *** indicate $p < 0.001$. Moreover, for a clearer presentation, the letter labeling method was used to compare the results of experiments with a larger number of groups. Bars with different letters indicate $p < 0.05$.

Results

The Immune Activity of Au NRs was Dramatically Increased by Coupling CpG ODNs

Au NRs were identified, and the results showed that their length was about 50 nm. Their width varied from about 10.2 to 18.6 nm, and the surface potential of the Au NRs was 24.6 mV (Figure 2A and B). The cytokine ELISA shows that CpG ODN raises IL-1R1, IL-6, IL-17, and TNF-α, whereas AuNRs alone raise IL-1R1 and IL-17 (Figure 2C–F). These data support the claim that CpG boosts inflammation more than AuNRs alone, and Au NRs-C is strongest. The results of immune cells analysis reveal that CpG ODN significantly stimulates the proliferation of positive immune cells, such as CD4+CD8+ double-positive

Table 1 List of Primers Used in This Study

Gene	F (5′-3′)	R (5′-3′)
GAPDH	TTGCCCTCAACGACCACTTT	TGGTCCAGGGGTCTTACTCC
FOXM1	AACAATAGCCTATCCAACA	CTCCATCTCTTGCTTGAT
HMOX1	GACTGCGTTCCTGCTCAA	CTCTGGTCCTTGGTGTCAT
UBE2C	ACATTGATAGTCCCTTGAA	TGCTTTGAGTAGGTTTCT
E2F1	CAGAGCAGATGGTTATGG	CTGAAAGTTCTCCGAAGA
PECAM1	AGTCATTACGGTCACAAT	CTGAGGACACTTGAACTT
FCGR3A	AACATCACCATCACTCAAG	TACCATCACCAAGCAGAA

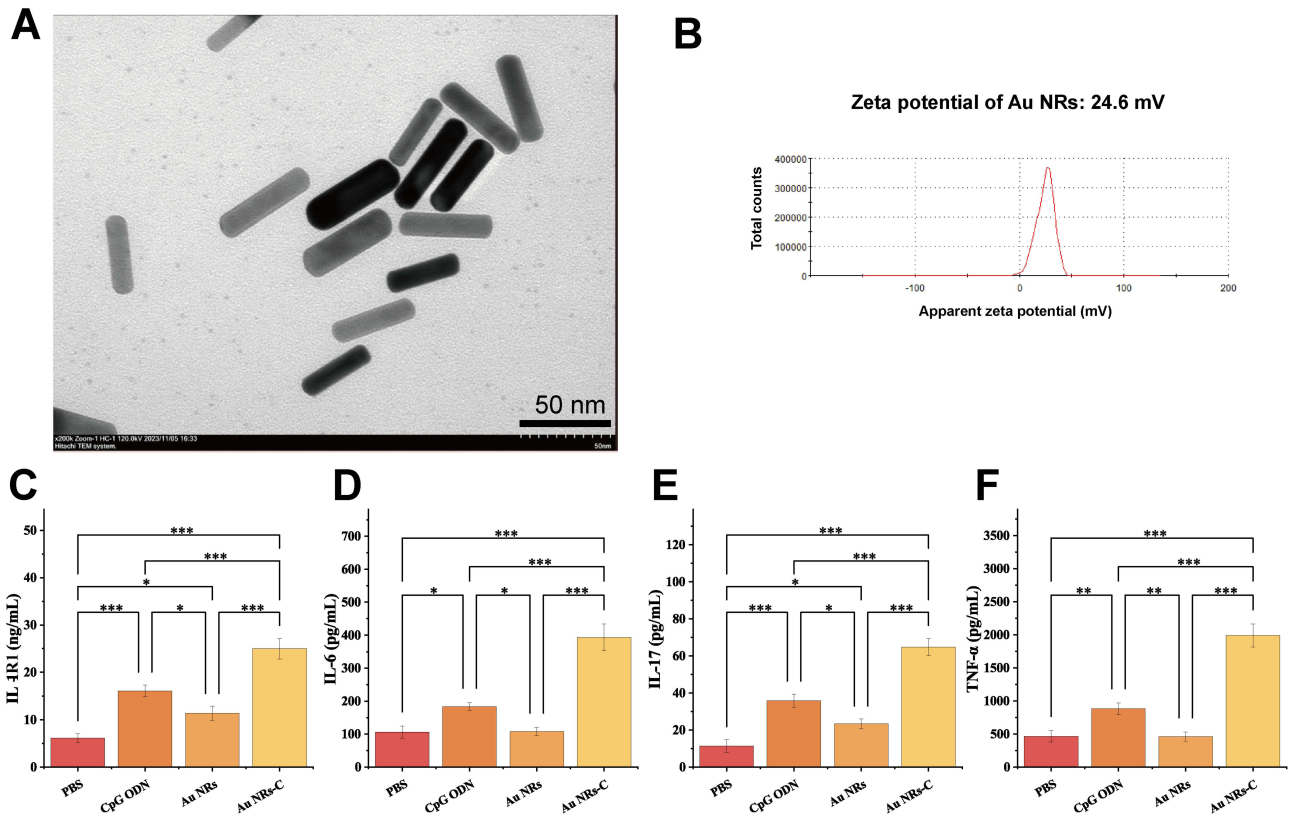

Figure 2 The preparation of gold nanorods. (**A**) Transmission electron microscopy analysis of gold nanorods. (**B**) Zeta potential analysis for Au NRs. (**C**–**F**) ELISA analysis to detect the expression of immune-promoting inflammatory factors, namely IL-1R1 (**C**), IL-6 (**D**), IL-17 (**E**), and TNF-α (**F**). Data were expressed as mean ± SD of three independent experiments. * indicate $p < 0.05$, ** indicate $p < 0.01$, *** indicate $p < 0.001$.

(DP) T cells, macrophages, NK cells, and Th17, while inhibiting the expression of the negative immune cell Treg (Figure 3A–E). Meanwhile, Au NRs did not have a significant effect on Th17 (Figure 3D), and significantly increased the expression of Treg (Figure 3E). Emphatically, the Au NRs-C further enhances all positives and suppresses the AuNR-induced Treg rise, yielding the lowest Treg levels (Figure 3A–E). These findings suggest that Au NRs-C not only greatly enhance immune activation capabilities but also compensate for some of the deficiencies of Au NRs in eliciting immune responses.

Analysis of Gene Expression Levels and Identification of DEGs

To further investigate the regulatory genes and pathways of immunity activation by Au NRs-C, high-throughput transcriptome sequencing was used for analysis. The datasets generated and/or analyzed during the current study are available in the NCBI repository, GSE285375. Sequencing of the ordered transcriptome of 12 samples yielded 80.82 G of CleanData, with Q30 bases distributed from 94.2 to 95.06% and an average GC content of 47.87%. The box plot (Figure 4A) illustrates a consistent distribution of FPKM values across the samples. These results affirm that the quality and accuracy of the data were adequate for further analysis. Principal Component Analysis (PCA) results (Figure 4B) reveal that the distances among the three experimental groups were relatively close, indicating improved parallelism between groups and enhancing the reliability of the obtained data, in line with the FPKM box plot results. Subsequently, pairwise comparisons of these three sample groups yielded DEGs, presented through heatmaps and volcano plots, adhering to $|\log_2(\text{Fold change})| \geq 1$ and q-value ≤ 0.05 criteria (Figure 4C–E). Specifically, the Au NRs_vs_PBS group revealed 2245 DEGs, with 864 up-regulated and 1381 down-regulated genes. In the Au NRs-C_vs_PBS group, 3270 DEGs were identified, with 1337 up-regulated and 1933 down-regulated genes. Lastly, the Au NRs-C_vs_Au NRs group found 1151 DEGs, consisting of 418 up-regulated and 733 down-regulated genes.

