Supporting Information

Combination of NIR therapy and regulatory T cell modulation using layer-by-layer hybrid nanoparticles for effective cancer photoimmunotherapy

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In vitro stability of IR-780

Meanwhile, the IR-780 stability of different adding layers was evaluated by measuring the absorbance using UV/visible spectrophotometer (PerkinElmer U-2800; Hitachi, Tokyo, Japan). To determine whether there are any losses of IR-780 after 4 coating layers, the loaded IR-780 before and after coating were analyzed by UV/visible spectrophotometer.

Isolation of the $CD4^+CD25^+$ Treg cells and $CD8^+$ T cells

To isolate the $CD4^+CD25^+$ Treg cells and $CD8^+$ T cells, single-cell suspensions were isolated from spleen and lymph nodes of 6-week-old C57BL/6 mice. $CD4^+$ T cells were purified with CD4 MicroBeads (Miltenyi Biotec). By adding CD8 MicroBeads (Miltenyi Biotec) into the collecting $CD4^-$ cells, purified $CD8^+$ T cells were obtained in Midi MACS sorting device (Miltenyi Biotec). To further purify the $CD4^+$ T cells, anti-mouse CD25-PE antibody (Biolegend) was added into the $CD4^+$ T cells, and then anti-PE magnetic beads (Miltenyi Biotec) were added into the cells. The purified $CD4^+CD25^+$ cells were sorted and the purity of $CD4^+CD25^+Foxp3^+$ cells were >95 %. The harvested $CD8^+$ T cells and Treg cells were seeded at the density of 2×10^5 in a flat-bottom 96-well plate coated with anti-CD28 (1 μg/mL; eBioscience) and anti-CD3 (5 μg/mL; Biolegend), together with different concentration of IMT and IMT loaded GITR-PLGA NPs. After incubation for 24 h, cells were collected and stained with Annexin V/PI kit before analyzing using flow cytometry (FACSCalibur; BD Biosciences, USA).

Coculture system

Briefly, Treg cells were seeded on the transwells (pore size: 0.4 μm) and B16 cells were added on the bottom of the 12-well plate. After 24 h, LBL hNPs ($GITR^+$), LBL hNPs (IgG isotype)

were premixed with cell culture medium and added to the 12-well. After 6 h incubation time, Treg cells on the transwells and B16 cells on the bottom were harvested separately and measured using flow cytometry.

In vitro cellular uptake of GITR-PLGA cores by Treg cells

The specific cellular uptake of GITR-PLGA cores by Treg cells were confirmed by incubating Treg cells with DiD labeled LBL hNPs (with GITR antibody modificantion on PLGA cores) or DiD labeled LBL hNPs (with IgG isotype modification on PLGA cores) at 0.1, 0.2 and 0.4 μg GITR or IgG antibody per 250,000 Treg cells. Immediately after adding indicated formulation, the culture medium was adjusted to 6.5 to mimic tumor acidic pH. For the GITR pretreatment study, Treg cells were treated with 1.0 μg GITR antibody per 250,000 Treg cells 1 h before adding the indicated formulation (0.4 μg GITR modification on PLGA cores). After 6 h, Treg cells were collected and cellular uptake of GITR-PLGA cores were measured by flow cytometry.

In vivo uptake of GITR-PLGA cores from LBL hNPs by intratumoral Treg cells

To further confirm the uptake of GITR-PLGA cores from LBL hNPs by intratumoral Treg cells *in vivo*, DiD labeled LBL hNPs with or without GITR antibody modification on the GITR-PLGA cores were intravenously injected into B16BL/6 tumor-bearing mice. At predetermined time points (0, 8, 24 h), single cells were harvested from tumor and intratumoral Treg cells were identified as $CD3^+CD4^+Foxp3^+$ T cells. Percentage of DiD labeled GITR-PLGA cores from LBL hNPs taken up by Treg cells were defined as the percentage of DiD^+Foxp3^+ cells.

In vivo evaluation of antitumor efficacy in MC-38 tumor-bearing mice model

Similar *in vivo* antitumor study was also performed in MC-38 tumor-bearing mice model, including treatment with PBS, free IMT (10.0 mg/kg), LBL hNPs (IMT), LBL hNPs (IMT/IR-780), LBL hNPs (IR-780, 8.0 mg/kg) (NIR irradiation) and LBL hNPs (IMT/IR-780) (NIR irradiation), n=6. The intravenous injection intervals were same with the study above and tumor volume and body weight were also monitored as described above. During treatment, blood was taken from the eyes of the mice every two days and the TGF-β, IFNγ and TNFα level in the blood was identified by ELISA kit (Thermo Fisher Scientific, USA). Further analysis of the intratumoral Treg cells and $CD8^+$ T cells were measured at day 20 following the methods described previously.

