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Article

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Posted Date: November 23rd, 2026

DOI: https://doi.org/10.21203/rs.3.rs-11543644/v1

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Additional Declarations: Competing interest reported. Sang Bong Lee and Yong Hyun Jeon declares no competing interests.

Cy5.5-labeled NMDAR antibody probe for fluorescence imaging of systemic inflammation and treatment response

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Article type: Article

Abstract

Fluorescence imaging agents may support repeated assessment of systemic inflammation and its response to pharmacological treatment. We prepared and evaluated N-Ab-NIR, a Cy5.5-labeled N-methyl-D-aspartate receptor (NMDAR) antibody conjugate, as a candidate inflammation imaging probe. Probe preparation was examined by separation analysis and optical spectroscopy. Cellular assays assessed viability, nitrite, inflammatory cytokines, and nuclear factor κB promoter activity. Dexamethasone and indomethacin reduced cellular inflammatory readouts without a marked reduction in viability under the tested conditions. In mice challenged with lipopolysaccharide (LPS), whole-body fluorescence was measured at 1, 4, and 24 h after LPS injection, followed by ex vivo organ imaging. Both treatments were associated with lower whole-body fluorescence than the LPS vehicle group, with clearer separation at 4 and 24 h, and lower lung-associated fluorescence at the final time point. These observations support further evaluation of N-Ab-NIR for fluorescence imaging of treatment-associated changes in experimental systemic inflammation. Additional characterization and specificity controls are required to separate target-related accumulation from altered probe distribution and clearance. The findings do not establish diagnostic performance in clinical sepsis.

Keywords: Fluorescence imaging; Antibody–dye conjugate; N-methyl-D-aspartate receptor; Systemic inflammation; Lipopolysaccharide; Treatment response

Introduction

Sepsis involves a dysregulated host response to infection and can lead to organ dysfunction [1, 2]. Its immune manifestations vary over time and include inflammatory activation and immune suppression [3, 4]. Experimental measurements that can be repeated in living animals may help characterize inflammatory responses and their pharmacological modulation. A systemic lipopolysaccharide (LPS) challenge provides a model of selected inflammatory features, while representing only part of the infectious and organ-dysfunction phenotype of clinical sepsis.

Macrophages respond to microbial products by activating inflammatory signalling and releasing cytokines and nitric oxide-related products [5–7]. These responses are coupled to changes in cellular metabolism [8, 9]. Cellular assays provide information on inflammatory activation, whereas in vivo imaging can add spatial and temporal information about an administered probe. The biological meaning of the imaging signal nevertheless requires independent validation.

Longer-wavelength fluorophores can reduce tissue autofluorescence and improve optical detection relative to visible probes [10, 11]. Antibody–dye conjugates combine a recognition element with a fluorescent label, but their tissue signals may reflect vascular retention, nonspecific uptake and clearance as well as target binding [12, 13]. Changes after drug treatment must therefore be interpreted in the context of both inflammation and probe disposition.

Fluorescent agents can provide complementary readouts of inflammatory processes, including tissue-associated signal and reactive oxygen species-related responses [14, 15]. NMDAR-associated macrophage

biology and antibody-based optical imaging provide a rationale for evaluating receptor-directed probe constructs in inflammatory models [16]. NMDAR-related calcium signalling has also been associated with cyclooxygenase 2 expression in macrophages [17]. A candidate imaging agent must nevertheless be evaluated in its specific formulation, disease model and acquisition setting, because labeling chemistry and probe disposition may influence the measured signal.

Dexamethasone (DEX) regulates glucocorticoid receptor-dependent inflammatory transcription [18], whereas indomethacin (Indo) inhibits cyclooxygenase-dependent prostaglandin synthesis [19, 20]. Here, we prepared and evaluated N-Ab-NIR, a Cy5.5-labeled NMDAR antibody conjugate, as a candidate fluorescence imaging agent for experimental systemic inflammation. The study examined whether DEX and Indo treatment were associated with changes in whole-body and organ fluorescence after systemic LPS challenge. Cellular inflammatory assays complemented the imaging measurements. Serial imaging and subsequent ex vivo analysis were used to characterize the probe-associated signal across the experimental groups. The in vivo workflow is summarized in Fig. 1.