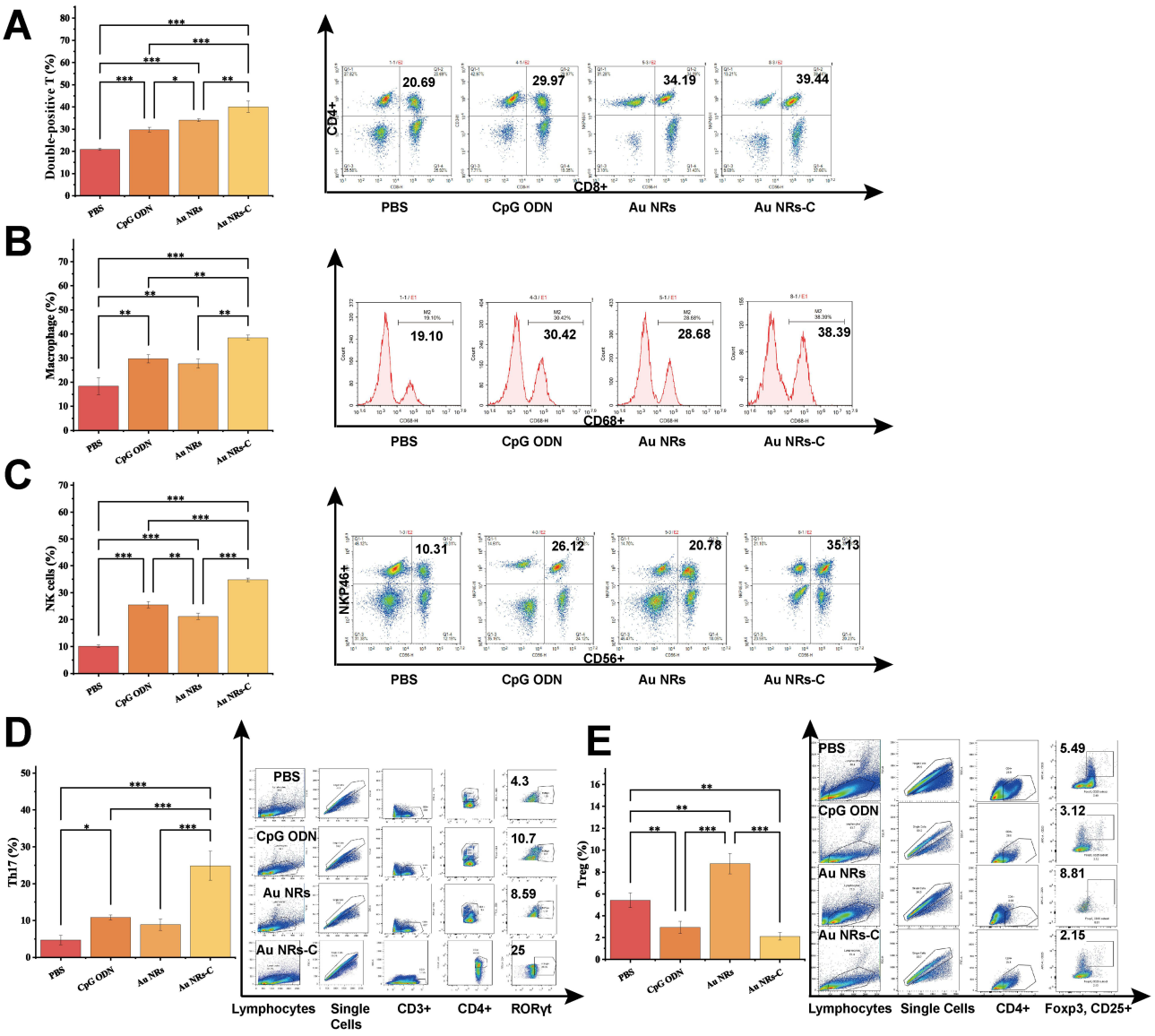

Figure 3 The immune activation assessment of Au NRs-C. (**A**–**E**) Flow cytometry detection of immune cell expression for double-positive T cells (**A**), macrophage (**B**), NK cells (**C**), Th17 (**D**), and Treg (**E**). Data were expressed as mean ± SD of three independent experiments. * indicate $p < 0.05$, ** indicate $p < 0.01$, *** indicate $p < 0.001$.

Differential Gene Enrichment Analysis

The GO and KEGG analyses of DEGs revealed a significant impact on immune system-related genes and pathways. The GO enrichment analysis (Figure 5A–C) categorized DEGs across three comparison groups into biological process (BP), cellular component (CC), and molecular function (MF). DEGs from each group showed varying degrees of enrichment in immune-associated GO terms. Notably, among the BP, four common terms emerged across all groups: leukocyte proliferation, macrophage activation, and response to lipopolysaccharide. DEGs of the Au NRs group were enriched in the regulation of the immune effector process, regulation of inflammatory response, and mononuclear cell proliferation. Distinctively, in the Au NRs-C group, DEGs were significantly enriched in immune-specific categories such as T cell, B cell, lymphocyte proliferation, and humoral immune response compared with the Au NRs group. Meanwhile, DEGs in the Au NRs-C_vs_PBS group were enriched in the antigen processing and presentation of peptide antigen via MHC class II and leukocyte chemotaxis. On MF, DEGs enhanced chemokine activity, cytokine activity, immune receptor activity, receptor-ligand activity, and signaling receptor activator activity. Moreover, DEGs from Au NRs-C were enriched for CXCR chemokine receptor binding.