To further demonstrate the efficacy of LBL hNPs in tumor eradication, MC-38 tumor model was used for *in vivo* antitumor study. Treatment with LBL hNPs (IMT/IR-780) or LBL hNPs (IMT) without NIR irradiation could significantly ($p<0.001$) slow down the fast growth trend of tumors compared to PBS or IMT group, but these antitumor effects were far less ($p<0.01$) than those triggered by LBL hNPs (IMT/IR-780) or LBL hNPs (IR-780) under NIR exposure (**Figure S16A** and **S16B**). LBL hNPs (IR-780) plus NIR group could greatly suppress tumor growth. However, no total tumor eradication was appeared in this treated group. LBL hNPs (IMT/IR-780) under NIR irradiation could induce significantly higher ($p<0.01$) tumor eradication compared to LBL hNPs (IR-780) plus NIR group, suggesting the necessity of combination of NIR treatment and immunotherapy. Meanwhile, the serum cytokine TGFβ that indicated the function of Treg cells were also measured (**Figure S16C**). The groups treated with IMT loaded LBL hNPs (IMT), LBL hNPs (IMT/IR-780) or LBL hNPs (IMT/IR-780) plus NIR could significantly reduce the serum TGFβ levels compared to PBS, IMT or LBL hNPs (IR-780) plus NIR, suggesting the necessity of IMT loaded inside LBL hNPs to inhibit the effect of Treg cells. In addition, the serum IFN-γ and TNF-α, which acted as inflammation

factor in indicating PDT-induced antitumor immunity, were also measured in this study. Significantly higher elevation of IFN-γ and TNF-α in mice were observed after LBL hNPs (IMT/IR-780) plus NIR treatment (**Figure S16D** and **S16E**), indicating successful cellular immunity initiated by our system. Further investigation of the intratumoral $CD8^+$/Treg cell ratio demonstrated that the strategy of combining inhibition of Treg cells with NIR treatment LBL hNPs (IMT/IR-780) plus NIR generated higher $CD8^+$ effector T cells again tumor compared to Treg cell inhibition alone group (LBL hNPs (IMT)) or NIR treatment alone group (LBL hNPs (IR-780) plus NIR) (**Figure S16F**).

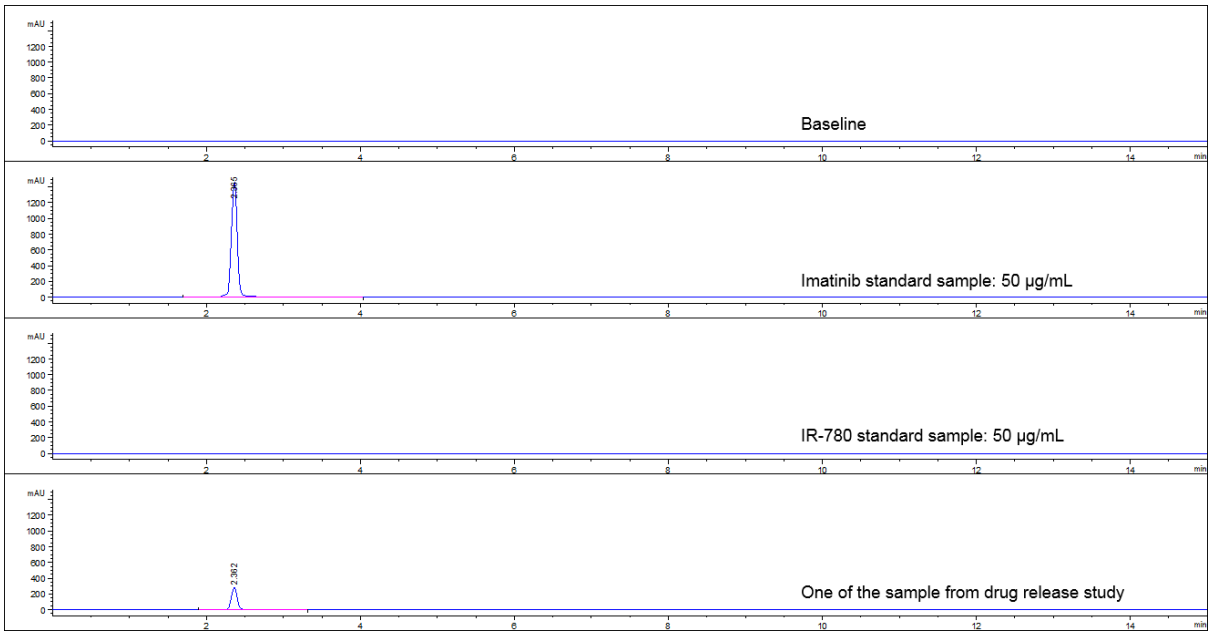

Figure S1. Comparison of HPLC peaks from baseline, IMT standard sample (50.0 µg/mL), IR-780 standard sample (50.0 µg/mL) and sample from drug release study.

