

Three-Dimensional Covalent Organic Frameworks with Cross-Linked Pores for Efficient Cancer Immunotherapy

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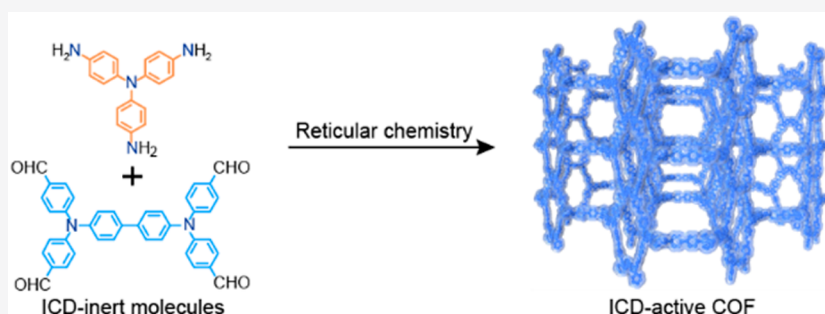
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ABSTRACT: We report the design and synthesis of a series of three-dimensional (3D) covalent organic frameworks (COFs) as immunogenic cell death (ICD) inducers for cancer immunotherapy. Three triple-topic amine building blocks, inactive to inducing ICD, were used to construct three COFs, COF-607, COF-608, and COF-609, with outstanding ICD eliciting efficiency. Mechanism studies revealed that after linking these ICD inert monomers into the COF backbone, the optical properties of these COFs could be systematically tuned to achieve excellent reactive oxygen species (ROS) production performance. This combined with 3D cross-linked pores, mimicking lung structure, favor the exchange and diffusion of oxygen and ROS, leading to excellent inducing ICD efficacy. One member, COF-609, is capable of triggering abscopal and long-lasting immune memory effects in a mouse model of breast cancer with >95% mice survival after being treated with COF-609+ α CD47 for 110 days.

KEYWORDS: 3D covalent organic frameworks, immunogenic cell death inducers, nanotherapeutics

Covalent organic frameworks (COFs), porous scaffolds constructed by connecting active building blocks with high multifunctional efficiency,^{1,2} provide precise control of pore environment and accurate interaction with guest molecules, thus ideally suited for biological applications.^{3–11} Recently, others and we showed that it is possible to utilize COF backbone absorb light to destroy the local tumor.^{12–15} However, such solely phototherapy mode only works for the local irradiated tumor.^{16–18} In practice, cancer cells usually exhibit extensive metastasis and recurrence behavior, which seriously restrict the therapeutic effect and result in more than 90% cancer-related deaths.^{19–21} The potentials to eliminate tumor metastasis and recurrence by pure COF materials has yet been explored. Herein, we report that COF could serve as an excellent platform to turn immunogenic cell death (ICD) inactive molecules to ICD active constructs for boosting antitumor immunity and inhibiting tumor metastasis.

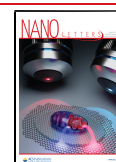
Immunotherapy has been recently recognized as a promising strategy to inhibit tumor metastasis and recurrence.^{22–24} However, their efficacy is severely limited by the low response rate. Some adjuvant therapy strategies, including phototherapy, radiotherapy, and chemotherapy, have the potential to cause

cancer cell deaths in an immunogenic mode, known as ICD, which could further promote the immune response rate to promise excellent therapeutic efficacy toward metastatic tumors.^{25–30} Their therapeutic efficacy is dictated by the efficiency of ICD inducers. The performance of traditional ICD inducers, such as indocyanine green (ICG) and oxaliplatin, is often limited by undesirable aggregation caused quench (ACQ).^{31,32} Linking molecular ICD inducers into porous structures, such as metal–organic frameworks (MOFs), proves to be as an effective way to solve the aggregation issue to effectively induce ICD and primed cancer immunotherapy.³³ Recently, COFs were used as nanocarriers to disperse the ICD inducer, which could also weaken the unwanted ACQ to promote their ICD eliciting performance.³⁴ However, in all of these studies, the molecular monomers utilized for structural