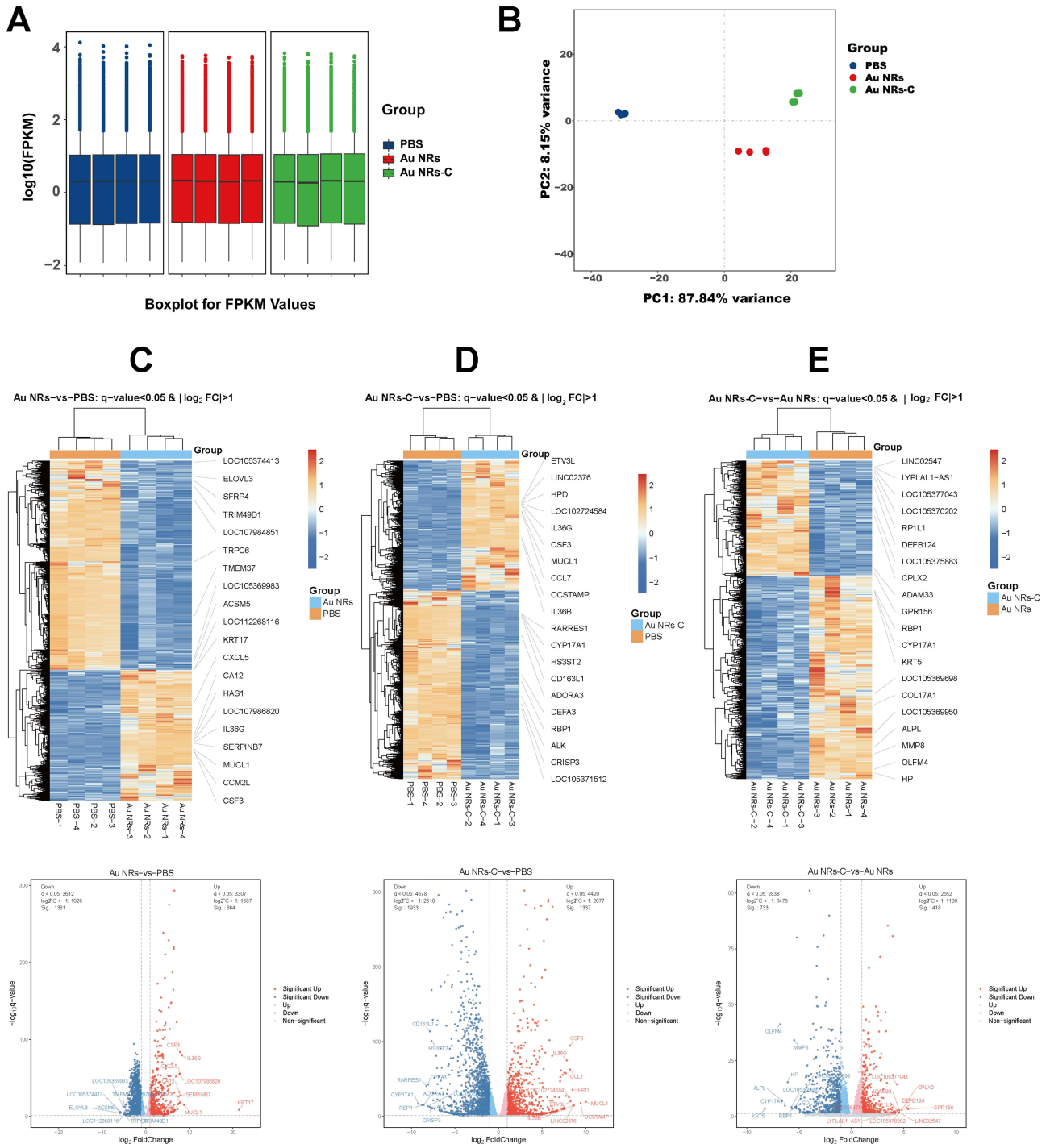

Figure 4 Analysis of functionalized gold nanorods gene expression levels and DEGs. (**A**) Analysis of gene expression levels of FPKM box line plot. (**B**) Principal Component Analysis plot. (**C**–**E**) Analysis of DEGs of heatmaps and volcano plots in groups Au NRs_vs_PBS (**C**), Au NRs-C_vs_PBS (**D**), and Au NRs_vs_Au NRs-C (**E**). Genes with non-significant differences are shown in gray, significantly up-regulated DEGs in red, and significantly down-regulated DEGs in blue.

The top 20 signaling pathways across three groups were classified through the KEGG level 1 hierarchy (Figure 5D–F). The DEGs of all three groups were enriched in cytokine-cytokine receptor interactions under environmental information processing, hematopoietic cell lineage within organismal systems, and rheumatoid arthritis within human diseases. In the organismal systems category, DEGs in Au NRs-C groups demonstrated greater enrichment in immune-responsive pathways, particularly B cell receptor signaling, IL-17, chemokine signaling, and Toll-like receptor signaling pathways.

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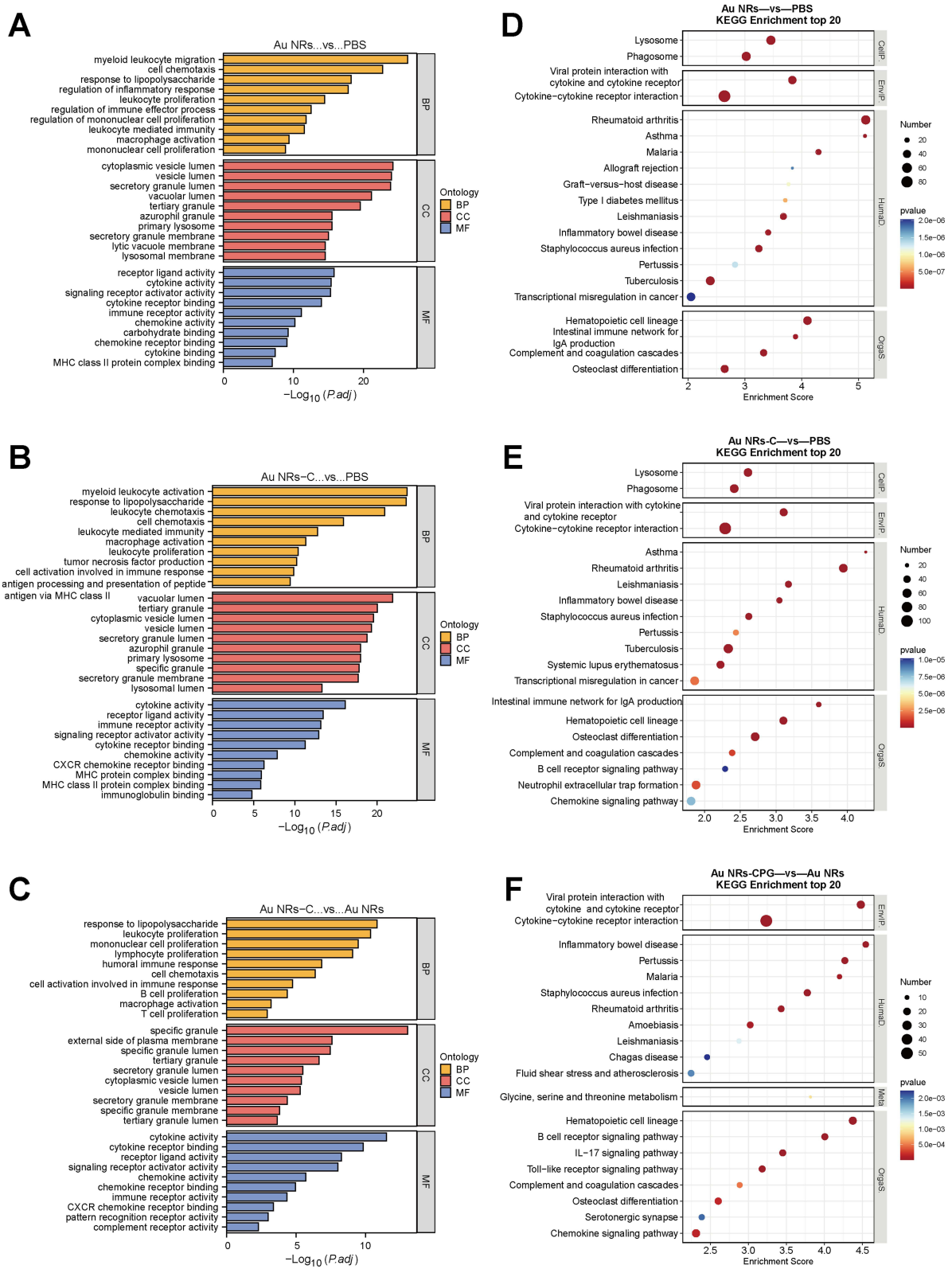

Figure 5 GO and KEGG enrichment analysis of DEGs. (**A**–**C**) GO enrichment analysis of DEGs in Au NRs_vs_PBS (**A**), Au NRs-C_vs_PBS (**B**), and Au NRs-C_vs_Au NRs (**C**). (**D**–**F**) KEGG enrichment analysis of DEGs in Au NRs_vs_PBS (**D**), Au NRs-C_vs_PBS (**E**), and Au NRs-C_vs_Au NRs (**F**).