Results

Probe preparation and optical measurements

N-Ab-NIR was prepared by coupling a Cy5.5 NHS ester to antibody primary amines. Figure 2a presents the supplier-based reagent structure and a representative antibody amide linkage [21]. The separation image distinguishes free dye, the crude conjugation mixture and purified material (Fig. 2b). The observed bands are consistent with a change in fluorescent species following conjugation and purification; they do not by themselves quantify conjugate purity or the dye-to-antibody ratio.

Excitation, emission and absorbance profiles are shown in Fig. 2c. These measurements provide optical information on the fluorescent preparation, but do not demonstrate retention of antigen-binding affinity. Quantitative purity, labeling efficiency and antigen recognition remain necessary for complete probe characterization.

Cellular viability and inflammatory readouts

Viability measurements showed no marked reduction in BJ6 cells or RAW264.7 macrophages under the tested LPS-positive or LPS-negative conditions (Fig. 3a–c). This observation supports interpretation of the accompanying cellular assays within the tested concentration range and exposure conditions; it does not establish in vivo safety.

Nitrite was higher in the LPS group and lower in the DEX- and Indo-treated groups under LPS-positive conditions (Fig. 3d). Basal nitrite changed little across the LPS-negative groups (Fig. 3e). DEX and Indo were also associated with lower tumor necrosis factor α (TNF-α), interleukin 6 (IL-6) and interleukin 1β (IL-1β) than LPS alone (Fig. 3f–h). DEX showed a larger numerical reduction in the cytokine panels, particularly IL-1β, but a comparative efficacy claim would require a direct statistical comparison between the two drugs.

Nuclear factor κB (NF-κB) promoter-driven reporter signals were lower with DEX or Indo than with LPS alone (Fig. 3i). Together, the cellular readouts are consistent with reduced inflammatory activation under the tested conditions. Their relationship to the cellular source or target specificity of whole-body fluorescence was not directly established.

Whole body fluorescence after anti-inflammatory treatment

Whole-body fluorescence was measured in LPS-challenged mice at 1, 4 and 24 h after LPS injection (Fig. 4a). Mean fluorescence was lower in both treatment groups than in the LPS vehicle group, with clearer separation at 4 and 24 h (Fig. 4b). The annotated comparisons at the later time points indicate $P < 0.05$ versus the LPS vehicle group. These observations describe treatment-associated differences in probe-related fluorescence during the observation period.

Ex vivo organ fluorescence

Ex vivo fluorescence was detected in several harvested organs and tissues (Fig. 5a). Lung-associated signal was lower in the DEX and Indo groups than in the LPS vehicle group, as reflected in the annotated comparisons (Fig. 5b). Liver, intestine, kidney and other tissues showed different distribution patterns across groups. These measurements describe tissue-associated probe fluorescence; they do not directly measure inflammation severity or tissue drug concentration.

Discussion

The main observation was an association between anti-inflammatory treatment and lower N-Ab-NIR-related fluorescence after systemic LPS challenge. Differences in whole-body signal were clearer at 4 and 24 h, and ex vivo imaging showed lower lung-associated fluorescence in the treated groups. The cellular assays separately showed reductions in inflammatory readouts under DEX and Indo treatment. Together, these findings motivate further evaluation of serial fluorescence measurements for assessing pharmacological responses in experimental systemic inflammation.

This interpretation is narrower than a claim of target-specific detection. Antibody-associated fluorescence reflects the amount and optical environment of labeled material within the sampled region. A treatment-related reduction could arise from altered target-associated uptake, but also from changes in vascular permeability, circulating probe concentration, nonspecific retention, degradation or clearance. The present experiments do not distinguish these contributions. A non-LPS imaging comparator, a non-targeting antibody–dye conjugate and blocking experiments would help test whether the observed differences depend on inflammation and NMDAR recognition.

The lung observations require the same caution. Residual blood and organ-dependent distribution can contribute to ex vivo fluorescence, and the imaging measurements alone cannot identify NMDAR-positive macrophages as the source. Histological assessment, receptor and macrophage colocalization, and standardized collection and normalization procedures would help connect organ fluorescence to the

underlying inflammatory process. The cellular and animal experiments provide complementary observations but do not establish a causal relationship between cytokine suppression and the imaging signal.

