In vivo molecular imaging for immunotherapy using ultra-bright near-infrared-I Ib rare-earth nanoparticles

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The near-infrared-I Ib (NIR-I Ib) (1,500–1,700 nm) window is ideal for deep-tissue optical imaging in mammals, but lacks bright and biocompatible probes. Here, we developed biocompatible cubic-phase (α-phase) erbium-based rare-earth nanoparticles (ErNPs) exhibiting bright downconversion luminescence at ~1,600 nm for dynamic imaging of cancer immunotherapy in mice. We used ErNPs functionalized with cross-linked hydrophilic polymer layers attached to anti-PD-L1 (programmed cell death-1 ligand-1) antibody for molecular imaging of PD-L1 in a mouse model of colon cancer and achieved tumor-to-normal tissue signal ratios of ~40. The long luminescence lifetime of ErNPs (~4.6 ms) enabled simultaneous imaging of ErNPs and lead sulfide quantum dots emitting in the same ~1,600 nm window. In vivo NIR-I Ib molecular imaging of PD-L1 and CD8 revealed cytotoxic T lymphocytes in the tumor microenvironment in response to immunotherapy, and altered CD8 signals in tumor and spleen due to immune activation. The cross-linked functionalization layer facilitated 90% ErNP excretion within 2 weeks without detectable toxicity in mice.

N in recent years, in vivo fluorescence- and luminescence-based molecular imaging in the second near-infrared window (NIR-II, 1,000–1,700 nm) under ~800–1,000 nm excitation have afforded high-resolution imaging at subcentimeter tissue depths, benefiting from suppressed photon scattering and diminished tissue autofluorescence in this spectral range1,2. Several classes of NIR-II probes have been developed, including small molecules3, carbon nanotubes (CNTs)4, polymer-encapsulated organic dyes5, quantum dots (QDs)6 and rare-earth lanthanide ion (Ln3+) doped nanoparticles7 (Table 1). Imaging at the long-wavelength end of the NIR-II window (NIR-I Ibs, 1,500–1,700 nm) increases penetration depth to subcentimeter and completely eliminates autofluorescence8–10. To date, CNTs5, inorganic QDs, including lead sulfide (PbS)9 and indium arsenide (InAs)10; and erbium-doped hexagonal-phase (that is, β-phase) rare-earth downconversion nanoparticles11 have been developed as NIR-I Ib probes for in vivo imaging. Nevertheless, the limited brightness of current NIR-I Ib probes, together with biocompatibility and toxicity concerns, limits their potential for clinical translation.

Much excitement has been generated by immunotherapy based on immune checkpoint blockade of programmed cell death-1 (PD-1) and PD-L1 (herein, PD pathway) for cancer treatment12–17. Blocking the PD pathway with anti-PD-L1 (or anti-PD-1) monoclonal antibodies (mAbs) can reverse cancer immune evasion and engender potent antitumor immunity in patients, resulting in durable cancer regression18–20. However, many challenges remain, including predicting patient therapeutic responsiveness and understanding how it is shaped by host and tumor components21,22. Clinical and preclinical works to predict response to anti-PD therapy have been relying on ex vivo biopsy with immunohistochemistry23 and in vivo positron-emission tomography24, single-photon emission computed tomography25 and fluorescence imaging26 to probe PD-L1 expression in tumor. An advantage of in vivo molecular imaging is the capability of dynamic monitoring and assessing of PD-L1 heterogeneity in tumor. It is, however, important to maximize imaging sensitivity, signal/background ratios, spatial and temporal resolution, and penetration depth. Also, although PD-L1 expression in tumor is a useful biomarker, it is not the only predictor and should be combined with other cellular and molecular signatures of the tumor microenvironment to investigate therapeutic responses and mechanisms26–28. Among various modalities, optical molecular imaging allows for high spatial resolution at the micrometer scale29, and possesses the potential of performing multiplexed imaging to follow several molecular targets simultaneously30,31. However, conventional optical molecular imaging based on fluorescence in the visible or near-infrared wavelengths <900 nm has been superficial in penetration depth with unsatisfactory spatial resolution due to light-scattering and autofluorescence problems32,33. PD-L1 molecular imaging has also been done using NIR-II fluorescence at ~1,100 nm (ref. 34), affording a relatively high tumor-to-normal tissue (T/NT) ratio of ~9.5. Molecular imaging of PD-L1 at the long end of the NIR-II range (NIR-I Ibs, 1,500–1,700 nm) could further increase penetration depth to subcentimeter and completely eliminate autofluorescence35–37.

Here, we report a Zn-doped α-phase (that is, cubic-phase; fluorite structure) ErNP with a core-shell structure of NaYbF4:2%Er,2%Ce,10%Zn@NaYF4 (Fig. 1a). An ~11-fold enhancement of the downconversion luminescence over the previous brightest
β-phase ErNPs was achieved through enhancing multi-phonon relaxation in α-phase ErNPs over β-phase and reducing crystal field symmetry through Zn\(^{2+}\) ion doping. A hydrophilic polymeric cross-linked network was developed to impart aqueous solubility and biocompatibility to the ErNPs, allowing fast biliary excretion of intravenously administrated nanoparticles within ~2 weeks without any discernable toxicity observed in mice. We also showed multiplexed molecular imaging in NIR-IIb using the ErNPs with milliseconds lifetime and our previously developed PbS QDs with a much shorter microsecond lifetime, both emitting at ~1,600 nm. Two-pixel molecular imaging with anti-PD-L1 mAb-labeled ErNPs (targeting PD-L1) and anti-CD8+ T-cells (targeting CD8\(^{+}\) T-cells) allowed in vivo noninvasive visualization of multiple targets in the same NIR-IIb emission window.

### Results

**Cubic-phase α-ErNPs with vastly enhanced downconversion ~1,550 nm luminescence.** Thus far, erbium-based downconversion ErNPs were all in β-phase (hexagonal phase)\(^{34,43}\) as an offspring of upconversion nanoparticles\(^{43}\). Earlier, we developed a β-phase ErNP (NaYbF\(_4\)-Er,Ce@NaYF\(_4\)) with Ce doping to suppress upconversion and increase downconversion\(^ {34}\). Here, we synthesized a different, α-phase (cubic-phase; fluorite structure) NaYbF\(_4\)-2%Er,2%Ce@NaYF\(_4\) nanocrystal (α-ErNP; same structure with β-phase ErNPs) by tuning the temperature during cothermolysis\(^ {38}\) and anti-CD8\(^{+}\) T-cells (Supplementary Fig. 1a). The core was encapsulated by epitaxial growth of an NaYF\(_4\) inert shell to reduce the aqueous quenching effect\(^ {39}\). The core-shell α-ErNPs were ~14 nm in size (Supplementary Fig. 1b), smaller than our previous β-phase ErNPs\(^ {34}\) (~18 nm). X-ray diffraction (XRD) (Supplementary Fig. 2) confirmed the α-phase fluorite structure (Joint Committee on Powder Diffraction Standards, JCPDS: 77-2043; space group: \(Fm\overline{3}m\)). The Raman scattering result (Fig. 1b) corresponded to cubic-phase NaLnF\(_4\) crystals, with phonon energies higher than those of β-phase\(^ {46}\).