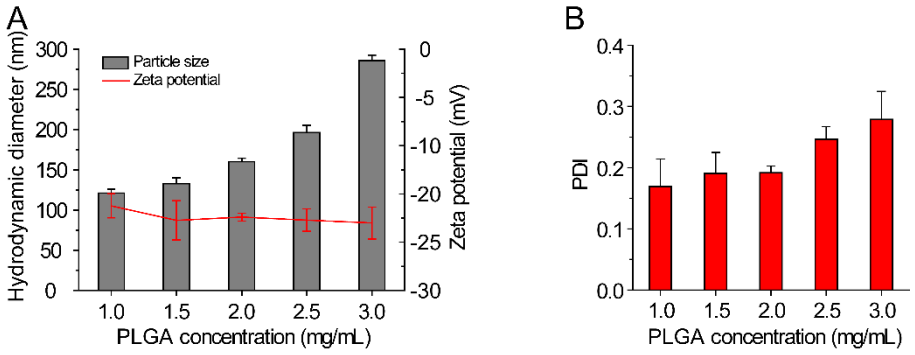

Figure S2. (A) Hydrodynamic diameter and zeta potential, and **(B)** PDI variations of PLGA nanoparticles at different PLGA concentrations, ranging from 1 to 3 mg/mL (n=3).

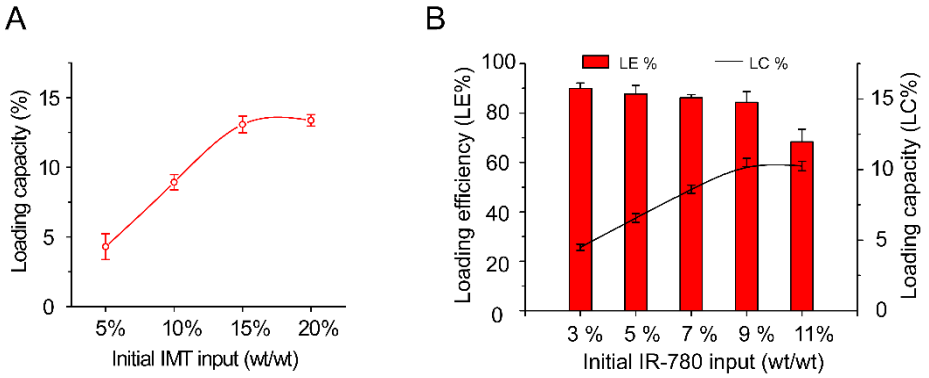

Figure S3. (A) Drug loading capacity (LC) of PLGA nanoparticles at various initial IMT input percentage, from 5% to 20%. **(B)** Loading efficiency (LE) and loading capacity (LC) of IR-780 in LBL hNPs. Initial IR-780 input ranged from 3 % to 11 % (n=3).

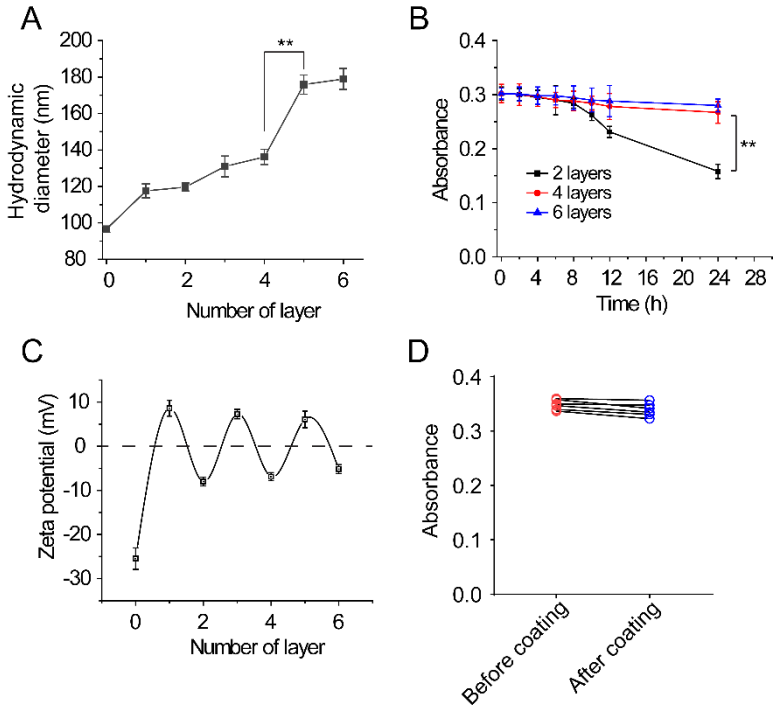

Figure S4. (A) Hydrodynamic diameter changes of LBL hNPs after adding different PLH and PEG-b-PLG layers (n=3), $^{**}p<0.01$. **(B)** Absorbance of IR-780 after different coating layers within 24 h (n=3), $^{**}p<0.01$. **(C)** Zeta potential of LBL hNPs after addition of alternate PLH and PEG-b-PLG layers (n=3). **(D)** Changes of IR-780 absorbance before and after the addition of 4 coating layers (n=3).