Furthermore, DEGs from the Au NRs-C_vs_Au NRs group were also enriched in Glycine, serine, and threonine metabolism in the metabolism classification.

Protein Interaction Network Analysis and the Identification and Validation of Hub Genes

To screen the hub genes, we examined protein-protein interactions (PPIs) involving the proteins encoded by DEGs. Under our research objectives, we focused on up-regulated and down-regulated DEGs in the Au NRs-C_vs_PBS group for PPIs, selecting the DEGs with the top 30 combined scores for interaction mapping (Figure 6B). Similarly, the results for groups Au NRs_vs_PBS and Au NRs-C_vs_ Au NRs are displayed in Figure 6A and C. Ultimately, six top-scoring interactive nodes were identified as hub genes in the Au NRs-C_vs_PBS group, which were up-regulated DEGs *FOXM1, HMOX1, UBE2C*, and *E2F1* and down-regulated genes *PECAM1* and *FCGR3A*. Subsequently, we employed RT-qPCR to further evaluate the expression levels of these six hub genes. Results indicated that *FOXM1, HMOX1, UBE2C*, and *E2F1* exhibited elevated expression in the Au NRs-C group, whereas *PECAM1* and *FCGR3A* displayed diminished expression (Figure 6D–I). These results are consistent with the high-throughput sequencing results, indicating that our transcriptome data are reliable.

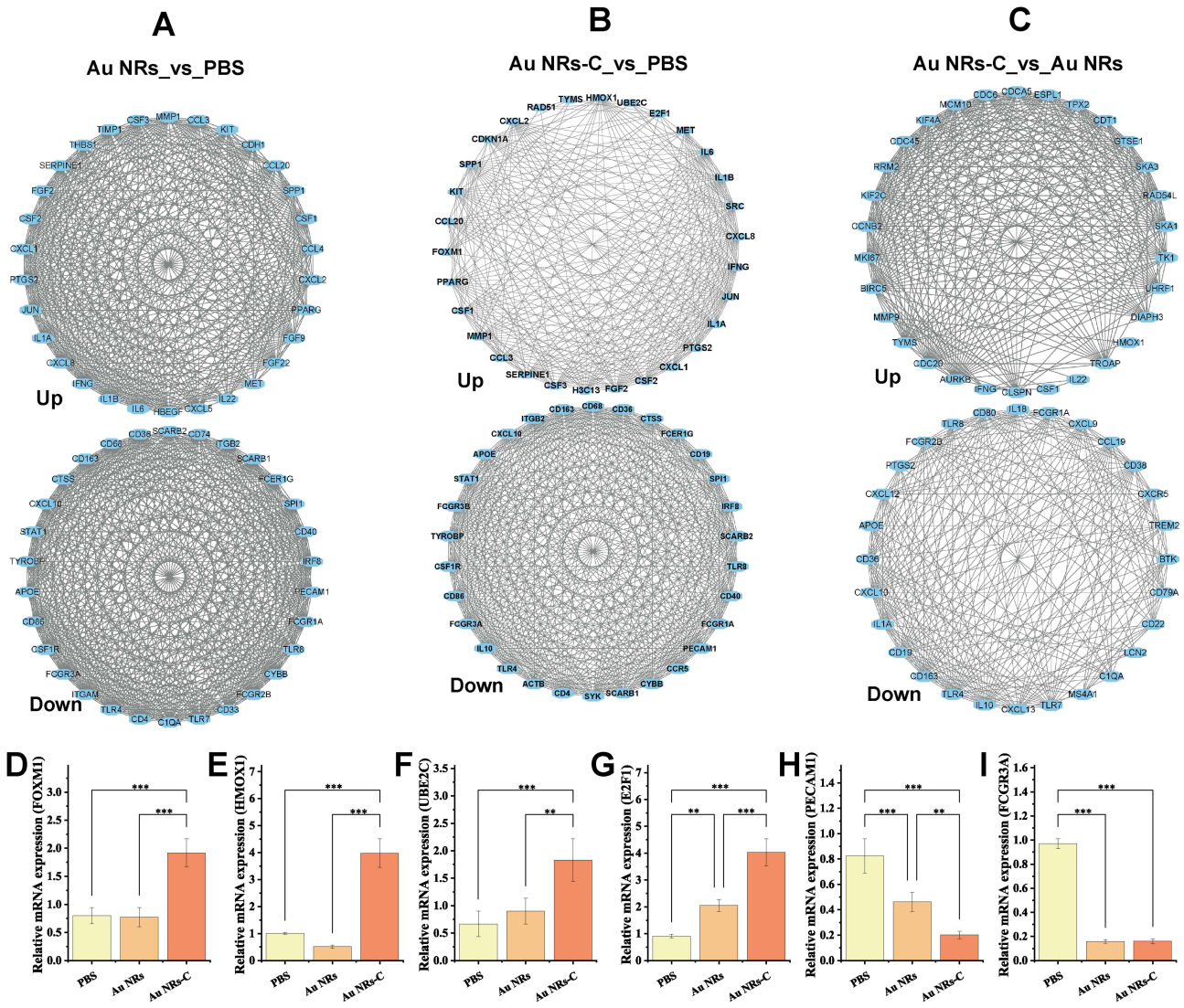

Figure 6 Protein interactions analysis and validation of hub genes. (**A**–**C**) Protein interactions analysis of groups Au NRs_vs_PBS (**A**), Au NRs-C_vs_PBS (**B**), and Au NRs-C_vs_Au NRs (**C**) of up-regulated genes (left) and down-regulated genes (right) in the top 30 of the combined scores. (**D**–**I**) Validation of mRNA expression levels of the hub genes: *FOXM1* (**D**), *HMOX1* (**E**), *UBE2C* (**F**), *E2F1* (**G**), *PECAM1* (**H**), and *FCGR3A* (**I**) by RT-qPCR. Data were expressed as mean ± SD of three independent experiments. ** indicate $p < 0.01$, *** indicate $p < 0.001$.