The downconversion NIR-IIb luminescence of α-ErNPs was about 7.6 times brighter than that of β-phase ErNPs on 980 nm excitation (Fig. 1c). As shown in Fig. 1d, Yb\(^{3+}\) served as the sensitizer to harvest 980 nm photons. The activator Er\(^{3+}\) extracted the excitation energy from the Yb\(^{3+}\) through efficient cross-relaxation [(\(^{2F_{5/2}}\)Yb, \(^{4I_{13/2}}\)Er) \(\rightarrow\) \(^{2F_{7/2}}\)Yb, \(^{4I_{15/2}}\)Er], populating the \(^{4I_{13/2}}\) state of Er\(^{3+}\). Subsequent nonradiative relaxation of Er\(^{3+}\) \(^{4I_{13/2}}\) \(\rightarrow\) \(^{4I_{15/2}}\), led to a population of \(^{4I_{15/2}}\) state, generating the 1,550 nm downconversion NIR-IIb luminescence by radiative transition from \(^{4I_{15/2}}\) to the ground state \(^{4I_{11/2}}\). In the cubic α-phase, the \(^{4I_{13/2}}\) \(\rightarrow\) \(^{4I_{15/2}}\) nonradiative transition by multiphonon orbit-lattice relaxation was enhanced over the hexagonal β-phase due to higher phonon energies (see Fig. 1b for Raman data) and Ln\(^{3+}\)-F\(^{–}\) interaction strength in cubic-phase NaLnF\(_4\) host lattice\(^ {38}\). This led to a higher population of the \(^{1I_{13/2}}\) state and a 7.6-fold enhancement of the \(^{1I_{13/2}}\) \(\rightarrow\) \(^{1I_{15/2}}\) downconversion 1,550 nm luminescence over the brightest β-phase ErNPs (~18 nm) previously made\(^ {44}\).

**Zn\(^{2+}\) doping enhances downconversion ~1,550 nm luminescence brightness and lifetime.** The luminescence intensity of free Ln\(^{3+}\) ions involved 4f–4f electric-dipole transitions, which are parity-forbidden due to quantum mechanical selection rules. Such prohibition can be partially broken due to the mixing of opposite parity states when Ln\(^{3+}\) ions are embedded in cubic lattices; a lower-symmetry lattice can facilitate this mixing of opposite parity configurations, resulting in luminescence enhancement of the rare earth ions\(^ {45}\). To enhance the downconversion luminescence, we doped Zn\(^{2+}\) ions (0.9 Å) into the α-ErNPs to make NaYbF\(_4\)-2%Er,2%Ce@NaYF\(_4\) (Fig. 1a); 10% Zn (nominal doping concentration) was found to be the optimal doping concentration (see Supplementary Table 1 for practical concentration of Zn\(^{2+}\) ions), giving 1.5-times higher downconversion luminescence in NIR-IIb than un-doped α-ErNPs (Fig. 1c). A total ~11-fold enhancement was achieved over the brightest β-phase ErNPs\(^ {34}\).

Energy dispersive X-ray (EDX) mapping of Zn-doped α-ErNPs revealed uniform distribution of Zn\(^{2+}\) ions in the particles (Supplementary Fig. 3). XRD patterns of Zn-doped α-ErNPs (Fig. 1f) showed (111) and (220) diffraction peaks shifting to larger angle values as Zn concentration increased (Supplementary Fig. 4a). This suggested shrinking of the unit cell when substituting the Ln\(^{3+}\) ions with smaller Zn\(^{2+}\) ions in the crystal lattice\(^ {46}\). The doping of Zn\(^{2+}\) could be accompanied by generating an F\(^{–}\) vacancy, or occupying an Na\(^{+}\) ion site and creating another Na\(^{+}\) vacancy at the same time to maintain the charge balance (Supplementary Fig. 4b). As a result, the deformation of crystal lattice in the Zn-doped α-ErNPs caused distortion of local symmetry around Er\(^{3+}\) ions, favoring 4f–4f transitions and the 1,550 nm downconversion luminescence. Accordingly, the upconversion luminescence of Zn-doped ErNPs was also enhanced by 0.33 times (Supplementary Fig. 4c). The absolute quantum yield (emission range, 1,300–1,800 nm) of the Zn-doped ErNPs in aqueous solutions was estimated to be ~5% (Supplementary Fig. 4d) under the laser excitation of 100 mW cm\(^{–2}\). Further increasing the Zn-doping concentration might generate excessive distortions and defects, leading to luminescence quenching (Fig. 1e)\(^ {41}\).

Importantly, the bright downconversion emission intensity of Zn-doped cubic ErNPs was accompanied by a prolonged luminescence lifetime (Fig. 1g). For Zn-doped α-ErNPs, the 1,550 nm luminescence lifetime, that is, the radiative part of the lifetime of the \(^{1I_{13/2}}\) state of Er\(^{3+}\), was measured to be ~7.0 ms (in cyclohexane) by time-resolved detection of emission from the particles. Such long-lived millisecond NIR-IIb luminescence of the ErNPs was useful for lifetime-based, time-resolved luminescence imaging, which can

### Table 1 | Comparison of current NIR-IIb fluorescence/luminescence probes

| Source          | Required in vivo imaging exposure time (ms) | In vivo imaging frame rates (fps) | Excitation power (mW cm\(^{–2}\)) | Constituent elements | Excretion | Emission lifetime (μs) |
|-----------------|-------------------------------------------|---------------------------------|----------------------------------|----------------------|-----------|------------------------|
| PbS QDs\(^ {46}\) | 2-5                                       | 30-60                           | 60-70                            | Pb\(^{2+}\), Cd\(^{2+}\), S | ~76% within 28 d | 46         |
| InAs QDs\(^ {46}\) | 5,000                                     | 0.2                             | 60                               | In, As\(^{3+}\), Cd\(^{3+}\), Se, S | ~b       | ~0.12                  |
| CNTs\(^ {43}\) | 200                                       | 4.6                             | 150                              | C                    | Un-excretable | ~b         |
| β-ErNPs\(^ {43}\) | 50-1,000                                  | 3                               | 100-140                          | Na, F, Yb, Y, Er     | ~b       | ~b         |
| Ce-doped β-ErNPs\(^ {34}\) | 20                                       | 25                             | 150                              | Na, F, Yb, Y, Er, Ce | ~b       | ~b         |
| α-ErNPs (this work) | 1.11-23.3                                | 30-90                           | 15-100                           | Na, F, Yb, Y, Er, Ce, Zn | ~90% within 14 d | 4,300      |

*Class I toxic metals defined by United States Pharmacopeia. *No data provided. *Long emission lifetime can be utilized for multiplexed imaging.*
be utilized for multiplexed molecular imaging since luminescence with a longer lifetime can be easily distinguished from microsecond fluorescence of NIR-IIb PbS QDs. Note that Zn-doped α-ErNPs (named ErNPs in short) were used for all in vitro/vivo experiments throughout this work.

**Biocompatible, ultra-bright and excretable ErNPs for real-time NIR-IIb imaging.** The bright 1,550 nm luminescence of ErNPs is ideal for in vivo imaging; however, such imaging hinges on imparting stability and biocompatibility to the ErNPs in aqueous and biological media without aggregation and associated toxicity. We devised hydrophilic, cross-linked coating layers on ErNPs (Fig. 2a) such that the probability of hydrophilic coating detaching from the particles was zero (see Methods for detailed reactions). The cross-linked coating was composited of four polymer layers, including an inner-most layer of hydrolyzed poly(maleic anhydride-alt-1-octadecene) (PMH) rich in –COOH groups, followed by an eight-arm branched polyethylene glycol amine (8Arm-PEG-NH₂) layer, a poly(acrylic acid) (PAA) layer and an outmost layer of mixed methoxy polyethylene glycol amine (mPEG-NH₂) ~14 nm.