N-Ab-NIR combines a Cy5.5 fluorophore with an NMDAR antibody in a candidate inflammation imaging construct. The present evaluation integrates probe preparation, cellular inflammatory readouts, serial whole-body imaging and ex vivo organ measurements within a systemic LPS model. Its practical focus is the relationship between pharmacological treatment and probe-associated fluorescence across the observation period. This framework can guide further studies of formulation, timing, biodistribution and quantitative performance. Head-to-head comparisons and independent validation would be needed to establish advantages over alternative imaging agents.

Probe characterization is a further limitation. The separation and optical measurements do not fully establish purity, dye loading, stability or preserved antigen recognition after conjugation. These properties can influence biodistribution and signal interpretation. The single representative amide linkage in Fig. 2a is a chemical schematic and does not identify the distribution of antibody labeling sites. Measurements at three discrete time points also support serial observations, rather than continuous real-time monitoring.

Finally, systemic LPS challenge is an experimental inflammation model and does not reproduce the full complexity of infection-associated sepsis. The study does not establish diagnostic sensitivity or specificity in patients, predict clinical outcomes, or demonstrate survival benefit or reversal of organ dysfunction. Within these limits, the observations support further investigation of treatment-associated fluorescence changes as a preclinical imaging endpoint. Establishing specificity and quantitative reproducibility is necessary before translating this endpoint into a validated biomarker.

Methods

Reagents and probe preparation

Anti-NMDAR1 antibody (Invitrogen/Thermo Fisher Scientific, PA3-102) was used as the antibody component. Cy5.5 NHS ester (Lumiprobe, catalog number 17020) was coupled to antibody primary amines to form the N-Ab-NIR conjugate. The reagent structure in Fig. 2a follows the manufacturer's molecular specification [21].

The antibody was transferred into an amine-free buffer, and the dye was dissolved in anhydrous dimethyl sulfoxide before addition. The reaction was conducted at room temperature with protection from light. Unreacted dye was removed, and the purified conjugate was designated N-Ab-NIR. LPS from Escherichia coli, DEX, Indo, phosphate-buffered saline, Griess reagent, viability reagents and cytokine assay kits were obtained from commercial suppliers.

Optical and separation measurements

Absorbance and fluorescence excitation and emission spectra were measured to characterize the fluorescent preparation. Free dye, the crude conjugation mixture and purified conjugate were compared by separation analysis (Fig. 2b); optical spectra are presented in Fig. 2c.

Cell culture and treatment

RAW264.7 mouse macrophages were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 1% penicillin–streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. The comparator cell line designated BJ6 was maintained under the reported culture conditions.

BJ6 cells and RAW264.7 macrophages under LPS-positive and LPS-negative conditions were evaluated in 96-well plates (Fig. 3a–c). DEX and Indo were each used at 10 μM. Cell viability was expressed relative to the vehicle group after absorbance measurement with a microplate reader.

Nitrite cytokine and reporter assays

Culture-supernatant nitrite was measured using the Griess reaction as an indirect readout of nitric oxide production [22], with LPS-positive and LPS-negative conditions evaluated separately (Fig. 3d, e). TNF-α, IL-6 and IL-1β were measured using enzyme-linked immunosorbent assays (Fig. 3f–h). NF-κB promoter activity was assessed using a luciferase reporter assay (Fig. 3i).

Animal ethics and experimental design

Animal procedures were approved by the Committee on the Ethics of Animal Experiments of the Laboratory Animal Center, K-medihub (approval number DGMIF-18070202-00). The approving institution adopted the English name K-medihub in place of DGMIF (Daegu-Gyeongbuk Medical Innovation Foundation); the approval number retains its original DGMIF prefix. Co-corresponding author Yong Hyun Jeon is affiliated with the K-medihub Pre-clinical center.

Specific pathogen-free, six-week-old C57BL/6 mice were used. Systemic inflammation was induced with LPS at 2 mg/kg. Immediately after LPS challenge, mice received vehicle, DEX or Indo; DEX and Indo were each administered at 10 mg/kg. N-Ab-NIR was administered through the tail vein at 1 mg/kg. The workflow is shown in Fig. 1.

Whole body and ex vivo fluorescence imaging

Whole-body images were acquired using an IVIS optical imaging system at 1, 4 and 24 h after LPS injection (Fig. 4a). Fluorescence and grayscale images were overlaid, and regions of interest were analyzed under consistent acquisition and display settings for group comparison (Fig. 4b).