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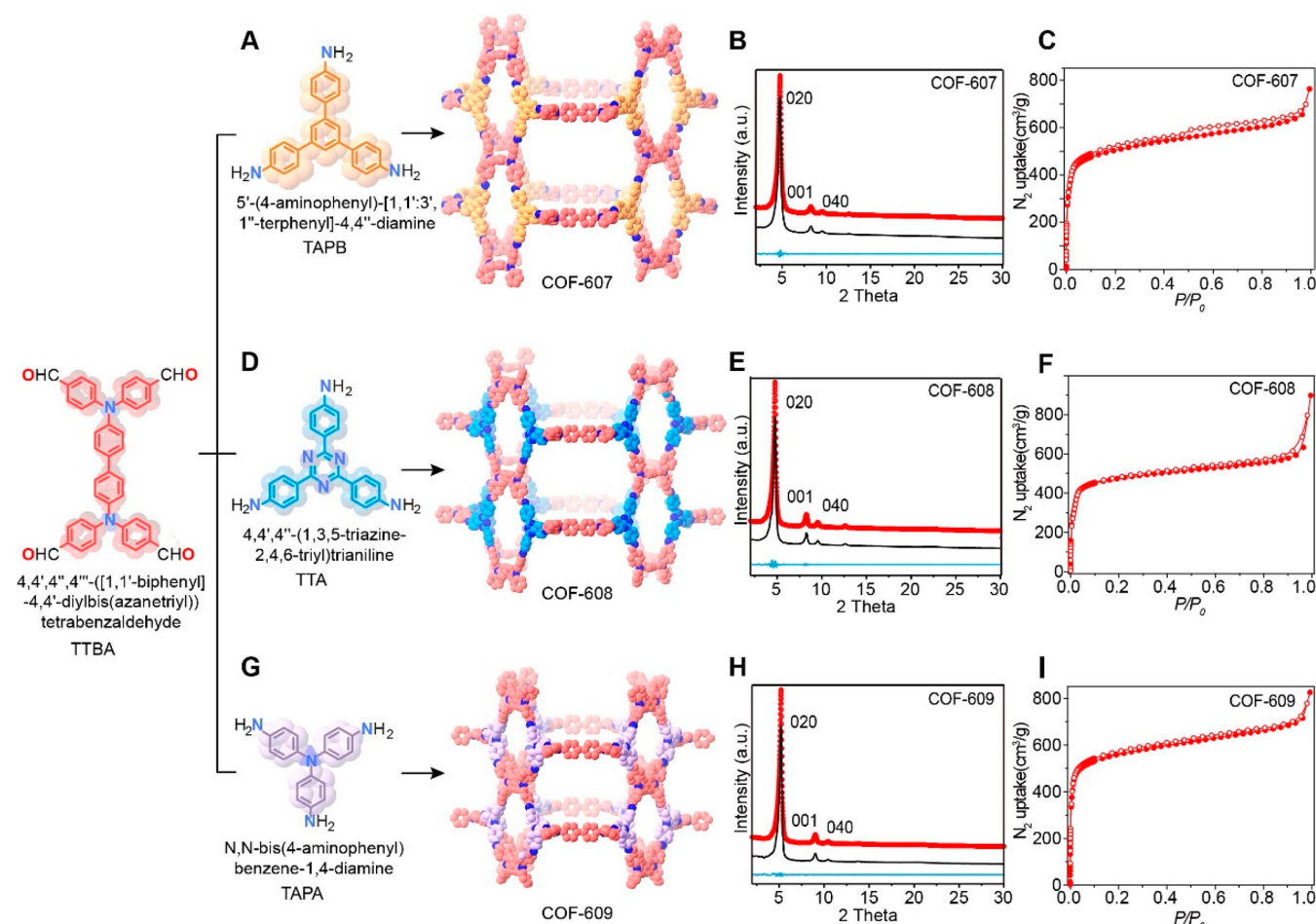


Figure 1. Construction and characterization of COF-607, COF-608, and COF-609. Synthesis route (A), PXRD patterns (B), and N_2 uptake of COF-607 (C). Synthesis route (D), PXRD patterns (E), and N_2 uptake of COF-608 (F). Synthesis route (G), PXRD patterns (H), and N_2 uptake of COF-609 (I).

design exhibited ICD eliciting activity on their own, while ICD inactive molecules were left largely unexplored. Furthermore, different from MOFs, the composition of COFs are light atoms, where the toxicity potentially induced by metal ions could be effectively obviated. Here, we demonstrated that for the first time organic molecules, inefficient in eliciting ICD on their own, could be used as molecular building blocks to construct 3D COFs with excellent ICD inducing efficiency. This was achieved by a pure 3D triphenylamine-based COF, COF-609, where their optical properties were finely tune to maximize their reactive oxygen species (ROS) generation and ICD eliciting efficiency. The triphenylamine-based COF exhibited outstanding ROS generation capability, which is three times that of the state-of-the-art 2D COF, COF-909, and five times that of the state-of-art porphyrin-based metal–organic framework (MOF), PCN-224, favorable for eliciting ICD.