Fig. 1 | Ultra-bright ~1,550 nm NIR-IIb luminescence of Zn-doped α-ErNPs. a, Schematic design of core-shell Zn-doped α-ErNPs (left) and corresponding large-scale transmission electron microscopy image (right, scale bar, 100 nm). b, Raman spectra of cubic-phase α-ErNPs and our previously reported hexagonal-phase ErNPs. c, Upconversion and downconversion luminescence spectra of α-ErNPs and β-phase ErNPs. The insets show NIR-IIb luminescence images of these two nanoparticles in cyclohexane. d, Simplified energy-level diagrams depicting the energy transfer involved in α-ErNPs on 980 nm excitation. e, Downconversion luminescence spectra of Zn-doped α-ErNPs with different Zn²⁺ concentrations (0%, 5%, 7.5%, 10%, 12.5% and 15%, nominal doping concentration). The insets show NIR-IIb luminescence images of α-ErNPs with 10% and 0% Zn²⁺ doping. f, g, XRD patterns (f) and lifetime decays (g) of cubic-phase α-ErNPs (10% Zn doping), cubic-phase α-ErNPs (0% Zn doping) and β-phase ErNPs. Similar results for n > 3 independent experiments. a.u., arbitrary units; R/G, red-to-green ratio; r, lifetime; Eₚ, phonon energy.
and 8Arm-PEG-NH₂ (in ~5:1 ratio; Supplementary Fig. 5). The multi-arm PEG-NH₂ stars and long PAA chains were responsible for cross-linking the polymeric layers. Dynamic light scattering (DLS) measurement showed an average hydrated size of ~35.5 nm in aqueous solution (Fig. 2b). On top of the PAA layer, the mixed mPEG-NH₂ and 8Arm-PEG-NH₂ outmost layer rendered the nanoparticles hydrophilic and water soluble, while imparting amine groups to allow conjugation of biological ligands for molecular imaging (Fig. 2a).

With the hydrophilic functionalized ErNPs intravenously injected into mice, we performed noninvasive NIR-IIb imaging in real time (defined as ≥30 frames per second (fps)) to glean mouse cerebro-vasculatures (Supplementary Video 1) through intact scalp/skull (Supplementary Video 2). Owing to the highly bright ErNPs, real-time imaging was possible by using a low-power 970 nm light-emitting diode (LED) lamp (15 mW cm⁻²) as the excitation source. We clearly resolved cerebral blood flow variations over repeated cardiac cycles with high temporal and spatial resolution (Fig. 2c) using an excitation power ~10 times lower than laser-based excitation sources used for previous ErNPs.⁴⁴.⁴⁵ Ventricular ejection

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**Fig. 2 | Biocompatible, rapid-excretable α-ErNPs for real-time NIR-IIb imaging under low-power LED excitation.** a, Schematic illustration of the hydrophilic ErNPs with cross-linking polymeric layers and amine groups on the surface as conjugation sites. b, DLS spectra of hydrophilic ErNPs with polymeric cross-linked network. c, NIR-IIb cerebral vascular image (left) by intravenous injection of 200 μl ErNPs (40 mg ml⁻¹) and excitation by a 970 nm LED (30 fps). The luminescence intensity of an inferior cerebral vein was plotted as a function of time (middle), showing the cardiac cycles (upper right) with a heartbeat frequency of 3.67 Hz by fast Fourier transformation (FFT, lower right). Scale bar, 5 mm. d, The wide-field images show the ErNP luminescence signals in liver and spleen at 1 d and 14 d p.i. Scale bar, 1 cm. e, The excretion of ErNPs from mouse (n = 3) liver and spleen can be seen by plotting the signal intensity in these organs (normalized to liver signal observed at 1 d p.i.) as a function of time over 2 weeks. f, Bio-distribution of ErNPs in main organs and feces of ErNP-treated mice (n = 3) at 14 d p.i. All data are presented as means ± s.d. Similar results for n > 3 independent experiments. ID, injection dose.
phases (Fig. 2c) were resolved within 50 s post-injection (p.i.), compared with previous attempts in which cardiac cycle waveforms were only resolved ~5 s p.i. (refs. 24, 25). Fast Fourier transformation showed a clear heart beat frequency of 3.67 Hz, corresponding to the 276-ms interval between every two consecutive intensity spikes in Fig. 2c. Under a higher and safe excitation power, ultrafast NIR-IIb hindlimb vasculature imaging (Supplementary Fig. 7a; on 980 nm diode laser excitation, 100 mW cm$^{-2}$) was carried out at a high frame rate of 90 fps (Supplementary Video 3).

NIR-IIb imaging at ~1,600 nm afforded deeper tissue penetration depths at subcentimeter scale$^{26}$, allowing for dynamic, noninvasive imaging of major organs and vasculatures in vivo (Supplementary Fig. 7b,c) for investigating the bio-distribution, pharmacokinetics and excretion of ErNPs tail-vein injected into mice (BALB/c, n = 3). Luminescence signals in liver and spleen gradually increased within 24 h p.i., suggesting accumulation of ErNPs from blood circulation into these organs (Fig. 2d and Supplementary Fig. 8c,d).

Importantly, we observed strong luminescence signals of ErNPs in the feces of mice over time (Supplementary Fig. 9b), indicating a biliary excretion pathway of ErNPs. Correspondingly, the signal intensity in the main organs of mice, including liver and spleen, kept decreasing within the monitored time period of 2 weeks (Figs. 2d,c). All of the feces excreted from mice were collected and measured by inductively coupled plasma optical emission spectrometry (ICP-OES), revealing that ~90% of injected α-ErNPs were excreted from the body in 2 weeks (Fig. 2f). This rapid, high-degree excretion of ErNPs could facilitate clinical translation of the nanoparticles (see Supplementary Fig. 10 for histological studies).

ErNPs-anti PD-L1 mAb for in vivo molecular imaging and immunotherapy. In vivo fluorescence imaging under excitation <1,000 nm and emission in the ~1,600 nm NIR-IIb window can benefit from greatly reduced photon scattering and an unusually large Stokes shift affording diminished autofluorescence/background interference$^{27,28}$. To investigate ErNPs as NIR-IIb probes targeting the PD-1/PD-L1 immune checkpoint, we conjugated anti-PD-L1 mAb (atezolizumab) to ErNPs (ErNPs-aPDL1; Supplementary Fig. 11c) with amine surface groups through 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) chemistry (see Methods). Successful conjugation of anti-PD-L1 mAb to ErNPs was first confirmed by live cell imaging of CT-26 colon cancer cells (PD-L1 over-expressed) and 4T1 murine breast cancer cells (PD-L1 low-expressed) in vitro (Supplementary Fig. 12a).

In vivo molecular imaging of PD-L1 was done after intravenous injection of ErNPs-aPDL1 into the tail vein of BALB/c mice (n = 5) bearing subcutaneous (s.c.) xenograft CT-26 tumors using a wide-field setup equipped with a two-dimensional (2D) InGaAs camera, by collecting emission photons at 1,500–1,700 nm under 980 nm excitation at a power density of 50 mW cm$^{-2}$ (exposure time ~5 s; Fig. 3a and Supplementary Fig. 13). About 200 μl ErNPs-aPDL1 solution was intravenously injected, comprising 250 μg anti-PD-L1 mAb (12.5 mg kg$^{-1}$) conjugated to ~2 mg ErNPs. The blood circulation time of $t_{1/2}$ ~ 5.5 h for ErNPs-aPDL1 in mice (Supplementary Fig. 14) was slightly longer than that of free ErNPs, $t_{1/2}$ ~ 5.2 h (Supplementary Fig. 8b). The luminescence intensity inside CT-26 tumor increased steadily post injection, indicating extravasation of ErNPs-aPDL1 from blood circulation and enrichment in tumor.