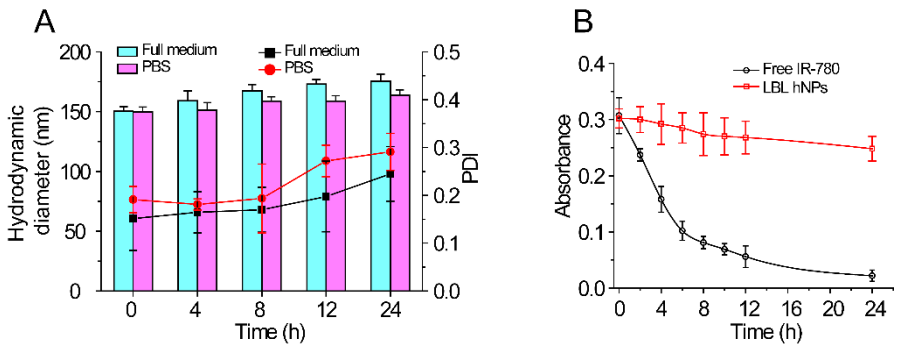

Figure S5. (A) Hydrodynamic diameter and PDI changes of LBL hNPs in RPMI full media (with 10% FBS) and PBS within 24 h (n=3). **(B)** UV absorbance of free IR-780 and LBL hNPs at 780 nm in PBS within 24 h (n=3).

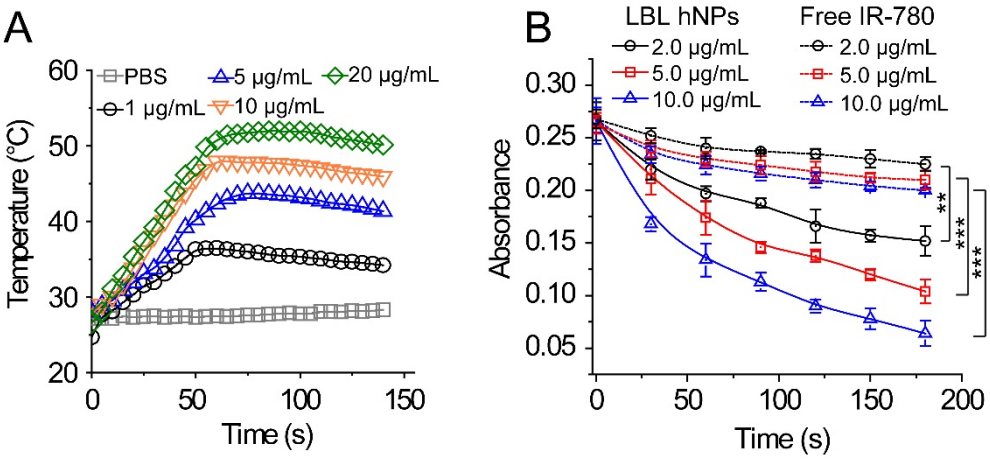

Figure S6. (A) Dose-dependent temperature increase of free IR-780 at different concentrations under 808 nm, 1.0 W/cm^2 NIR irradiation. **(B)** Decay curves of DPBF absorption at 410 nm after treatment with free IR-780 and LBL hNPs at increasing IR-780 concentrations (2.0, 5.0, 10.0 μg/mL) under 808 nm, 1.0 W/cm^2 NIR irradiation, indicating the production of singlet oxygen which acted as a potent ROS.

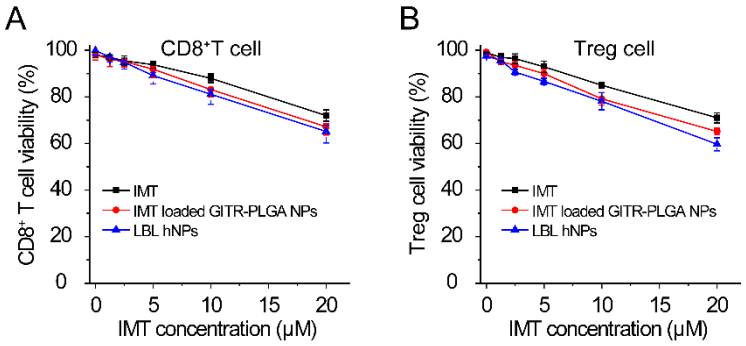

Figure S7. (A) Cell viability of IMT, IMT loaded GITR-PLGA NPs and LBL hNPs against $CD8^+$ T cell at different IMT concentrations (n=6). **(B)** Cell viability of IMT, IMT loaded GITR-PLGA NPs and LBL hNPs against Treg cells at different IMT concentrations (n=6).