Spectroscopic studies showed that their excellent ROS efficiency shall be attributed to the fine tuning of their optical behaviors, including light absorption, steady-state photoluminescence (PL), time-resolved PL, and PL quantum yield (PLQY). Furthermore, the covalent linkage in 3D COFs here effectively avoids unwanted active site aggregation and offers sufficient exchange and diffusion with ROS, thus achieving excellent ROS generation efficacy. More importantly, COF-609 with excellent ROS generation performance was further

demonstrated to be efficient in triggering endoplasmic reticulum (ER) stress, which is critical for inducing robust ICD. Mechanism studies revealed that three representative ICD parameters, including calreticulin (CRT), adenosine triphosphate (ATP), and high mobility group protein B1 (HMGB1), were found to be drastically increased after treated with COF-609, where their corresponding molecular building blocks and the representative PDT COF, COF-909, are inefficient in eliciting ICD under the same conditions (Figure 3E–G), demonstrating that COF could serve as an excellent platform to turn ICD inactive molecules into ICD active constructs. In addition, the strong ICD, elicited by COF-609, was further demonstrated to be efficient in reducing of immunosuppressive myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Tregs) number in the macroenvironment (spleens and draining lymph nodes), as well as the increase of T cells infiltration in the microenvironment, favoring durable antitumor immune response, which could drastically increase the efficacy of CD47 blockade therapy, a blockade which is originally inefficient in treating 4T1 tumor-bearing mice. This combinational therapy mode could effectively elicit abscopal and long-lasting immune memory effects in breast cancer model, yielding >95% mice survival after treatment with COF-609+ α CD47 for 110 days.

The typical synthesis of COF-607 to COF-609 were achieved by self-assembly of triple-topic amine building blocks