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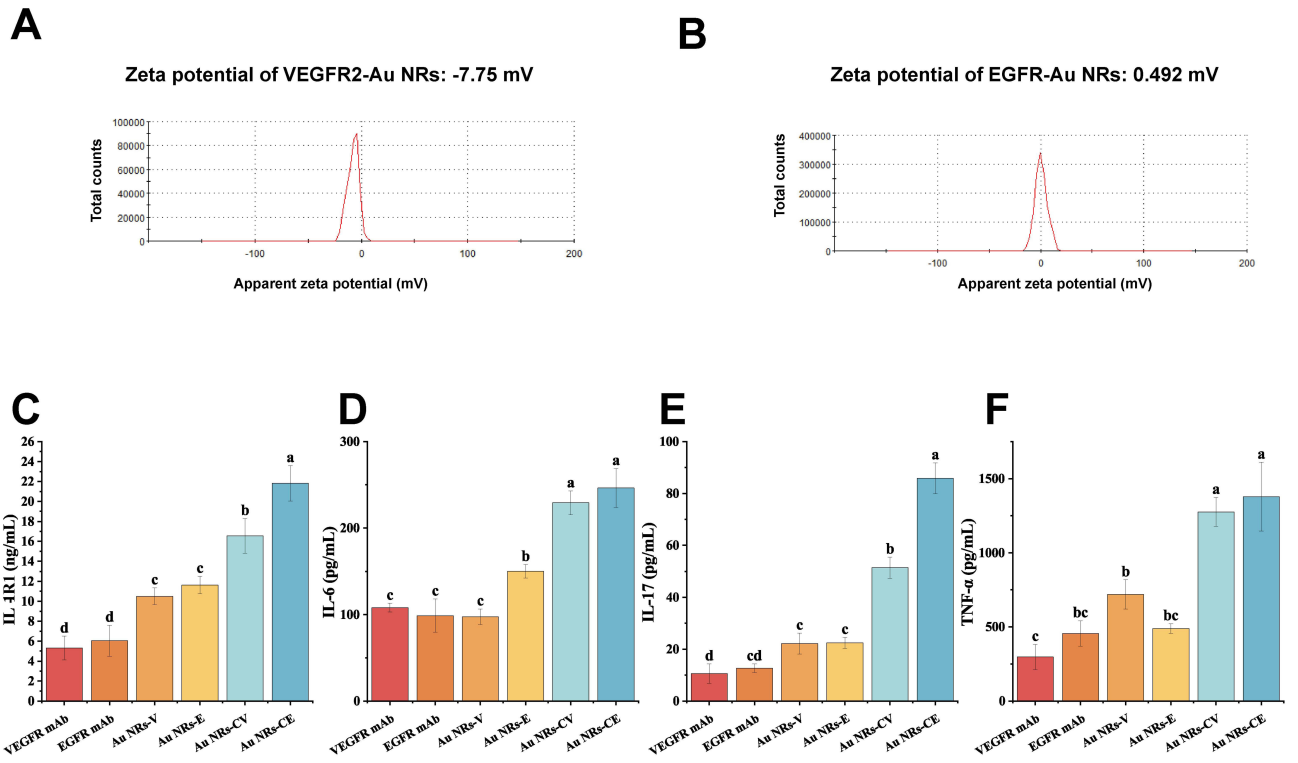

Figure 7 The preparation of antibody-functionalized gold nanorods. (**A** and **B**) Zeta potential analysis for VEGFR2-functionalized Au NRs (**A**) and EGFR-functionalized Au NRs (**B**). (**C**–**F**) ELISA analysis to detect the expression of immune-promoting inflammatory factors, namely IL-1R1 (**C**), IL-6 (**D**), IL-17 (**E**), and TNF-α (**F**). Data were expressed as mean ± SD of three independent experiments. The statistical significance of the differences is indicated by the letter marking method (a, b, c, d). Bars with different letters indicate $p < 0.05$ by ANOVA with Tukey's post-hoc.

Antibody-Functionalized Au NRs Bind CpG ODN with Greatly Improved Immune Function

Our previous studies have reported the use of antibody-conjugated Au NRs for targeted therapy. Transcriptomic sequencing has also revealed that CpG ODN-conjugated Au NRs influence angiogenesis, suggesting that their combined effects may yield favorable outcomes. Subsequently, we prepared functionalized Au NRs by coupling VEGFR2 or EGFR antibodies with Au NRs. Zeta potential analysis indicated that the surface potential of the Au NRs was 24.6 mV (Figure 2B), while the VEGFR2-conjugated Au NRs exhibited a surface potential of −7.75 mV (Figure 7A), and the EGFR-conjugated Au NRs displayed a potential of 0.492 mV (Figure 7B). There was a large decrease in the surface potential of the modified Au NRs, which indicated that we had successfully prepared the functionalized Au NRs. ELISA results demonstrated that both VEGFR2- and EGFR-AuNRs alone enhanced certain cytokines and increased DP T/NK cells while reducing Tregs. Notably, when CpG ODN was added to either antibody-AuNR, cytokine levels and effector cells increased further (Figures 7C–F and 8A–D). However, adding CpG ODN to EGFR-AuNR restored Treg levels (Figure 8E), indicating this combination did not suppress regulatory cells as effectively. Overall, antibody-functionalized Au NRs demonstrate superior immunostimulatory effects when combined with CpG ODN.

Discussion

Au NRs are frequently utilized in cancer therapy. However, off-target effects and immune evasion remain significant challenges in their application. In the previous study, we successfully prepared functionalized conjugates of EGFR mAb and Au NRs and found that they had the effect of promoting apoptosis in squamous carcinoma cells.[5,29] This approach has partially mitigated the off-target effects associated with Au NR treatment. Additionally, the researchers found that Janus nanomotors (AuNR/PMO@CPG) promoted tumor permeability to exert positive tumor therapeutic effects.[30] Furthermore, CPG@Au NRs/m-R848 treatment inhibited tumor growth by activating CD8+ T cells.[20] Immune cells,