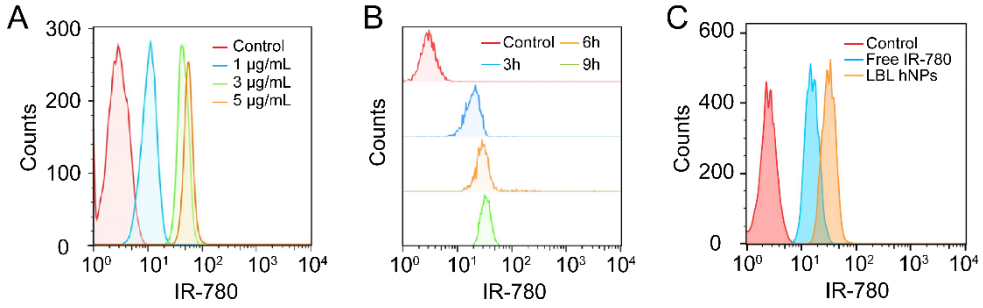

Figure S8. (A) *In vitro* dose-dependent cellular uptake of IR-780 from LBL hNPs after incubation for 3 hours. Concentration of loaded IR-780 varied from 1 μg/mL to 5 μg/mL (n=3). **(B)** *In vitro* cellular uptake of IR-780 in B16BL/6 cells treated with free IR-780 dye after incubation for 3 h, 6 h and 9 h. Concentration of IR-780 was 3 μg/mL (n=3). **(C)** Further comparison of IR-780 cellular uptake in free IR-780 and LBL hNPs treated groups using flow cytmetry. The concentration of IR-780 was 3 μg/mL (n=3).

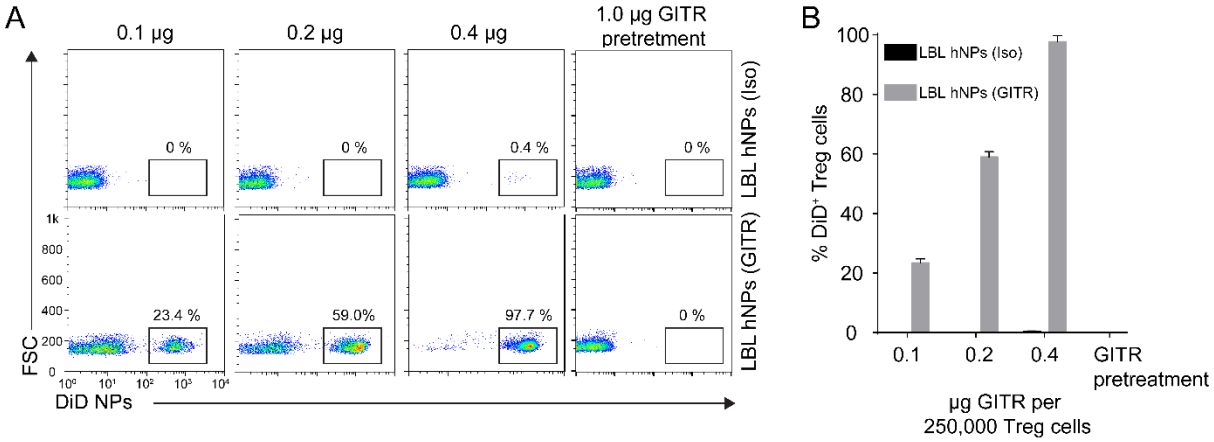

Figure S9. (A) *In vitro* specific binding of DiD labeled GITR-PLGA cores from LBL hNPs to Treg cells at 0.1, 0.2 and 0.4 µg GITR per 250,000 Treg cells. IgG isotype modified PLGA cores from LBL hNPs were used as the control, n=3. **(B)** Quantification of DiD^{+}Treg cells.

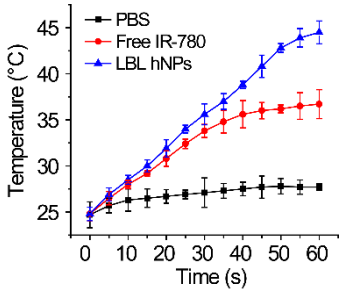

Figure S10. *In vitro* temperature changes of the B16BL/6 tumor cells treated with PBS, free IR-780 and LBL hNPs under 1.0 W/cm^2 NIR irradiation for 1 min (n=3).