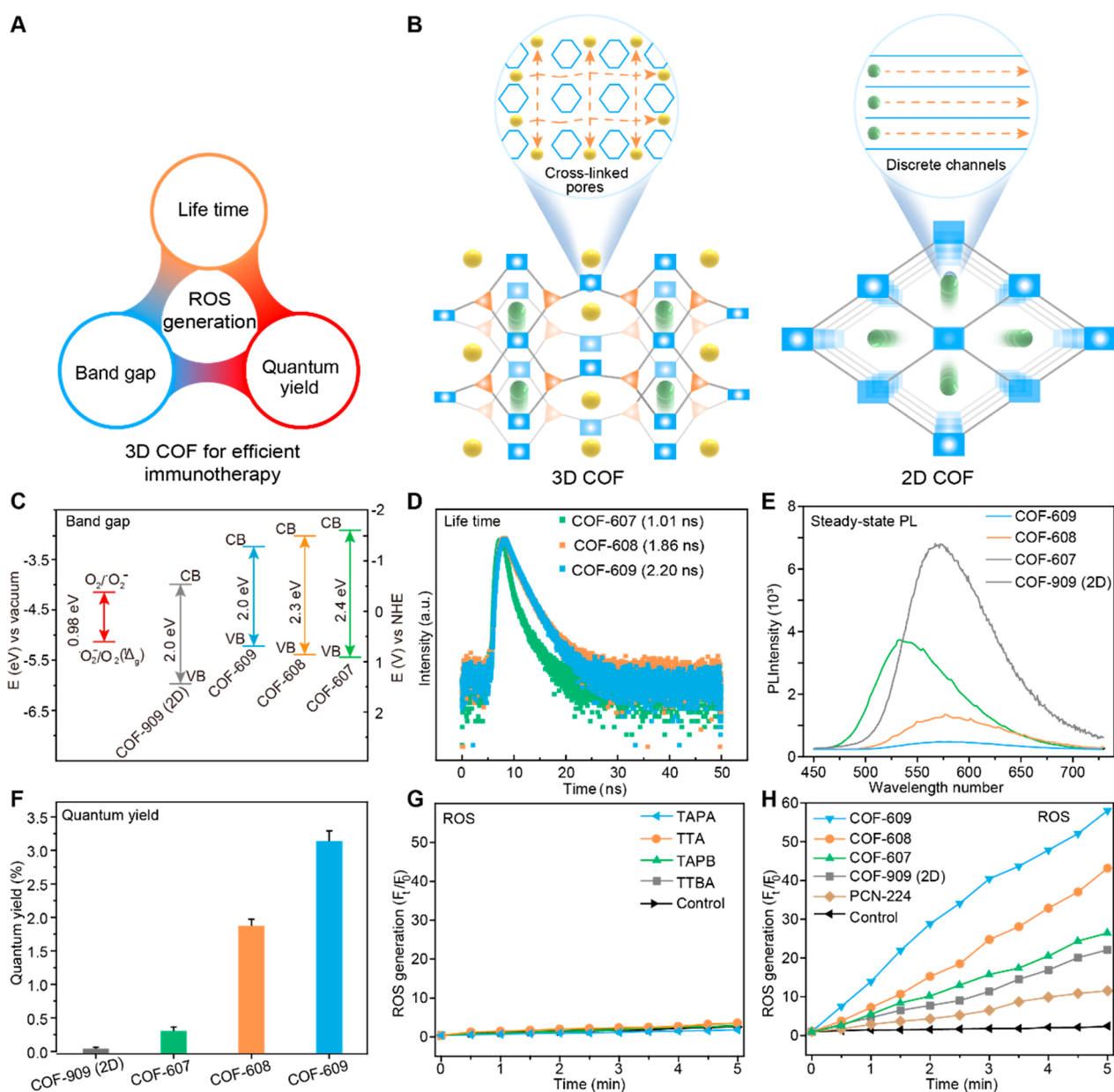


Figure 2. Optical properties and ROS generation capability of COF-607, COF-608, and COF-609. (A) Illustration of tuning the ROS generation performance of 3D COFs by finely tuning three key factors, including bandgap, lifetime, and quantum yield value. (B) Schematic illustration of the rational designed of 3D COF with cross-linked pores and 2D COFs with discrete channels. Compared to 2D COFs, the isolated alignment of photosensitizers in the cross-linked pores of 3D COFs effectively avoid unwanted aggregation-induced quench aggregation and offer sufficient contact with reactive oxygen species, thus achieving excellent ROS generation performance. (C) Band gap structure of COF-607 to COF-609. (D) Time-resolved PL spectra of COF-607 to COF-609. (E) Steady-state PL spectra contrast of COF-607 to COF-609. (F) Quantum yield of COF-607 to COF-609. (G) ROS generation efficiency of different monomers in comparison to TCPP. (H) ROS generation efficiency of COF-607, COF-608, and COF-609 in comparison to PCN-224, tested under the identical conditions.

with a tetra-topic aldehyde, tetrabenzaldehyde (TTBA) (Figure 1A,D,G). To characterize the crystalline structure of these new COFs, a series of spectroscopic studies were performed, and here we use COF-609 as an example. The high crystallinity of COF-609 could be reflected in their sharp powder X-ray diffraction (PXRD) peaks (Figure 1H). In this study, the imine condensations of square and trigonal monomer strategy, which were recently reported and demonstrated as an effective way to formed 3D COFs with *ffc* topology,^{35,36} were used to construct three new 3D COFs with identical topology. The experimental PXRD patterns matched well with those calculated from the simulated

structures in terms of both peak intensities and positions, confirming the successful formation of the targeted COFs. The sharp PXRD pattern was refined against the simulated noninterpenetrated and two-fold-interpenetrated structures by Pawley refinement. The calculated PXRD pattern of two-fold-interpenetrated *ffc* topology does not match with the experimental data, where the noninterpenetrated simulated PXRD patterns showcases good agreement with experimental data. The crystal lattice in *C2/m* space group ($a = 56.7 \text{ \AA}$, $b = 56.0 \text{ \AA}$, $c = 17.8 \text{ \AA}$, $\alpha = \beta = 90^\circ$ and $\gamma = 110^\circ$) was observed with acceptable residues ($R_{wp} = 2.24\%$, $R_p = 2.06\%$) (Figure 1H, S19). The construction of imine linkage was proven by the