The ErNPs-aPDL1 nanoparticles primarily accumulated in tumor, liver, spleen and intestine at 24 h p.i., with little retention in other organs including heart, lung, kidney and brain (Supplementary Fig. 15). The NIR-IIb T/NNT signal ratio increased sharply during the initial hours post injection, and peaked at T/NNT ~ 42.2 ± 3.7 at 24 h p.i. (Fig. 3b). This T/NNT ratio was remarkably high compared with previous fluorescence-based molecular tumor imaging using fluorophores emitting in the entire optical range (T/NNT ratios are typically ~2–4 in the 700–900 nm NIR-I range$^{29,30}$, and ~8–12 in the ~1,100 nm NIR-IIa range$^{25,31}$). Without anti-PD-L1 mAb, the nontargeted free ErNPs showed much weaker signal in the CT-26 tumor (mice n = 3), with a lower peak T/NNT ~ 11.0 ± 1.1 at 24 h p.i. (Fig. 3b) due to passive accumulation through the enhanced permeability and retention effect. To further confirm specific in vivo targeting of ErNPs-aPDL1 to CT-26 tumor, we performed free anti-PD-L1 antibody blocking experiments (Supplementary Fig. 16) and observed much lower peak T/NNT ~ 7.15 ± 0.55 at 24 h p.i., confirming highly specific ErNPs-aPDL1 targeting of CT-26 tumor. Importantly, the signal intensity of ErNPs-aPDL1 in the tumor mice liver and spleen kept decreasing within the monitored time period of 2 weeks (Supplementary Fig. 17a), similar to the free ErNPs in the healthy mice, indicating fast excretion of intravenously administrated ErNPs-aPDL1 from tumor mice. The aspartate/alanine aminotransferase measurements also demonstrated no discernable toxicity of ErNPs-aPDL1 in vivo (Supplementary Fig. 17b).

Next, we imaged BALB/c mice bearing s.c. murine breast 4T1 tumors (mice n = 3) with low PD-L1 expression using the same intravenously injected ErNPs-aPDL1 (Fig. 3a). The peak T/NNT ratio reached 12.3 ± 1.2 at 24 h p.i. (Fig. 3b), much lower than T/NNT ~ 42 for ErNPs-aPDL1-injected mice bearing CT-26 tumor, which was consistent with ex vivo tumor PD-L1 expression analysis based on flow cytometry (Supplementary Fig. 12b). The high T/NNT ratio of >40 with the ErNPs-aPDL1 probe for CT-26 tumor resulted from the combined effects of the high specificity of ErNPs-aPDL1 toward the PD-1 immune checkpoint, the vastly boosted luminescence brightness of Zn-doped cubic-phase α-ErNP NIR-IIb probes and the near-zero autofluorescence of biological tissues under a large ~600 nm Stokes shift from 980 nm excitation to ~1,600 nm emission.

We reduced the amount of anti-PD-L1 mAb by an order of magnitude (from 250 μg to 20 μg mAb), conjugated to the same 2 mg of ErNPs (ErNPs-1/10$^4$ aPDL1) for each ~200 μl injection (antibody dose ~1 mg kg$^{-1}$) and still obtained excellent results for molecular imaging of PD-L1 in tumors (mice n = 3; Fig. 3c). The peak T/NNT ratio was 31.1 ± 2.1 at 24 h p.i. for CT-26 tumors on BALB/c mice (Fig. 3b). Molecular imaging using low doses of PD-L1 antibody (therapeutic doses were up to ~10–20 mg kg$^{-1}$ in the clinic)$^{44,45}$ would be preferred for therapeutic response assessment due to lower cost and, more importantly, reduced potential side-effect risks$^{44,46}$. When zooming in to the tumor (Fig. 3d), we imaged noninvasively through the skin to glean ErNPs-aPDL1 NIR-IIb signals circulating in the tortuous tumor vasculature at 5 min p.i., resolving vessels in CT-26 tumor down to micrometer spatial resolution. Leakage of ErNPs-aPDL1 from blood vessels into tumor tissue was also observed (Fig. 3d), indicating the start of ErNPs-aPDL1 extravasation and binding to cancer cells within the tumor. Interestingly, we observed that anti-PD-L1 mAbs conjugated to ErNP surfaces retained the PD-1/PD-L1 checkpoint blocking capability of free antibodies for therapeutic cancer treatment (see Fig. 3e,f and Supplementary Figs. 18–20), suggesting ErNPs-aPDL1 as a theranostic agent for both molecular PD-L1 imaging and immunotherapy.

Two-plex in vivo molecular imaging at ~1,600 nm for PD-L1 markers and CD8$^+$ cytotoxic T lymphocytes (CTLs). Activated CD8$^+$ CTLs in response to antibody treatment play critical roles in immune checkpoint blockade therapy by infiltrating into tumor and inducing apoptosis of cancer cells$^{47}$. In vivo imaging and evaluating the bio-distribution of CTLs in relation to PD-L1 expression could provide insights into activation and migration of T cells in response to antibody immunotherapeutic treatment. To this end, we developed a two-plex NIR-IIb molecular imaging approach to simultaneously mapping out CD8$^+$ CTLs and PD-L1 in vivo.

Exploiting the long-lived ~1,550 nm luminescence (4.3 ms in aqueous solution) of ErNPs, we devised time-resolved imaging to
differentiate ErNP luminescence from short-lived fluorescence of QDs for two-plex molecular imaging in the same 1,500–1,700 nm NIR-IIb window (Fig. 4a,b). Our recently developed PbS QDs exhibited shorter-lived 1,600 nm fluorescence with a lifetime of ~46 μs (Fig. 4c) and were combined with ErNPs as the second NIR-IIb imaging probe. For imaging PbS emission without ErNP luminescence (Fig. 4a), we used 808 nm continuous-wave (CW) laser for excitation that was absorbed only by PbS QDs (Fig. 4d,e) and not by ErNPs (absorption in ~900–1,000 nm range; Fig. 4d). For imaging ErNP luminescence without PbS emission (Fig. 4b), we used a 980 nm pulse (pulse duration ~1 ms) for excitation and set a delay time of 1 ms to the InGaAs CCD (charge-coupled device) camera through computer control to allow full fading of the short-lived fluorescence (46 μs) of PbS QDs before recording. Subsequent recording with the camera collected the 1,550 nm long-lived luminescence (4.3 ms) signals from ErNPs, affording a distinct ErNP detecting channel (Fig. 4b) without any PbS fluorescence.

To track the CD8+ T cells in vivo, we conjugated anti-CD8α mAb (clone 2.43) to PbS QDs (PbS-aCD8) for targeting CD8+ CTLs. BALB/c mice (n = 3) bearing CT-26 s.c. tumors were intravenously injected with a mixture of ErNPs-aPDL1 and PbS-aCD8 (Fig. 4f).