After the final imaging time point, mice were anesthetized by inhalation of 2% isoflurane (Hana Pharm Co., Ltd., Republic of Korea) delivered using an XGI-8 Gas Anesthesia System (PerkinElmer, Inc., Waltham, MA, USA), and were then euthanized by cervical dislocation under anesthesia. Following euthanasia, major organs or tissues were collected for ex vivo fluorescence imaging to assess biodistribution (Fig. 5a). The

tissue order was heart, lung, tissue labeled lipid, stomach, liver, spleen, intestine, kidney, bone, muscle and brain. Regions of interest were analyzed under consistent settings to compare the LPS vehicle, DEX and Indo groups (Fig. 5b).

Statistical analysis

Data are expressed as means ± standard deviations. Group comparisons were performed using unpaired Student's t tests and one-way analysis of variance, with $P < 0.05$ taken as the threshold for statistical significance. Statistical analyses were performed using GraphPad Prism version 5.

Assistance with manuscript and figure preparation

ChatGPT (OpenAI; used in October 2026) assisted with language editing, manuscript organization, reference formatting and cross-checking for author review. AI-assisted code was used for schematic layouts and figure-label corrections. Figure 1 was generated using GPT Image (OpenAI) from the experimental workflow described in the manuscript. The authors remain responsible for verifying the content against the source records and for approving the final submission.

Data availability

The datasets generated and/or analysed during the current study are available from the corresponding authors on reasonable request.

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Acknowledgements

This work was supported by the National Research Foundation of Korea (grant number 2022R1FA1063012). The funder had no role in study design, data collection and analysis, the decision to publish, or manuscript preparation.

Author contributions

Sang Bong Lee conceived the study, designed and performed the experiments, analyzed the data, prepared the figures, wrote and revised the manuscript, and obtained funding. Yong Hyun Jeon was responsible for compliance with animal experimentation regulations, performed animal experiments, and contributed to manuscript writing.

Additional information

Competing interests

Sang Bong Lee and Yong Hyun Jeon declares no competing interests.

Consent declarations

Consent to participate and consent for publication are not applicable; this study did not involve human participants or identifiable human participant information.