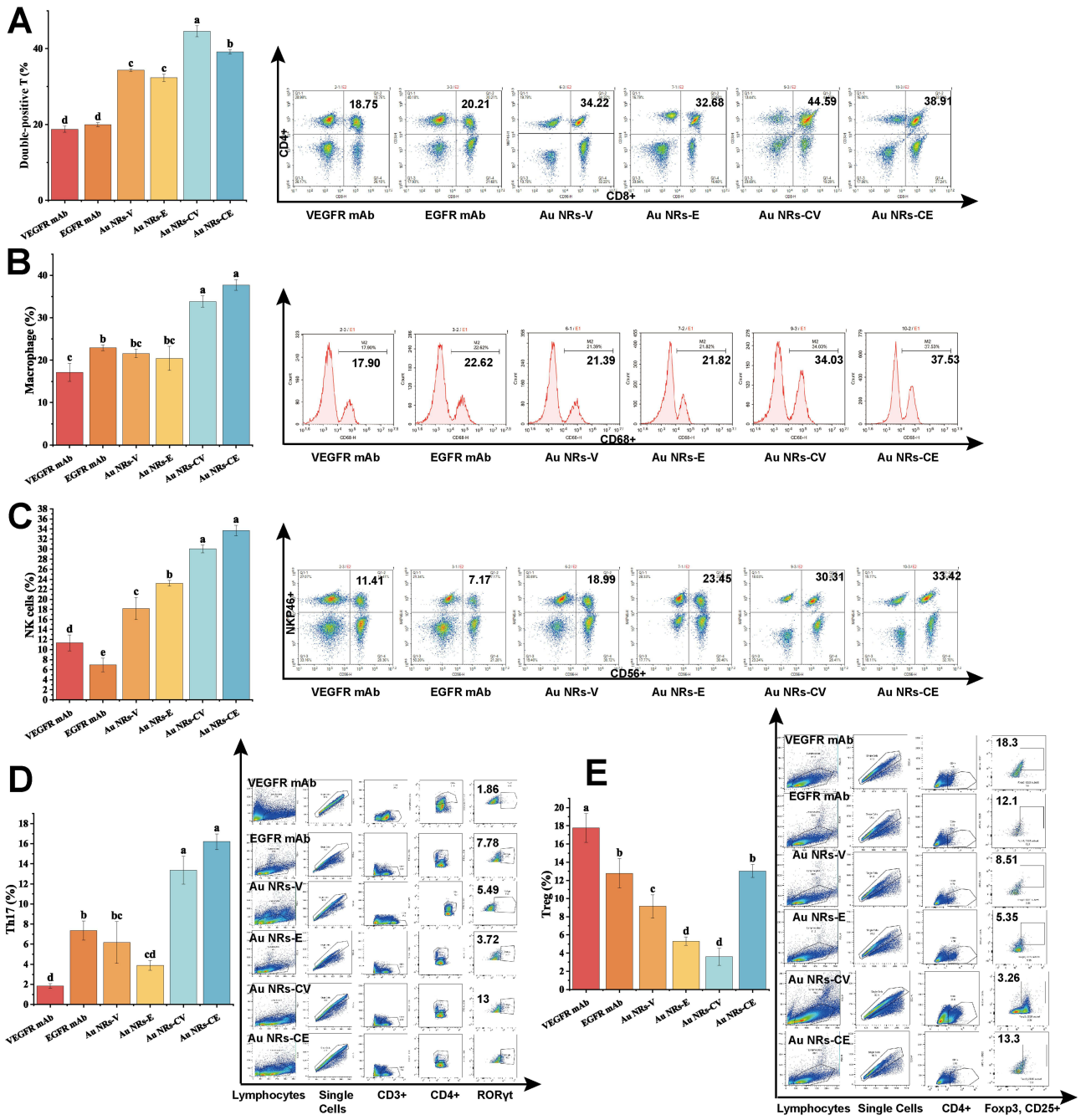

Figure 8 The immune activation assessment of antibody-functionalized gold nanorods. (**A**–**E**) Flow cytometry detection of immune cell expression for double-positive T cells (**A**), macrophages (**B**), NK cells (**C**), Th17 (**D**), and Treg (**E**). Data were expressed as mean ± SD of three independent experiments. The statistical significance of the differences is indicated by the letter marking method (a, b, c, d, e). Bars with different letters indicate $p < 0.05$ by ANOVA with Tukey's post-hoc.

which are prevalent in the tumor microenvironment (TME), play a crucial role in tumor progression and prognosis.[31] CpG ODN, a well-known immune adjuvant, can initiate immune regulatory cascades, leading to the production of pro-inflammatory cytokines and chemokines.[32] Nevertheless, it remains unclear whether combining CpG ODN with Au NRs can act synergistically to activate immune responses and the underlying mechanisms.

This study investigates the immune activation and potential mechanisms of action of Au NRs-C. Our results indicate that both Au NRs and CpG ODN possess the ability to promote the proliferation of immune-related inflammatory factors and immune activation. Notably, the Au NRs-C group, which combines both agents, exhibited superior effects on four

https://doi.org/10.2147/JIR.S528371

pro-immune inflammatory factors and immune stimulation compared to their individual applications. IL-1 has a wide range of biological activities and plays an important role in the immune system. It not only promotes the activity of cells of the innate immune system but also activates and enhances the function of polarized T cells.[8] It has been found that IL-1 signaling depends on the IL-1 receptor, and the control of IL-1 receptor signaling and expression is relevant to the differentiation of Th17 cells and autoimmune diseases. Moreover, the IL-1R1 assay offers greater stability and a more comprehensive reflection of cellular status.[33] Therefore, IL-1R1 was used to study the mechanism of cellular response to IL-1 signaling and T cell-mediated immune responses in this study. Furthermore, our research revealed that Au NRs stimulate the expression of Treg cells, consistent with previous studies.[34] However, we found that the combination of Au NRs with CpG ODN significantly inhibited this stimulatory effect, resulting in lower Treg expression levels than those observed with CpG ODN alone. This suggests that coupling CpG ODN with Au NRs not only greatly enhances the immune activation capacity of Au NRs but also mitigates the unintended Treg increase by AuNRs. In addition, our study observed a significant increase in Au NRs-C CD4+CD8+ DP T cell activity relative to the other groups. Previous studies have shown that significant overexpression of DP T cells is associated with inhibition of Treg expression and cytotoxic effects.[35] This is consistent with our findings that Au NRs-C significantly suppressed Treg expression and enhanced immune function.

To delve deeper, transcriptome sequencing was employed to elucidate its potential immune mechanisms. Apparently, these DEGs are enriched in leukocyte and immune cell-associated pathways. Notably, these DEGs were also associated with functions such as antigen processing and presentation via MHC class II and MHC protein complex binding. DP T cells were found to have enhanced cytotoxicity against patient-derived cells in an MHC-restricted manner.[35] The findings observed in this study, Au NRs-C significantly promoted the expression of DP T cells, and DEGs were significantly enriched in MHC antigen-presenting functions, reaffirming this conclusion. Additionally, AuNRs-E5, which refers to Au NRs enhanced with the CXCR4 antagonist E5, showed the ability to activate immune anti-tumor responses.[36] Similarly, we found that the DEGs were enriched in CXCR chemokine receptor binding. To further investigate, six hub genes were revealed. They were up-regulated DEGs *FOXM1, HMOX1, UBE2C*, and *E2F1*, and down-regulated genes *PECAM1* and *FCGR3A*. Notably, combined with previous studies for synthesis, we hypothesized that the up-regulated gene *E2F1* and down-regulated gene *PECAM1* may significantly influence immune activation triggered by Au NRs-C. *E2F1* is involved in the regulation of DNA damage repair and cell cycle[37] and has shown strong links to various tumor developments.[38–40] Moreover, *E2F1* expression correlates positively with various immune cells,[41] especially affecting the activation of CD8+ T cells.[42] Furthermore, it has been found that in the presence of LPS, *E2F1* was activated to activate TLR4 to play an immune role.[43–45] This was consistent with our results that DEGs are enriched in response to lipopolysaccharide and toll-like receptor signaling pathways. Additionally, another hub gene, *PECAM1*, belongs to the immunoglobulin superfamily. Its immune receptor tyrosine inhibitory motifs designate it as an immune receptor. *PECAM1* is expressed in 95% of splenic lymphocytes and functions primarily in the negative regulation of immune responses.[46,47] Besides, we found that *E2F1*[48] and *PECAM1*[49,50] were both associated with angiogenesis, a process intricately tied to tumor progression.[51] We further investigate the antibody-functionalized Au NRs (VEGFR2 and EGFR) by integrating these results with our previous research, specifically examining the immune activation characteristics of Au NRs functionalized with these two antibodies in conjunction with CpG ODN. The results of the study showed that, except for the unsatisfactory performance of EGFR-functionalized Au NRs in terms of Treg cells after binding CpG ODN. In general, antibody-functionalized Au NRs possessed better immune activation after binding CpG ODN.