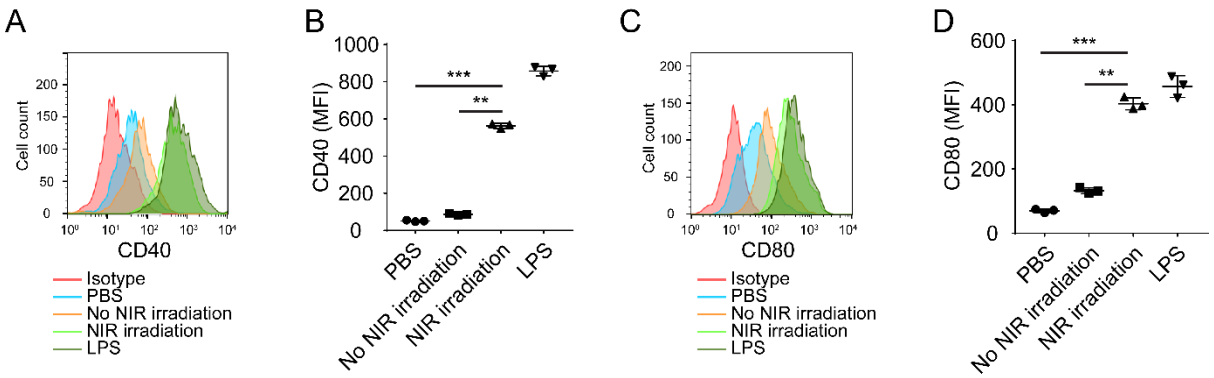

Figure S11. (A) *In vitro* CD40 expression and corresponding statistical analysis **(B)** of non-matured BMDCs exposed to tumor-associated antigen derived from B16BL/6 cells treated with PBS or LBL hNPs (without or with NIR, 808 nm, 1.0 W/cm^2, 1 min). The concentration of IR-780 was 3 μg/mL. LPS was used as a positive control. **(C)** *In vitro* CD80 expression and corresponding statistical analysis **(D)** of non-matured BMDCs exposed to tumor-associated antigen derived from B16BL/6 cells treated with PBS or LBL hNPs (without or with NIR, 808 nm, 1.0 W/cm^2, 1 min). The concentration of IR-780 was 3 μg/mL. n=3, $^{**}p<0.01$, $^{***}p<0.001$.

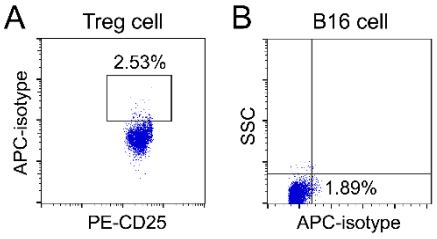

Figure S12. (A-B) Competitive cellular uptake of IgG isotype modified PLGA cores from LBL hNPs between Treg cells **(A)** and B16BL6 cells **(B)** in the coculture system.

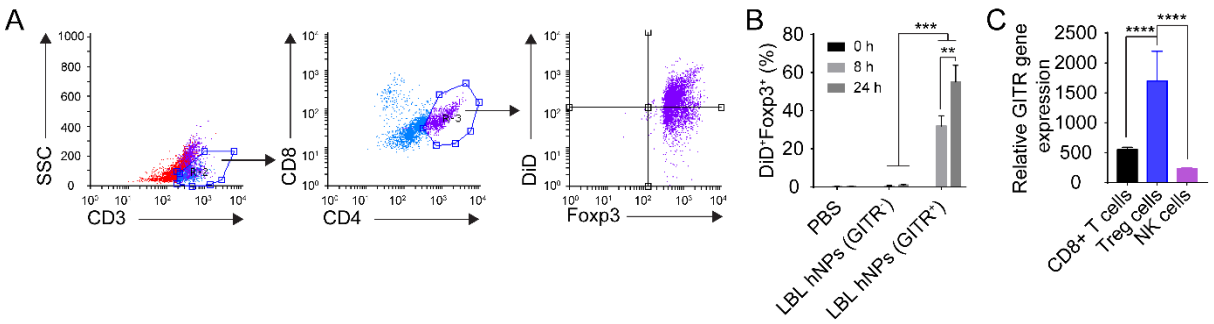

Figure S13. (A) Gating strategy of *in vivo* DiD labeled PLGA cores from LBL hNPs taken up by intratumoral Treg cells. **(B)** Percentage of DiD labeled GITR-PLGA cores from LBL hNPs or DiD labeled PLGA cores (no GITR modification) from LBL hNPs taken up by Treg cells during 0, 8 and 24 h. $^{**}p<0.01$, $^{***}p<0.001$. **(C)** Relative GITR gene expression in $CD8^+$ T cells, Treg cells, and NK cells (data originated from BioGPS). $^{****}p<0.0001$.

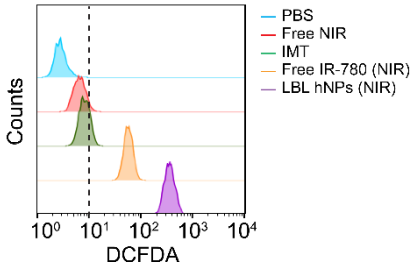

Figure S14. *In vivo* ROS generation in tumor areas after treating with PBS, free NIR, IMT, free IR-780 (NIR) and LBL hNPs (NIR) using DCFDA probe (n=6).