We first performed in vivo 360° rotation imaging of mouse whole-body to glean the bio-distribution of ErNPs-aPDL1 and PbS-aCD8 in tumor, liver, spleen and other organs at 24 h p.i. (Fig. 4g and Supplementary Video 4). A strong signal in CT-26 tumor appeared in the ErNP channel (green, Fig. 4f,g) due to targeting of ErNPs-aPDL1. Meanwhile, we also observed accumulation of PbS-aCD8-labeled CD8+ T cells within the tumor in the PbS channel (red, Fig. 4f,g). When zoomed in to the tumor for high-magnification imaging, the shape of the CT-26 tumor was clearly visualized in the ErNP channel with a relatively even signal distribution through the tumor (Supplementary Fig. 21). However, for the PbS-aCD8 channel, the signals were higher around the periphery of the tumor and extending inward (see Fig. 4f and Supplementary Figs. 21b and 22b), a result consistent with ex vivo analysis by flow cytometry (Supplementary Fig. 22c–e). This suggested the infiltration of immune-competent CD8+ CTLs starting primarily from the periphery region of the CT-26 tumor, which was limited by vascular hyper-permeability and shortage of functional lymphatic vessels inside solid tumors48.
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injected with ErNPs-aPDL1 and free anti-CD8α mAb (same dose as for the antibody on the PbS-aCD8). At 48 h p.i., the ErNPs-aPDL1 accumulated in CT-26 tumors with a consistently high T/NT ratio of ~40.1 (Fig. 5b). Then, PbS-aCD8 was intravenously injected at 48 h post co-injection of ErNPs-aPDL1 and free anti-CD8 mAb. A strong signal from PbS-aCD8 was only detected in the liver and spleen at 24 h after the second injection (Fig. 5a,b), with little signal from PbS-aCD8 in the CT-26 tumor. This suggested blocking of CD8α receptors on the T cells from the first injection of free anti-CD8 mAb, validating the specificity of PbS-aCD8 toward CD8+ T cells. The PbS-aCD8 nanoparticles (in healthy mice) primarily accumulated in liver, spleen and intestine at 24 h p.i., with little...
Fig. 5 | In vivo two-plex NIR-IIb molecular imaging of PDL1 and CD8+ CTLs for assessing immune activation and responses. a, Schematic of the CD8α blocking experiment. A CT-26 tumor mouse was first intravenously injected with mixed ErNPs-aPDL1 and free anti-CD8α mAb (left wide-field image, 48 h post first injection), followed by a second intravenous injection of PbS-aCD8 (right wide-field image, 24 h post second injection). b, The signals of tumor and spleen to background ratios were plotted to reveal the bio-distributions of ErNPs-aPDL1 (left) and PbS-aCD8 (right) with CD8 and without blocking (mice = 3 for each group). c–e, Wide-field images of mice from different directions (left arm, belly and right arm) reveal the in vivo bio-distributions and spleen to background ratios were plotted to reveal the bio-distributions of ErNPs-aPDL1 (left) and PbS-aCD8 (right) with CD8 and with no blocking (mice = 3 for each group). f, Corresponding (T/spleen)_CD8 ratios in these mice (n = 3 for each group). Green color, ErNPs-aPDL1; red color, PbS-aCD8, for all of these images. All data are presented as means ± s.d. All scale bars, 5 mm. Data in b and f are presented as box plots (center line, median; box limits, upper and lower quartiles; whiskers, 1.5 interquartile range; points, outliers). ****P = 0.0001, t = 14.4971, degrees of freedom (df) = 4; *****P = 0.0001, t = 15.0393, df = 4.

We observed that for CT-26 tumors on mice that received both PbS-aCD8 and anti-PD-L1 treatment (with ErNPs-aPDL1), accumulation of PbS-aCD8 in the tumor was high but weak in main organs, including liver and spleen (Fig. 5c), suggesting that the clonally expanded activated effector T cells in spleen were trafficking to and infiltrating the tumor bed, and killing the cancer cells (Fig. 3e)43. Contrary to this immune-activated group with good response to immunotherapy, mice (n = 3) bearing CT-26 tumor injected with PbS-aCD8 alone without any PD-L1 antibody blockade treatment (Fig. 5d and Supplementary Video 5) showed prominent PbS-aCD8 signals in the liver and spleen (spleen signal/background ~19.3 versus ~2.1 in the immune-activated case; Supplementary Fig. 24a,b), with a lower PbS-aCD8 signal T/NT ratio of ~6.5 (Supplementary Fig. 24b) than immune-activated mice treated by PD-L1 blockade therapy (PbS-aCD8 signal T/NT ratio ~ 10.2; Supplementary Fig. 24a). This was consistent with ex vivo flow cytometry results (Supplementary Fig. 25). Further, for 4T1 tumor-bearing mice (n = 3) injected with ErNPs-aPDL1 and PbS-aCD8 (Fig. 5e and Supplementary Video 6) and 4T1 tumor-bearing mice (n = 3) injected with free ErNPs and PbS-aCD8 (Supplementary Fig. 26), no therapeutic response/tumor regression was observed and CD8+ T cells were primarily in the liver and spleen, similar to the case for mice bearing CT-26 tumor without any anti-PD-L1 treatment or immune activation.

Analysis of in vivo rotating NIR-IIb images recorded at various angles allowed plotting of CD8+ T-cell signal ratio between...
tumor and spleen (T/spleen) for analysis of immune system activation and responses to immunotherapy (Fig. 5f). We found that the (T/spleen)_CD8 ratio of ~5.3 in immune-activated, ErNPs-aPD-L1-treated, CT-26-bearing mice was >15 times higher than the (T/spleen)_CD8 ratio for CT-26 mice without anti-PD-L1 treatment ((T/spleen)_CD8 ratio ~0.31), and >30 times higher than the (T/spleen)_CD8 ratio for 4T1 mice treated by anti-PD-L1 but without responses ((T/spleen)_CD8 ratio ~0.14). The result suggested that a robust immune response to antibody treatment involved a large proportion of CTLs being activated and migrating from lymphoid organs rich in immune cells (for example, spleen) into tumor to eradicate cancer cells (see Supplementary Fig. 18 for therapeutic effect). Thus, noninvasive in vivo two-plex NIR-II imaging to glean tumor PD-L1 level and CD8+ T-cell distribution in tumor versus spleen could provide useful parameters for assessing response to immunotherapy. We note that the bio-distribution and expression level of a single biomarker can be properly evaluated based on NIR-II signals of the nanoparticles; however, the parallel quantification of two biomarkers based on the duplex signals from two nanoparticles could be affected by the difference in the nanoparticle extravasation into the tumor and may be subject to less accuracy.

**Discussion**

While upconversion nanoparticles have been investigated for decades, the investigation of downconversion luminescence of erbium-doped ErNPs at ~1,600 nm for in vivo NIR-II imaging with subcentimeter tissue penetration and micrometer image resolution is more recent12,13,14. This work developed ultra-bright cubic-phase ErNPs for noninvasive NIR-II imaging of biomarkers important to immunotherapy. These ErNPs are of low toxicity15,16, without toxic elements such as Pb, Cd and Hg. Also important to biocompatibility is the hydrophilic coating on ErNPs formed by cross-linking polymer layers, forming a capping network to prevent detachment of the coating. The ~90% biliary excretion of intravenously administered ErNPs from mice in 2 weeks alleviates concerns of long-term toxicity due to retention. It is therefore potentially possible to combine with other novel probes to image multiple immune targets in the same NIR-II window simultaneously and with more accuracy for immunotherapeutic response.

Combining ~1,600-nm-emitting ErNPs and PbS QDs, our two-plex molecular imaging revealed heterogeneous bio-distributions of PD-L1 and CD8+ CTLs. We observed high T/NT ratios of PD-L1 in CT-26 colon tumors with favorable therapeutic responses to anti-PD-L1 therapy, and much lower T/NT ratios of PD-L1 in nonresponding 4T1 tumors. For mice bearing CT-26 tumors without antibody therapy or nonresponding 4T1 tumors with antibody treatment, (T/spleen)_CD8 ratio is low with a high proportion of CD8+ immune cells in lymphoid tissues. For CT-26 tumors treated with anti-PD-L1 mAb, potent antitumor immunity was generated accompanied by the observation of high (T/spleen)_CD8 ratios, indicating the vast majority of activated CTLs accumulating in tumor to recognize and eradicate the tumor. A low (T/spleen)_CD8 ratio observed under co-administration of anti-PD-L1 and a CD8 probe could be an indicator of ineffective blockade of PD-1/PD-L1 signaling-mediated tumor immunity dysfunction. Such in vivo noninvasive bio-distribution assessments of tumor cells and immune cells in the whole body could complement ex vivo biopsy-based diagnostic methods. Thus, it is possible to develop a specific scoring algorithm combining in vivo tumor PD-L1 expression level and immune cell status to provide a more accurate prediction for immunotherapeutic response.