In conclusion, this study suggests that CpG ODN combined with Au NRs can more effectively enhance immune activation and explore its underlying mechanism. Subsequent research on antibody-functionalized Au NRs also suggests that Au NRs-C plays a beneficial role in the field of tumor therapy. It is crucial to emphasize that we did not perform functional studies on the *E2F1* and *PECAM1* genes. Moreover, the results of the mechanism were based on bioinformatics analysis, which needs to be validated by further studies, such as in vivo tests. The data were from PBMC culture, not a disease model. In addition, all results are from ex vivo PBMC experiments, and sample size and donor variability issues need to be addressed. Besides, the potential cytotoxicity of Au NRs or CpG ODN to PBMC was not assessed. Despite this limitation, our findings suggest that CpG ODN conjugation can tilt AuNR therapy towards pro-immune

effects. Meanwhile, this study provides a highly valuable theoretical basis and direction for the study and improvement of Au NRs in the field of immune activation and may play a key role in tumor therapy.

Abbreviations

NRs, Nanorods; Au NRs, Gold nanorods; CpG ODN, CpG oligodeoxynucleotide; Au NRs-C, Au NRs combined with CpG ODN; PBMCs, Peripheral blood mononuclear cells; Au NRs-V, Au NRs functionalized with VEGFR mAb; Au NRs-E, Au NRs functionalized with EGFR mAb; Au NRs-CV, Au NRs functionalized with both CpG ODN and VEGFR mAb; Au NRs-CE, Au NRs functionalized with both CpG ODN and EGFR mAb; FSC, Forward-scattered light; SSC, Side-scattered light; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; DP T cells, CD4+CD8+ double-positive T cells; PCA, Principal Component Analysis; DEGs, Differently expressed genes; BP, Biological process; CC, Cellular component; MF, Molecular function; PPIs, protein-protein interactions; TME, Tumor microenvironment.

Data Sharing Statement

The datasets generated and/or analyzed during the current study are available in NCBI at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE285375, reference number GSE285375. Other data will be made available by the corresponding author on request.

Ethical/Copyright Corrections

Clinical samples: All procedures involving human subjects were approved by the Yunnan Cancer Hospital Ethics Committee (acceptance No. SLKYCS2024-046) and comply with the Declaration of Helsinki.

Funding

This study was supported by Yunnan Province "ten thousand people plan" famous doctor special project (grant No. YNWR-MY-2019-050), Yunnan Provincial Health Commission - Medical Leading Talents Program (grant No. L-2019012), and the Science and Technology Department of Yunnan Province - Key project of Basic Research Program (grant No. 202301AY070001).

Disclosure

The authors have declared that they have no conflicts of interest.

References

1. Zheng J, Cheng X, Zhang H, et al. Gold nanorods: the most versatile plasmonic nanoparticles. *Chem Rev*. 2021;121:13342–13453. doi:10.1021/acs.chemrev.1c00422
2. Li J, Zhu B, Zhu Z, et al. Simple and rapid functionalization of gold nanorods with oligonucleotides using an mPEG-SH/Tween 20-assisted approach. *Langmuir*. 2015;31:7869–7876. doi:10.1021/acs.langmuir.5b01680
3. Hwang SY, Tao AR. Biofunctionalization of gold nanorods. *Pure Appl Chem*. 2011;83:233–241. doi:10.1351/PAC-CON-10-09-35
4. Li Z, Huang P, Zhang X, et al. RGD-conjugated dendrimer-modified gold nanorods for *in vivo* tumor targeting and photothermal therapy. *Mol Pharm*. 2009;7:94–104. doi:10.1021/mp9001415
5. Zhang Y, Cong L, He J, et al. Photothermal treatment with EGFRmAb-AuNPs induces apoptosis in hypopharyngeal carcinoma cells via PI3K/AKT/mTOR and DNA damage response pathways. *Acta Biochim Biophys Sin*. 2018;50:567–578. doi:10.1093/abbs/gmy046
6. Mitchell WA, Aspinall R. The immune system. In: Aging H, editor. *HIV and Aging*. CRC Press; 2008:1–22.
7. Chaplin DD. Overview of the immune response. *J Allergy Clin Immunol*. 2010;125:S3–23. doi:10.1016/j.jaci.2009.12.980
8. Sims JE, Smith DE. The IL-1 family: regulators of immunity. *Nat Rev Immunol*. 2010;10:89–102. doi:10.1038/nri2691
9. Tanaka T, Narazaki M, Kishimoto T. IL-6 in inflammation, immunity, and disease. *Cold Spring Harb Perspect Biol*. 2014;6:a016295. doi:10.1101/cshperspect.a016295
10. Cua DJ, Tato CM. Innate IL-17-producing cells: the sentinels of the immune system. *Nat Rev Immunol*. 2010;10:479–489. doi:10.1038/nri2800
11. Jin W, Dong C. IL-17 cytokines in immunity and inflammation. *Emerg Microbes Infect*. 2013;2:1–5. doi:10.1038/emi.2013.58
12. Calzascia T, Pellegrini M, Hall H, et al. TNF-α is critical for antitumor but not antiviral T cell immunity in mice. *J Clin Invest*. 2007;117:3833–3845. doi:10.1172/JCI32567
13. Mellman I, Coukos G, Dranoff G. Cancer immunotherapy comes of age. *Nature*. 2011;480:480–489. doi:10.1038/nature10673
14. Zhang Y, Wang T, Tian Y, et al. Gold nanorods-mediated efficient synergistic immunotherapy for detection and inhibition of postoperative tumor recurrence. *Acta Pharm Sin B*. 2021;11:1978–1992. doi:10.1016/j.apsb.2021.03.035