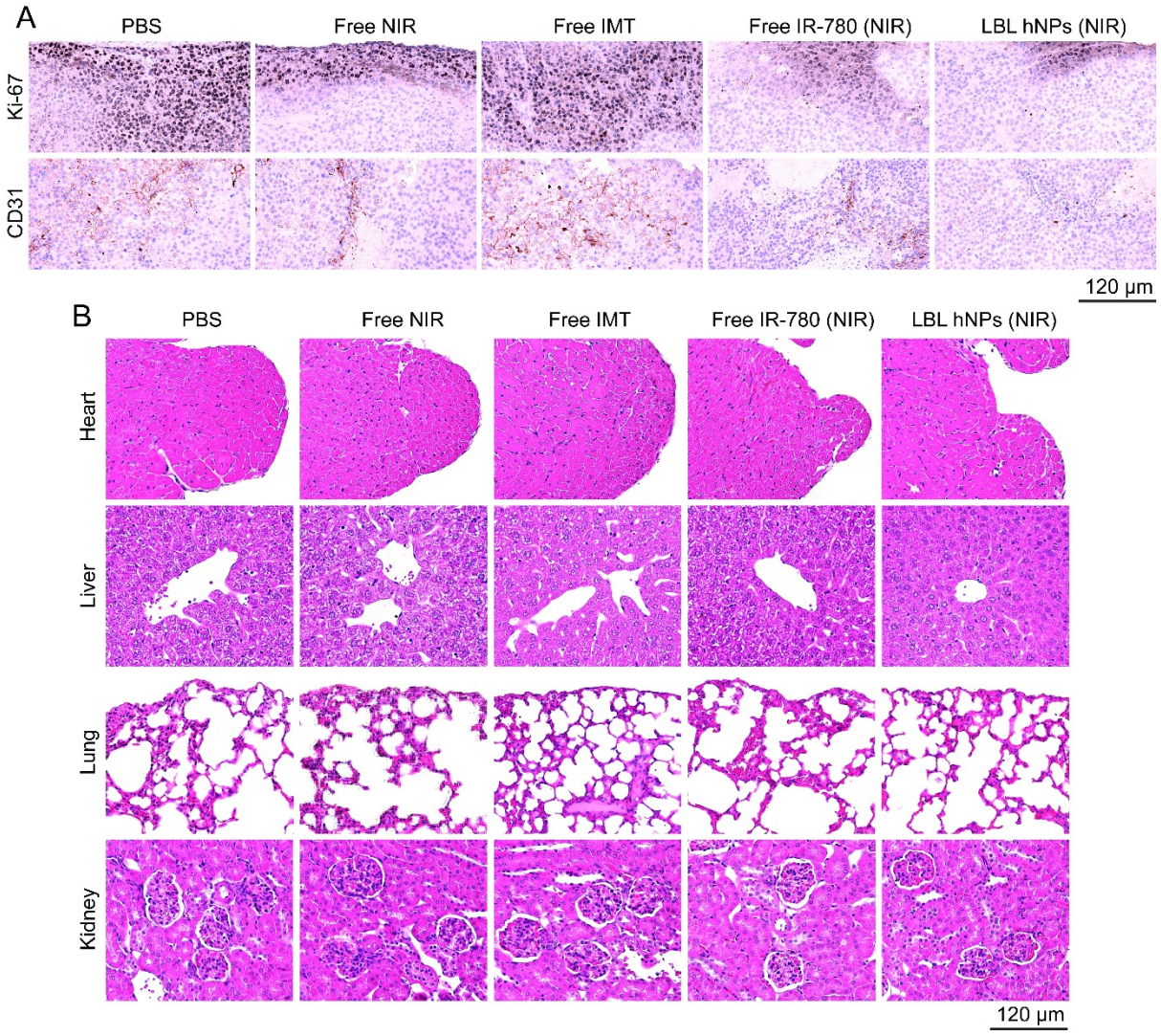

Figure S15. (A) Immuno-histochemical evaluation for intra-tumoral Ki-67 and CD 31 expression after 3 dosages treatment with PBS, free NIR, IMT, free IR-780 (NIR) and LBL hNPs (NIR). **(B)** *In vivo* histopathological observation in heart, liver, lungs, and kidneys collected from groups with PBS, free NIR, IMT, free IR-780 (NIR) and LBL hNPs (NIR) treatment.