Figures and legends

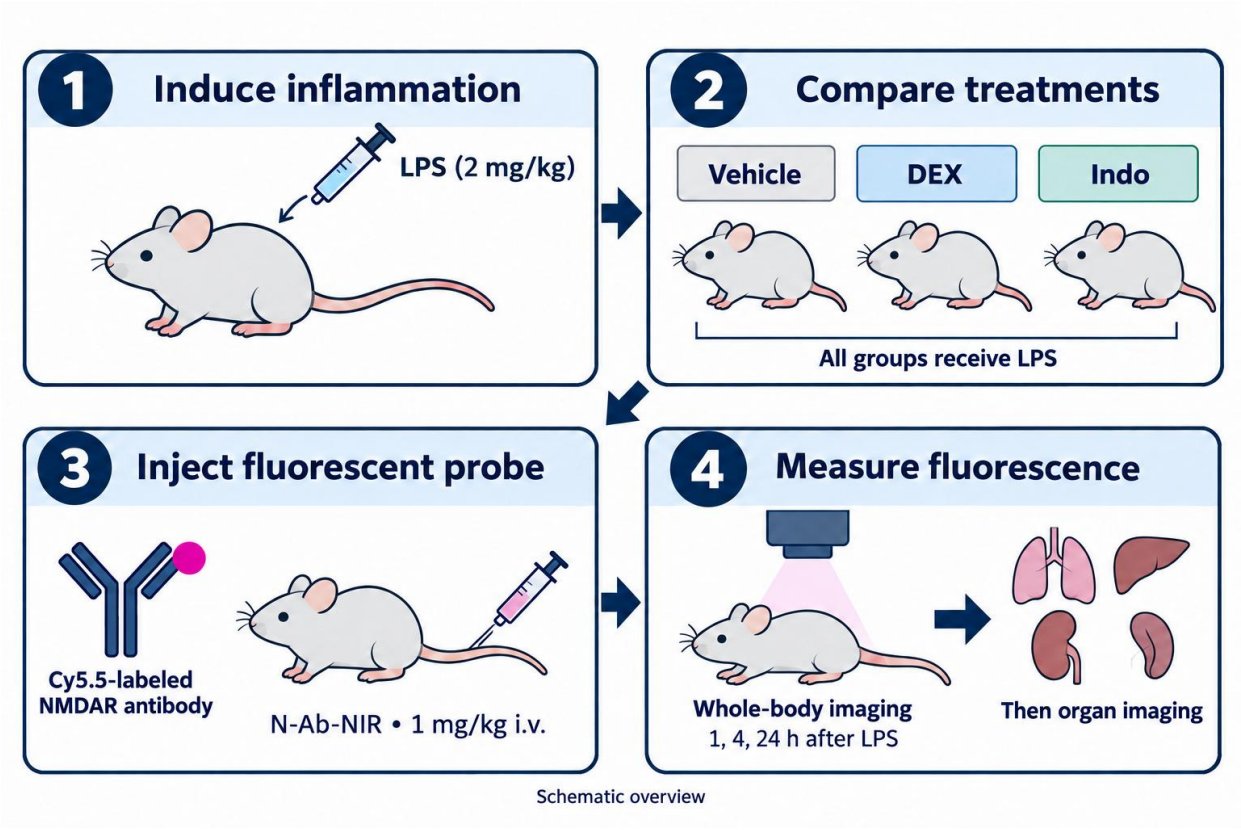

Figure 1. In vivo experimental workflow. (1) Systemic inflammation was induced using lipopolysaccharide (LPS; 2 mg/kg). (2) Mice received vehicle, dexamethasone (DEX; 10 mg/kg) or indomethacin (Indo; 10 mg/kg). (3) All groups received the Cy5.5-labeled NMDAR antibody (N-Ab-NIR; 1 mg/kg) intravenously. (4) Whole-body fluorescence was measured at 1, 4 and 24 h after LPS injection, followed by ex vivo organ imaging. The icons are schematic and do not represent acquired experimental images. The single magenta dot is illustrative and does not specify a measured dye-to-antibody ratio. This workflow illustration was generated using GPT Image (OpenAI) from the experimental study design.