**Online content**

Any methods, additional references, Nature Research reporting summaries, source data, statements of code and data availability and associated accession codes are available at https://doi.org/10.1038/s41587-019-0262-4.

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Author contributions

H. Dai and Y.Z. conceived and designed the experiments. Y.Z., Z.M., F.W., X.W., Y.Y., Y.L., Z.M. and L.J. performed the experiments. H. Dai and Y.Z. conceived and designed the experiments. Y.Z., Z.M., F.W., X.W., Y.Y., Y.L., Z.M., H. Du, M.Z., Q.C., S.Z., Q.S., H.W., Y.T., Q.L., W.W., K.C.G. and H. Dai analyzed the data and wrote the manuscript. All authors discussed the results and commented on the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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Methods

Reagents. Rare-earth(III) acetate hydrate (RE: Yb, Er, Ce), zinc acetate, sodium trifluorooacetate, ODE, sodium hydroxide, ammonium fluoride, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), PAA (average molecular weight: 10,000), PMH (average molecular weight: 30,000), 4-morpholineneethanesulfonic acid (MES), 4-(dimethylamino)pyridine (DMAP), cyclohexane, chloroform, 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris-base), fetal bovine serum, PBS and RPMI-1640 medium were purchased from Sigma-Aldrich and used without further purification. Tritium(I) oxides and trifluoroacetic acid (99%) were purchased from Alfa Aesar and were used as received. mPEG-NH₂ (average molecular weight: 5,000) was purchased from Laysan-Bio. 8Arm-PEG-NH₂ (average molecular weight: 40,000) was purchased from Advanced Biochemicals. Anti-PD-L1 mAb (Atezolizumab, Clone SP142) was purchased from Selleckchem. Anti-CD8α mAb (Clone 2.43) was purchased from Bio X Cell. EZ-Link Sulfo-NHS-LC-Biotin and DRAQ5 Fluorescent Probe (15 mmol) and ODE (37.5 mmol) in a two-necked flask at room temperature. The solution was heated to 150 °C for 30 min under argon gas flow with vigorous magnetic stirring then heated to 120 °C under vacuum for 30 min to remove water and ensure the reagents and temperatures to afford the final PMH. The supernate was washed by centrifugal filter (100kDa) four times to remove excess PEG. The final ErNPs (~16mg) with cross-linked polymer network were dispersed in 1.6ml 1xPBS solution at 4°C for long-term storage.

Conjugation of anti-PD-L1 mAb on ErNPs (ErNPs-sPD-L1). The above ErNPs were used as-dispersed polymer network dispersed in 1xPBS solution (200μl, containing 2 mg ErNPs), anti-PD-L1 mAb (250μg), EDC (1.5 mg) and 800 μl MES solution (10 mM, pH = 8.5) were mixed and shaken for 3h. The solution was centrifuged at 4,400 r.p.m. for 30 min to remove potential large floccules. The supernate was washed by centrifugal filter (100kDa) twice, and then dispersed in 200 μl 1xPBS solution (for one injection).

Conjugation of anti-CD8α mAb on PbS QDs (PbS-cCD8). The water-dispersible PbS QDs were prepared by our previously developed method [6]. PbS QDs (0.25 mg) in 50 μl 1xPBS solution, EDC (0.75 mg) and streptavidin (50μg) were added to 500 μl MES solution (10mM, pH = 6.5). The solution was stirred at room temperature for 3 h, and then washed by centrifugal filter (100kDa) four times to obtain anti-CD8α mAb-biotin (dispersed in 100 μl 1xPBS solution). Meanwhile, anti-CD8α mAb (150 μg) was dissolved in 300 μl 1xPBS solution. Then, 6 μl EZ-Link Sulfo-NHS-LC-Biotin (1.7 mg ml⁻¹ in dimethylsulfoxide) was added. The solution was stirred at room temperature for 1.5 h, and then washed by centrifugal filter (100kDa) four times to obtain anti-CD8α mAb-biotin (dispersed in 100 μl 1xPBS solution). The PD-L1 and streptavidin were mixed, and then stirred at room temperature for 2 h. The solution was washed by centrifugal filter (100kDa) twice, and then dispersed in 200 μl 1xPBS solution (for one injection).

Mouse handling. All vertebrate animal experiments were performed under the approval of the Stanford University’s Administrative Panel on Laboratory Animal Care. All experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. BALB/c female mice were purchased from Charles River. The surrounding relative humidity level was 55–65% and the temperature was ~25 °C. The hair of mice was carefully removed using a hair removal tool avoiding wounds to the skin. Before NIR-Ib in vivo imaging, a rodent anesthesia machine with 2 l min⁻¹ O₂ gas flow mixed with 2.5% isoflurane was used to anesthetize the mice. During the dynamic imaging, the mouse was kept anesthetized by a nose cone delivering 2 l min⁻¹ O₂ gas mixed with 2.5% isoflurane. For in vivo dynamic imaging (real-time and ultrafast), a 1xPBS solution of ErNPs (40 mg ml⁻¹, 200 μl) was injected. Mice were randomly selected from cages for all experiments. All groups within the study contained n = 3–5 mice. CT-26 tumors were generated by s.c. injection of 2×10⁶ CT-26 cells in 50 μl PBS. 4T1 tumors were generated by s.c. injection of 2×10⁴ 4T1 cells in 50 μl PBS. The mice were used for imaging and treatment when the volume of the tumor reached 20 mm³ (about 3 d post inoculation).

Dynamic fluorescence imaging in the NIR-Ib window. A water-cooled, 640 × 512-pixel 2D InGaAs array (detection range: 400–1,700 nm; Raptor Photonics) was used to carry out in vivo imaging of mouse brain and hindlimb. For LED-excited real-time in vivo imaging of mouse cerebral vessels, the excitation light was provided by a 970 nm LED lamp (100 W) equipped with an aluminum heatsink cooling fan and a 60-degree lens. The excitation power density at the imaging plane was 15 mW cm⁻². The emitted luminescence was allowed to pass through a 1,100 nm and a 1,500 nm long-pass filter (Thorlabs) to ensure that the NIR-Ib images were taken in the NIR-Ib region of 1.500–1.700 nm. The upper bound at 1,700 nm was determined by the sensitivity profile of the InGaAs detector. A lens pair consisting of two achromats (200 mm and 100 mm; Thorlabs) was used to focus the image onto the detector with a field of view of 25 × 25 mm². The exposure time for each image acquisition was 23.3 ms, while the overheat time of the camera was 10 ms. Therefore, the frame rate we used for real-time imaging was 44 fps (for one injection). For ultrafast in vivo imaging of mouse hindlimb vessels, the excitation light was provided by a 980 nm CW laser coupled to a collimator (F240LSA-980; Thorlabs). The excitation power density at the imaging plane was 100 mW cm⁻². The imaging setup is the same as above. The exposure time for each image acquisition was 1.11 ms; thus, the frame rate we used for ultrafast imaging was 1/(23.3 ms × 10 ms) = 90 Hz.

Optical emission spectrometry (ICP-OES) measurement. Tissue and cells samples were digested in concentrated nitric acid (68%) overnight, followed by heating in concentrated nitric acid and hydrogen peroxide for 2 h using a hot plate, to obtain clear solutions. The Yb contents in the solutions were determined by ICP-OES (Thermo Scientific ICAP 6300 Duo View Spectrometer), and the Yb contents in the mouse tissues and feces were subsequently calculated.