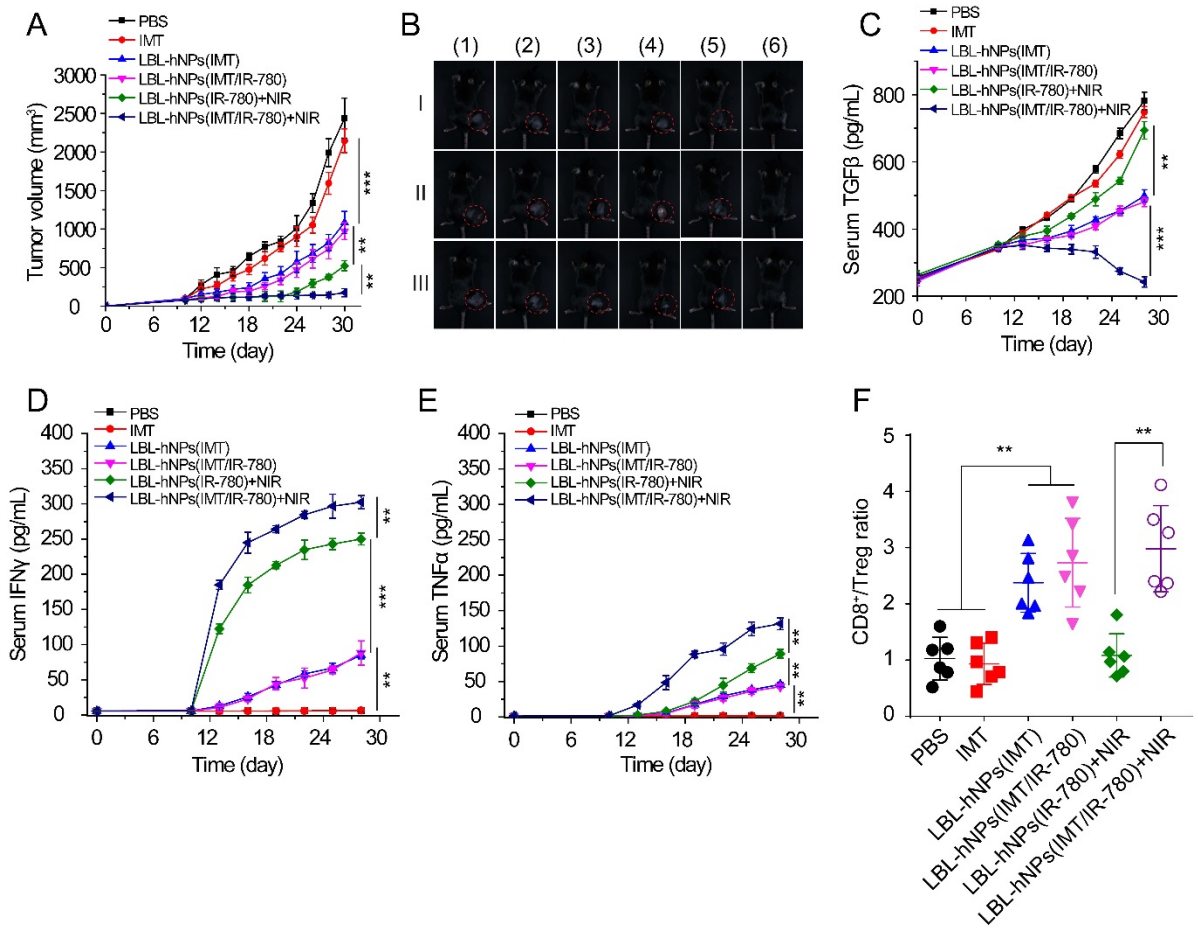

Figure S16. (A) Average tumor growth curves for mice after indicated treatments. The intravenous injection began at day 10 after random group division (n=6). **(B)** Representative images of MC-38 tumor-bearing C57BL/6 mice taken at 28 days. (1) PBS, (2) IMT, (3) LBL hNPs (IMT), (4) LBL hNPs (IMT/IR-780), (5) LBL hNPs (IR-780) +NIR, (6) LBL hNPs (IMT/IR-780) +NIR. Mean serum TGF-β **(C)**, IFNγ **(D)** and TNFα **(E)** levels in mice treated with PBS, IMT, LBL hNPs (IMT), LBL hNPs (IMT/IR-780), LBL hNPs (IR-780) plus NIR and LBL hNPs (IMT/IR-780) plus NIR during study days (n=6). **(F)** Ratio of intratumoral $CD8^+$ effector T cells to Treg cells after treatment with indicated formulations (n=6). $^{**}p<0.01$, $^{***}p < 0.001$, one-way ANOVA.

Table S1. Histomorphometrical analysis of tumor masses, taken form B16/BL6 allograft C57BL/6 mice

Items / Groups	Tumor cell volumes (%/mm^2)	Immunoreactive cell percentages (%/mm^2 of tumor mass)		Immunoreactive cell numbers (cells/mm^2 of tumor mass)	
		Ki-67	CD31 (PECAM-1)	CD4 (Th)	CD8
Control (G1)	77.39±10.10	56.94±10.31	40.70±5.62	3.50±1.05	8.83±2.32
Treatment					
G2	59.82±6.90[a]	27.58±4.94[f]	28.89±3.59[f]	3.33±1.37	8.33±3.27
G3	73.66±11.16[c]	53.65±14.71[g]	36.84±6.10[h]	4.00±1.41	10.17±4.62
G4	45.90±6.65[acd]	18.80±2.42[fgi]	19.60±3.09[fgi]	61.00±14.14[fgi]	690.33±249.49[fgi]
G5	17.55±7.18[abde]	8.61±2.51[fgij]	3.63±1.73[fgij]	265.83±105.25[fgij]	1513.67±399.25[fgij]

Values are expressed as mean ± SD of six tumor mass histological fields

Groups: G1 = PBS treated B16/BL6 murine melanoma tumor cell-allograft control masses; G2 = Free NIR irradiation treated tumor cell-allograft masses; G3 = Free IMT treated tumor cell-allograft masses; G4 = Free IR-780 with NIR treated tumor cell-allograft masses; G5 = LBL hNPs with NIR treated tumor cell-allograft masses.

CD31 = Platelet endothelial cell adhesion molecule 1 (PECAM-1); CD = Cluster of differentiation; PBS = Phosphate buffered saline; IMT = Imatinib; LBL = Layer by layer hybrid nanoparticles

[a] $p<0.01$ as compared with G1 by LSD test

[b] $p<0.01$ and [c] $p<0.05$ as compared with G2 by LSD test

[d] $p<0.01$ as compared with G3 by LSD test

[e] $p<0.01$ as compared with G4 by LSD test

[f] $p<0.01$ as compared with G1 by MW test

[g] $p<0.01$ and [h] $p<0.05$ as compared with G2 by MW test

[i] $p<0.01$ as compared with G3 by MW test

[j] $p<0.01$ as compared with G4 by MW test