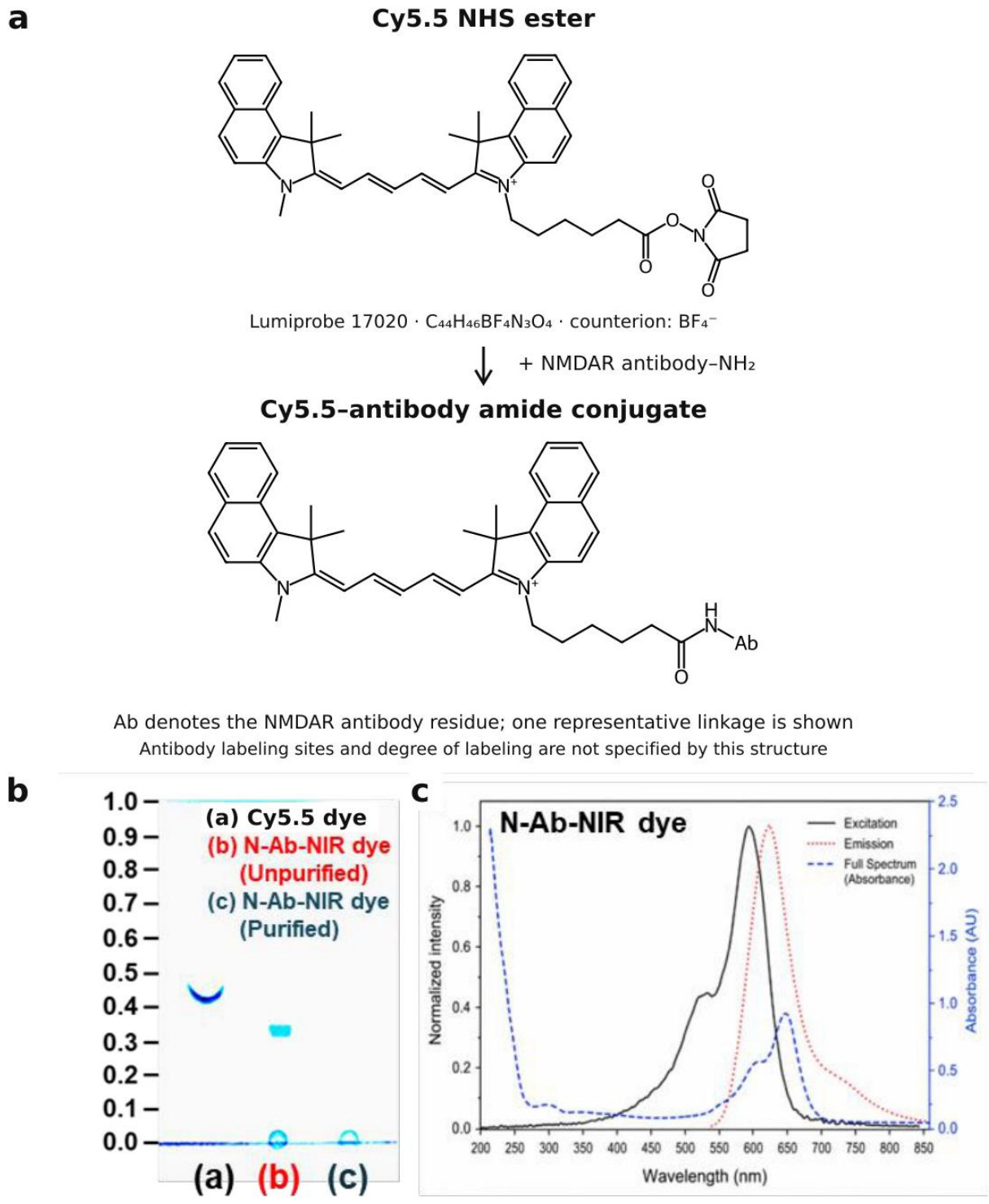

Figure 2. Probe preparation and optical measurements. (a) Cy5.5 NHS ester structure based on the supplier's molecular specification [21] and a representative antibody amide conjugate. Ab denotes the antibody residue. One amine-derived linkage is shown; attachment sites and degree of labeling are not defined. The reagent counterion is tetrafluoroborate. (b) Separation analysis of free dye, crude conjugation mixture and purified conjugate, identified by internal sample labels (a), (b) and (c). (c) Excitation, emission and absorbance profiles.