15. Durgeau A, Virk Y, Corgnac S, Mami-Chouaib F. Recent advances in targeting CD8 T-cell immunity for more effective cancer immunotherapy. *Front Immunol*. 2018;22:14. doi:10.3389/fimmu.2018.00014
16. Gliwiński M, Piotrowska M, Iwaszkiewicz-Grześ D, Urban-Wójciuk Z, Trzonkowski P. Therapy with CD4+CD25+ T regulatory cells-should we be afraid of cancer? *Współczesna Onkol*. 2019;23:1–6. doi:10.5114/wo.2019.84110
17. Huang X, El-Sayed IH, Qian W, El-Sayed MA. Cancer cell imaging and photothermal therapy in the near-infrared region by using gold nanorods. *J Am Chem Soc*. 2006;128:2115–2120. doi:10.1021/ja057254a
18. Jurk M, Vollmer J. Therapeutic applications of synthetic CpG oligodeoxynucleotides as TLR9 agonists for immune modulation. *BioDrugs*. 2007;21:387–401. doi:10.2165/00063030-200721060-00006
19. Lu W, Cui C, Wang Y, et al. CpG ODN as an adjuvant arouses the vigor of B cells by relieving the negative regulation of surface TLR9 to enhance the antibody response to vaccine. *Appl Microbiol Biotechnol*. 2021;105:4213–4224. doi:10.1007/s00253-021-11316-9
20. Jia Y, Shi K, Dai L, et al. Gold nanorods and polymer micelles mediated dual TLR stimulators delivery system CPG@Au NRs/M-R848 regulate macrophages reprogramming and DC maturation for enhanced photothermal immunotherapy of melanoma. *Small Methods*. 2023;7:2201087. doi:10.1002/smtd.202201087
21. Wang H, Qin S, Zhang L, et al. EGFRmAb-modified gold nanorods reduce autophagy by disrupting the lysosomal structure for the treatment of head and neck squamous cell carcinoma. *Am J Transl Med*. 2022;6:113–123.
22. Chen N, Wei M, Sun Y, et al. Self-assembly of poly-adenine-tailed CpG oligonucleotide-gold nanoparticle nanoconjugates with immunostimulatory activity. *Small*. 2014;10:368–375. doi:10.1002/smll.201300903
23. Pattanapanyasat K, Kyle DE, Tongtawe P, Yongvanitchit K, Fucharoen S. Flow cytometric immunophenotyping of lymphocyte subsets in samples that contain a high proportion of non-lymphoid cells. *Cytometry*. 1994;18:199–208. doi:10.1002/cyto.990180403
24. Alvarez DF, Helm K, Degregori J, Roederer M, Majka S. Publishing flow cytometry data. *Am J Physiol Lung Cell Mol Physiol*. 2010;298:L127–30. doi:10.1152/ajplung.00313.2009
25. Anders S, Huber W; European Molecular Biology Laboratory. Differential expression of RNA-Seq data at the gene level-the DESeq package. 2012. Available from: https://api.semanticscholar.org/CorpusID:1084010. Accessed August 25, 2025.
26. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol*. 2014;15:550. doi:10.1186/s13059-014-0550-8
27. The Gene Ontology Consortium, The gene ontology resource: 20 years and still GOing strong. *Nucleic Acids Res*. 2018;47:D330–8. doi:10.1093/nar/gky1055
28. Kanehisa M, Araki M, Goto S, et al. KEGG for linking genomes to life and the environment. *Nucleic Acids Res*. 2007;36:D480–4. doi:10.1093/nar/gkm882
29. Hai Y, Wang H, Qiu Y, et al. Optimization of endothelial growth factor receptor monoclonal antibody-gold nanorods photothermal therapy for laryngeal squamous cell carcinoma. *Bioengineered*. 2022;13:3262–3274. doi:10.1080/21655979.2022.2025517
30. Zhang Y, Xing Y, Zhou H, et al. NIR-activated Janus nanomotors with promoted tumor permeability for synergistic photo-immunotherapy. *Acta Biomater*. 2024;190:463–475. doi:10.1016/j.actbio.2024.10.040
31. Oliva M, Spreafico A, Taberna M, et al. Immune biomarkers of response to immune-checkpoint inhibitors in head and neck squamous cell carcinoma. *Ann Oncol*. 2019;30:57–67. doi:10.1093/annonc/mdy507
32. Klinman DM. Use of CpG oligodeoxynucleotides as immunoprotective agents. *Expert Opin Biol Ther*. 2004;4:937–946. doi:10.1517/14712598.4.6.937
33. Kim DH, Lee -W-W. IL-1 receptor dynamics in immune cells: orchestrating immune precision and balance. *Immune Netw*. 2024;24:e21. doi:10.4110/in.2024.24.e21
34. Safina I, Nima Al Sudani ZA, Hashoosh A, et al. Gold nanorods enhance different immune cells and allow for efficient targeting of CD4+ Foxp3+ Tregulatory cells. *PLoS One*. 2021;16:1–19. doi:10.1371/journal.pone.0241882
35. Schad SE, Chow A, Mangarin L, et al. Tumor-induced double positive T cells display distinct lineage commitment mechanisms and functions. *J Exp Med*. 2022;219:e20212169. doi:10.1084/jem.20212169
36. Meng Y, Zhou J, Liu X, et al. CXC chemokine receptor type 4 antagonistic gold nanorods induce specific immune responses and long-term immune memory to combat triple-negative breast cancer. *ACS Appl Mater Interfaces*. 2023;15:18734–18746. doi:10.1021/acsami.3c03130
37. González-Romero F, Mestre D, Aurrekoetxea I, et al. E2F1 and E2F2-mediated repression of CPT2 establishes a lipid-rich tumor-promoting environment. *Cancer Res*. 2021;81:2874–2887. doi:10.1158/0008-5472.CAN-20-2052
38. Xiong Y, Shi L, Li L, et al. CDCA5 accelerates progression of breast cancer by promoting the binding of E2F1 and FOXM1. *J Transl Med*. 2024;22:639. doi:10.1186/s12967-024-05443-w
39. Enjoji S, Yabe R, Tsuji S, et al. Stemness is enhanced in gastric cancer by a SET/PP2A/E2F1 axis. *Mol Cancer Res*. 2018;16:554–563. doi:10.1158/1541-7786.MCR-17-0393
40. Wang J, Bi W, Lv R, et al. SMEK1 promotes clear cell renal cell carcinoma progression via EGFR tyrosine-kinase dependent pathway. *Cancer Lett*. 2024;1:217148. doi:10.1016/j.canlet.2024.217148
41. Dong W, Zhan C. Bioinformatic-based mechanism identification of E2F1-related ceRNA and E2F1 immunoassays in hepatocellular carcinoma. *J Gastrointest Oncol*. 2022;13:1915–1926. doi:10.21037/jgo-22-674
42. Sui Q, Liu D, Jiang W, et al. Dickkopf 1 impairs the tumor response to PD-1 blockade by inactivating CD8+ T cells in deficient mismatch repair colorectal cancer. *J Immunother Cancer*. 2021;9:e001498. doi:10.1136/jitc-2020-001498
43. Lim C-A, Yao F, Wong JJ-Y, et al. Genome-wide mapping of RELA(p65) binding identifies E2F1 as a transcriptional activator recruited by NF-κB upon TLR4 activation. *Mol Cell*. 2007;27:622–635. doi:10.1016/j.molcel.2007.06.038
44. Ishii KJ, Koyama S, Nakagawa A, Coban C, Akira S. Host innate immune receptors and beyond: making sense of microbial infections. *Cell Host Microbe*. 2008;3:352–363. doi:10.1016/j.chom.2008.05.003
45. Takeuchi O, Akira S. Pattern Recognition Receptors and Inflammation. *Cell*. 2010;140:805–820. doi:10.1016/j.cell.2010.01.022
46. Wilkinson R, Lyons AB, Roberts D, Wong M-X, Bartley PA, Jackson DE. Platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31) acts as a regulator of B-cell development, B-cell antigen receptor (BCR)-mediated activation, and autoimmune disease. *Blood*. 2002;100:184–193. doi:10.1182/blood-2002-01-0027

47. Newton-Nash DK, Newman PJ. A new role for platelet-endothelial Cell Adhesion Molecule-1 (CD31): inhibition of TCR-mediated signal transduction1. *J Immunol*. 1999;163:682–688. doi:10.4049/jimmunol.163.2.682
48. Chen M, Capps C, Willerson JT, Zoldhelyi P. E2F-1 regulates nuclear factor-κB activity and cell adhesion. *Circulation*. 2002;106:2707–2713. doi:10.1161/01.cir.0000038706.30661.86
49. Newman PJ. The biology of PECAM-1. *J Clin Invest*. 1997;100:S25–9.
50. Newman PJ. The role of PECAM-1 in vascular cell biology. *Ann N Y Acad Sci*. 1994;714:165–174. doi:10.1111/j.1749-6632.1994.tb12041.x
51. Leoncini E, Ricciardi W, Cadoni G, et al. Adult height and head and neck cancer: a pooled analysis within the INHANCE Consortium. *Head Neck*. 2014;36:1391.