Lifetimal measurement. The lifetime measurement was performed on a microscope mounted with a ×100 (NA = 0.8) objective to focus the 980 nm laser.
The 980 nm excitation and fluorescence signal were separated by a dichroic mirror with cut-off wavelength of 980 nm. After being collected by the x100 objective, the fluorescence signal was focused by a 200 mm tube lens, filtered by a 1,500 nm long-pass filter and transmitted to an InGaAs photomultiplier tube (PMT, H12397-75, Hamamatsu) through a multimode fiber. Fluorescence signal recording was realized by synchronously controlling the PMT and a 980 nm laser using LabVIEW software through a data acquisition card (NI USB-6210). The fluorescence signal was immediately recorded by the PMT 1 ms after excitation was turned off.

Two-plex molecular imaging in the NIR-Ib window. A water-cooled, 640 × 512-pixel 2D InGaAs array (detecting range: 400–1,700 nm; Nikonix 640, Raptor Photonics) was used to carry out two-plex imaging of tumor-bearing mice\(^1\). A lens pair consisting of two achromats (200 mm and 75 mm; Thorlabs) was used to focus the image onto the detector with a field of view of 65 × 50 mm\(^2\). For the P68 channel, the excitation light was provided by an 808 nm diode laser with the CW model and filtered by two short-pass filters with cut-off wavelengths at 850 nm and 1,000 nm. The excitation power density at the imaging plane was 50 mW cm\(^{-2}\). The emitted luminescence was allowed to pass through a 1,100 nm long-pass filter (Thorlabs) to ensure that the NIR images were taken in the NIR-IIb region of 1,500–1,700 nm.

For the ErNP channel, the excitation light was provided by a 980 nm diode laser with the modulation model to generate the laser pulse (duration: 1 ms, peak power density: 50 mW cm\(^{-2}\)). In the lifetime-resolved imaging system, synchronous control of the camera and laser was realized using LabVIEW software through a data acquisition card (NI USB-6210). First, a 1 ms laser pulse was triggered to excite the nanoparticles, before waiting for 1 ms; then, a luminescence image with exposure time of 5 ms was captured by the camera. The emitted luminescence was allowed to pass through a 1,100 nm and a 1,500 nm long-pass filter (Thorlabs) to ensure that the NIR images were taken in the NIR-Ib region of 1,500–1,700 nm. For high-magnification two-plex molecular imaging in the NIR-Ib window, the lens pair was changed to an optical lens system (Optem Zoom 70XL) to provide a field of view of 1.2 x 1 mm\(^2\).

High-magnification molecular imaging in the NIR-Ib window. A water-cooled, 640 × 512-pixel 2D InGaAs array (detecting range: 400–1,700 nm; Raptor Photonics) equipped with an optical lens system (Optem Zoom 70XL) was used to carry out high-magnification molecular imaging. The emitted NIR-Ib signal was allowed to pass through a 1,100 nm and a 1,500 nm long-pass filter (Thorlabs) to ensure that the NIR images were taken in the NIR-Ib region of 1,500–1,700 nm. The high-magnification molecular imaging with the maximum magnification provided a field view of 1.2 x 1 mm\(^2\).

In vitro profiling of PD-L1. CT-26 cancer cells (high PD-L1 expression), 4T1 cancer cells (low PD-L1 expression) and HEK293 human cells (no PD-L1 expression) were seeded at 1 × 10\(^5\) cells per well in chambered slides as three groups, with 400 μl RPMI-1640 cell medium. After incubation in a humidified atmosphere of 5% CO\(_2\) at 37°C for 12 h, the cells were washed twice with 400 μl cold 1xPBS buffer (4°C). ErNPs–aPDL1 was subsequently added into each group, respectively, at the same dose (1 mg ml\(^{-1}\), 200 μl) and interacted with the cells for 30 min at 4°C, following by washing twice with cold PBS buffer. The cells were then fixed with 4% paraformaldehyde for 30 min at room temperature and nuclei were stained using 100 μl DRAQ5 (1 x 10\(^{-5}\) M). At the end, the fixed cells were imaged under a home-built NIR-I microscope with both NIR-I and NIR-II channels.

Flow cytometry. For collecting cells from the CT-26 and 4T1 tumors, the tumors were dissected out and cut into small pieces. The tumor tissues were dissociated using the Miltenyi mouse tumor dissociation kit according to the manufacturer’s instructions. The preparations were passed through a 70 μm cell strainer and washed thoroughly with PBS buffer supplemented with 0.5% BSA (PBS-BSA buffer). Finally, the cells were resuspended in PBS-BSA buffer and stained with 10 μg mAb (KT15)-FITC (for CD8\(^+\) T cells) or CD27 (PD-L1, B7-H1) mAb (MIH5), Super Bright 780, eBioscience (for PD-L1 expression levels) for flow cytometry on Beckman Coulter CytoFLEX flow cytometer and analyzed using FlowJo. For collecting cells from spleen tissues, the spleen was minced finely with scissors and scalpel, and mashed on a 70 μm cell strainer to create a single-cell suspension. The red blood cells were lysed by incubating cells in ACK lysis buffer for 10 min and mononuclear cells were washed thoroughly with PBS-BSA buffer. Finally, the cells were resuspended in PBS-BSA buffer and stained with CD8α mAb (KT15)-FITC (for CD8\(^+\) T cells) for flow cytometry on Beckman Coulter CytoFLEX flow cytometer and analyzed using FlowJo.

Absolute quantum yield measurement. The absolute quantum yield measurement was performed by following a literature protocol\(^4\) with slight modifications. The NIR-Ib probes were excited by a 980 nm laser (for Zn-doped α-ErNPs and β-ErNPs) or an 808 nm laser (for PbS QDs). The laser power density was 100 mW cm\(^{-2}\). An integrating sphere (Thorlabs; IS200) was used to spread the incoming light by multiple reflections over the entire sphere surface. The outcome lights, including laser excitation light and NIR fluorescence of NIR-Ib probes, were taken using a home-built NIR spectrograph with a spectrometer (Acton SP2300i) equipped with a liquid-nitrogen-cooled InGaAs linear array detector (Princeton OMA-V). Note that the excitation light has to be attenuated by a neutral density filter (Newport; optical density = 2.0) before being detected. According to the equation (1), the absolute quantum yield of NIR-Ib probes was calculated:

\[
QY = \frac{\text{photons emitted}}{\text{photons absorbed}} = \frac{E_{\text{sample}}}{L_{\text{[blank]}} - L_{\text{[sample]}}}
\]

where QY is the quantum yield, \(E_{\text{sample}}\) is the emission intensity, and \(L_{\text{[blank]}}\) and \(L_{\text{[sample]}}\) are the intensities of the excitation light in the presence of the water and the NIR-Ib probe sample, respectively.

Statistics and data analysis. Data analysis was performed in Origin 9.0.0. Means ± s.d. were calculated by Origin 9.0.0. In Figs. 3b and 5b, background was measured from a randomly selected area without vasculature or tumor. T/NT is the ratio of fluorescence signals in the whole tumor area over the randomly selected background. For each representative experimental result, the number of successful independent experiments performed is indicated in the corresponding figure legend.

Characterization. Transmission electron microscopy images were taken with a JEOL-2100F transmission electron microscope (JEOL) operating at 200 kV. EDX mapping images were obtained on a JEOL-2100F equipped with an EDX analyzer. ICP-OES was performed on a Thermo Scientific ICAP 6300 Duo View Spectrometer. DLS and acta potential measurements were performed on a Malvern Zetasizer Nano ZS90. XRD patterns were recorded on a Philips Xpert PRO MPD X-ray diffractometer operated at 35 kV and 45 mA with Cu-Kα radiation. The upconversion luminescent properties were studied using a Horiba Jobin Yvon FluoroLog3 spectrometer equipped with a 980 nm diode laser as excitation. The downconversion luminescent properties were studied using an Acton SP2300i spectrometer equipped with an InGaAs linear array detector (Princeton OMA-V) and using a 980 nm diode laser as excitation. NIR fluorescence images of the downconversion emission were obtained using 2D InGaAs array (Ninox 640, Raptor Photonics) with 640 × 512 pixels using a 980 nm diode laser as excitation. Raman spectra were obtained with polarized incident laser light (wavelength \(\lambda = 532\) nm) on a Jobin Yvon T64000.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
The data that support the findings of this study are available from the corresponding author upon reasonable request.