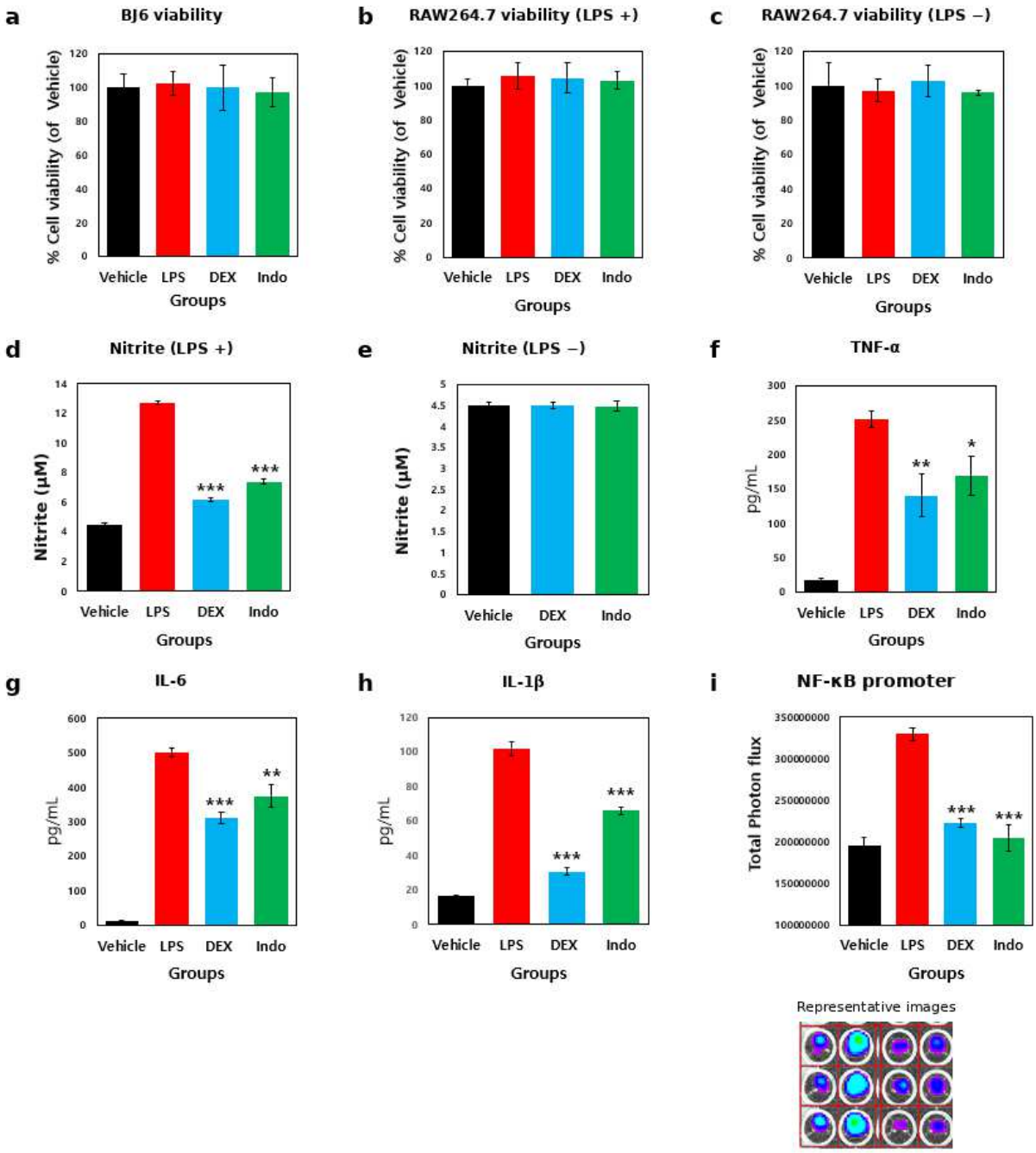

Figure 3. Cellular viability and inflammatory readouts. (a) BJ6 viability. (b, c) RAW264.7 viability under LPS-positive and LPS-negative conditions, respectively. (d, e) Nitrite under LPS-positive and LPS-negative conditions, respectively. (f–h) Tumor necrosis factor α (TNF-α), interleukin 6 (IL-6) and interleukin 1β (IL-1β), respectively. (i) Nuclear factor κB (NF-κB) promoter-driven reporter signal and representative luminescence images. Dexamethasone (DEX) and indomethacin (Indo) were each used at 10 μM. Data are displayed as mean ± s.d.; *P < 0.05, **P < 0.01 and ***P < 0.001 versus the LPS group for the annotated comparisons. Panel placement and headings were reformatted; the plotted data and acquired luminescence image array were retained.