References
51. Mai, H.-X. et al. High-quality sodium rare-earth fluoride nanocrystals: controlled synthesis and optical properties. J. Am. Chem. Soc. 128, 6426–6436 (2006).
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54. Boyer, J.-C. & van Veggel, F. C. J. M. Absolute quantum yield measurements of colloidal NaYF\(_4\) Er\(^{3+}\), Yb\(^{3+}\) upconverting nanoparticles. Nanoscale 2, 1417–1419 (2010).
Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Research policies, see Authors & Referees and the Editorial Policy Checklist.

Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

| Item | Confirmation |
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| n/a  | Confirmed    |
| 1. | The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement |
| 2. | A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly |
| 3. | The statistical test(s) used AND whether they are one- or two-sided |
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| 5. | A description of all covariates tested |
| 6. | A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons |
| 7. | A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals) |
| 8. | For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted Give P values as exact values whenever suitable. |
| 9. | For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings |
| 10. | For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes |
| 11. | Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated |

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection

- LabView2009 was used to synchronously control the laser pulse and image record.

Data analysis

- ImageJ 1.52a was used to process the imaging data obtained by our NIR imaging system;
- Jade5.5.26 was used to process and analyze the original XRD data;
- Origin9.0.0 was used to draw the curves and analyze the standard deviation;
- FlowJo10 was used to process and analyze the original flow cytometry data;
- Zetasizer Software7.12 was used to process and analyze the original DLS data.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

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All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
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All summary or representative data generated and supporting the findings of this study are available within the paper. Raw data that support the findings of this study are available upon request.
Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size: The sample size was not calculated before experiments. In these experiments, an appropriate sample size (3-5 mice) is determined by the reproducibility of molecular imaging.

Data exclusions: No data were excluded from the analyses.

Replication: All attempts at replication were successful.

Randomization: Mice were randomly selected from cages for all experiments, and then divided into groups for further treatments.

Blinding: Not applicable, since no group was allocated.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

| Materials & experimental systems | Methods |
|----------------------------------|---------|
| n/a | n/a |
| [x] Antibodies | [x] ChiP-seq |
| [x] Eukaryotic cell lines | [ ] Flow cytometry |
| [x] Palaeontology | [x] MRI-based neuroimaging |
| [x] Animals and other organisms | |
| [ ] Human research participants | |
| [ ] Clinical data | |

Antibodies

Antibodies used:

- anti-PD-L1 (Selleckchem, Cat#: A2004, clone SP142; Lot #: 02; Dilution: 150 µg / 200 µL);
- anti-CD8a (Bio X Cell; Cat #: BE0051; Clone 2.43; Lot #: 7051182; Dilution: 150 µg / 200 µL);
- CD274 (PD-L1, B7-H1) Monoclonal Antibody (MIH5), Super Bright 780, eBioscience™ (ThermoFisher; Cat #: 78-5982-82; Clone MIH5; Lot #: 1955731; Dilution: 1 to 200);
- CD8 alpha Monoclonal Antibody (KT15), FITC (ThermoFisher; Cat #: MAS-16759; Clone KT15; Lot #: UC2744993; Dilution: 1 to 200).

Validation:

- anti-PD-L1 (Selleckchem, Cat#A2004, clone SP142, Lot#02). Application statement in manufacturer's website as following: "Atezolizumab can apply to humanized mice, non-humanized mice (e.g: C57BL/6 mice), peripheral blood and other related assays (Only for Reference)." https://www.selleckchem.com/products/Atezolizumab.html?gclid=EAIaIQobChMgbvp9IM6EAAYAASAAel1S5yO_BwE.

- anti-CD8a (Bio X Cell, Cat#BE0051, clone 2.43, Lot#7051182). Please see the manufacturer’s website link for application. https://bxcell.com/product/m-cd8a-2/.

- CD274 (PD-L1, B7-H1) Monoclonal Antibody (MIH5), Super Bright 780, eBioscience™ (ThermoFisher, Cat#78-5982-82, clone MIH5, Lot#1955731). Application statement in manufacturer’s website as following: "This MIH5 antibody has been reported for use in flow cytometric analysis." https://www.thermoscientific.com/antibody/product/CD274-PD-L1-B7-H1-Antibody-clone-MIH5-Monoclonal/78-5982-82.

- CD8 alpha Monoclonal Antibody (KT15), FITC (ThermoFisher, Cat#MAS-16759, clone KT15, Lot#UC2744993). Please see the manufacturer’s website link for application. https://www.thermoscientific.com/antibody/product/CD8-alpha-Antibody-clone-KT15-Monoclonal/MAS-16759.
Eukaryotic cell lines

Policy information about cell lines

Cell line source(s) All the cell lines (CT-26 and 4T1) were purchased from ATCC.

Authentication None of the cell lines used were authenticated.

Mycoplasma contamination The cell lines were not tested for mycoplasma contamination.

Commonly misidentified lines (See ITAG register) No commonly misidentified cell lines were used.

Animals and other organisms

Policy information about studies involving animals: ARRIVE guidelines recommended for reporting animal research

Laboratory animals Female BALB/c mice 6–7 weeks of age were purchased from Charles River.

Wild animals The study did not involve wild animals.

Field-collected samples The study did not involve samples collected from the field.

Ethics oversight Mouse handling was approved by Stanford University’s administrative panel on Laboratory Animal Care. All experiments were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Flow Cytometry

Plots

Confirm that:

- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a ‘group’ is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation For harvesting cells from the CT-26 and 4T1 tumor, the tumor was dissected out, cut into small pieces. The tumor tissues were dissociated using the Miltenyi mouse tumor dissociation kit according to the manufacturers’ instructions. The preparations were passed through a 0.07 mm cell strainer and washed thoroughly with PBS buffer supplemented with 0.5% BSA (PBS-BSA buffer). Finally, the cells were resuspended in PBS-BSA buffer and stained with CD8 alpha Monoclonal Antibody (KT15), FITC (for CD8+ T cells) or CD274 (PD-L1, B7-H1) Monoclonal Antibody (MIHS), Super Bright 780, ebioscience™ (for PD-L1 expression levels) for flow cytometry. For harvesting cells from spleen tissues, the spleen was minced finely with scissors and scalpel, and mashed on a 0.07 mm cell strainer to create a single-cell suspension. The red blood cells were lysed by incubating cells in ACK lysis buffer for 10 minutes and mononuclear cells were washed thoroughly with PBS-BSA buffer. Finally, the cells were resuspended in PBS-BSA buffer and stained with CD8 alpha Monoclonal Antibody (KT15), FITC (for CD8+ T cells) for flow cytometry.

Instrument Beckman Coulter CytoFLEX flow cytometer.

Software Flowjo10.

Cell population abundance No cell sorting was performed.

Gating strategy Based on the pattern of FSC-A/SSC-A, cells in the lymphocyte gate were used for analysis of CD8+ T cell subsets; cells in the tumor cell gate were used for analysis of PD-L1 expression level. Singlets were gated according to the pattern of FSC-A vs. FSC-H. Positive populations were determined by the specific antibodies, which were distinct from negative populations.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.