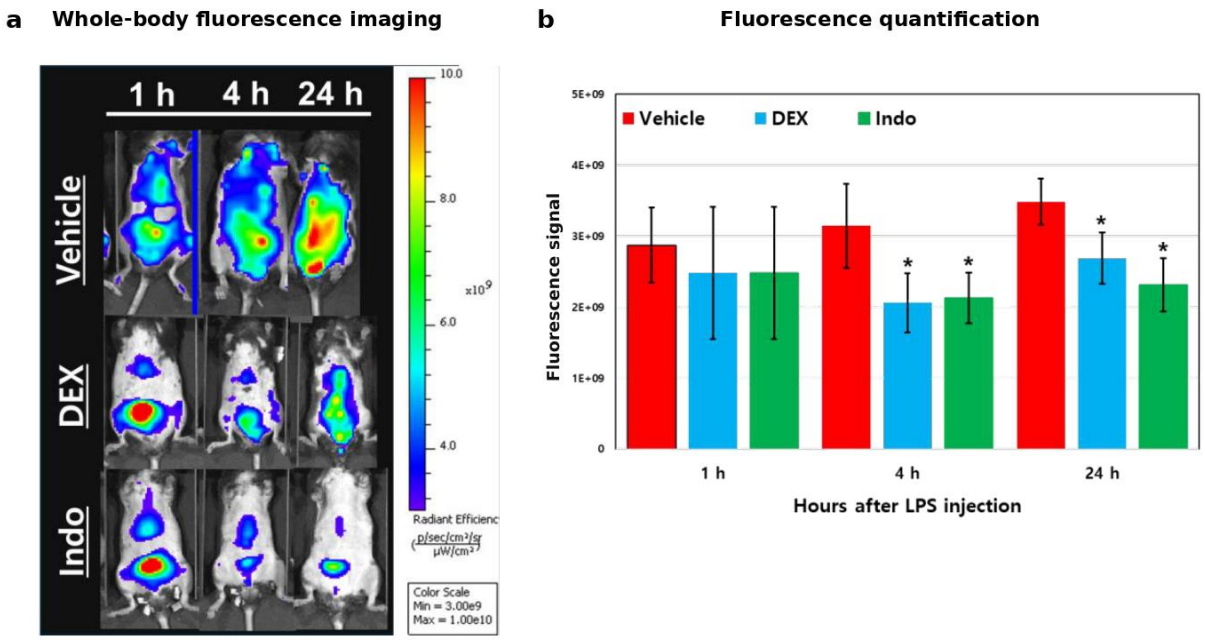

Figure 4. Whole-body fluorescence after systemic LPS challenge. (a) Representative images at 1, 4 and 24 h after LPS injection. (b) Group fluorescence quantification. LPS was administered at 2 mg/kg, dexamethasone (DEX) or indomethacin (Indo) at 10 mg/kg, and N-Ab-NIR at 1 mg/kg. Vehicle denotes the LPS-challenged group receiving drug vehicle. Data are displayed as mean ± s.d.; *P < 0.05 versus the LPS vehicle group at the corresponding time point. The complete acquired image array and graph were rearranged without changing their data or color scale.

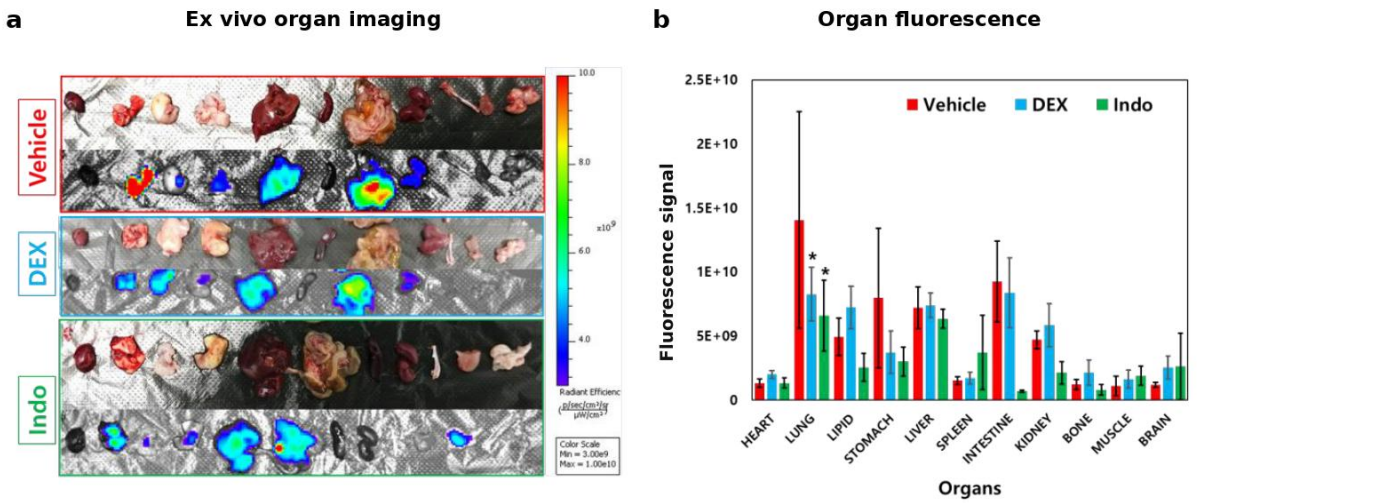

Figure 5. Ex vivo organ fluorescence after LPS challenge and drug treatment. (a) Photographs and fluorescence overlays of harvested organs or tissues. (b) Quantification in the order heart, lung, tissue labeled lipid, stomach, liver, spleen, intestine, kidney, bone, muscle and brain. Vehicle denotes the LPS vehicle group; DEX (dexamethasone) and Indo (indomethacin) denote the treatment groups. Data are displayed as mean ± s.d.; *P < 0.05 versus the LPS vehicle group for the annotated lung comparisons. The image array and graph were rearranged without changing their data or color scale.