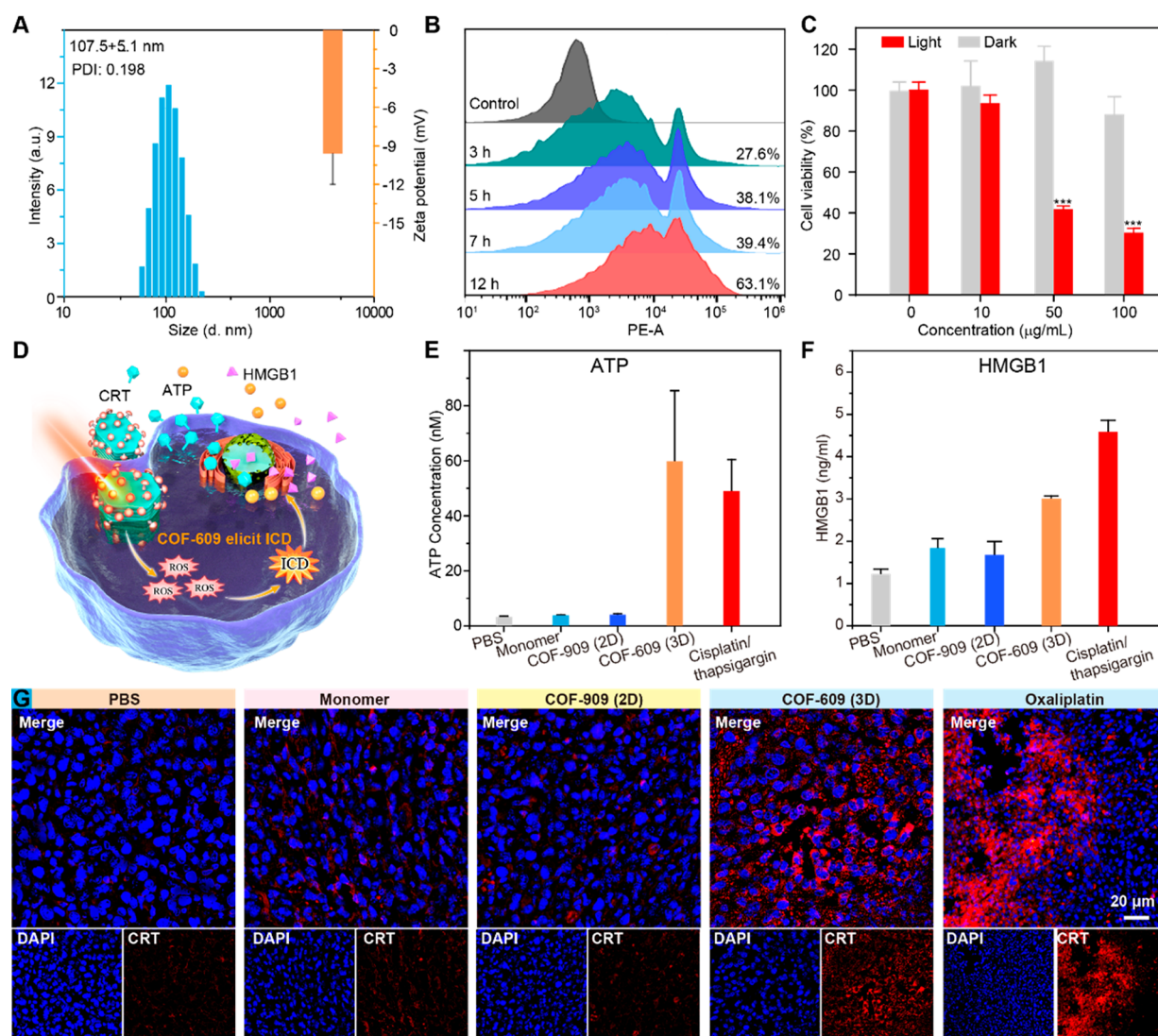


Figure 3. Immunogenic cell death induces the capability of COF-609. (A) Particle size and zeta potential of COF-609. (B) Uptake of COF-609 nanoparticles by 4T1 cells, measured through flow cytometry. (C) Cytotoxicity of COF-609 nanoparticles at different concentrations. (D) Illustration of inducing ICD by COF-609 mediated PDT, where the danger associated molecular patterns, such as calreticulin (CRT), adenosine triphosphate (ATP), and high mobility group protein B1 (HMGB1), were found to be drastically increased. (E) ATP released from 4T1 cells with different treatment, which measured at 1 and 4 h. (F) HMGB1 released from 4T1 cells after COF-609 mediated PDT at 24 h. (G) Immunofluorescence staining of CRT after different treatments. All of the *in vitro* cell experiments were conducted with three replicates each time and repeated three times.

emergence of a 1622 cm^{-1} peak in the Fourier transform infrared (FT-IR) spectrum (Figure S3) and a 398.79 eV peak in the X-ray photoelectron spectroscopy (XPS) spectrum (Figure S10). Similar phenomena were observed in both COF-607 and COF-608 synthesized in this study (Figures 1A–F and S1–S9). The particle size of these COFs was evidenced by scanning electron microscopy (SEM), transmission electron microscopy (TEM), and dynamic light scattering (DLS), where all of these COFs exhibited uniform structures with sizes of approximately 100 nm (Figure 3A and S11 to S13). The permanent porosity of these COFs was verified by the N_2 isotherm result with Brunauer–Emmett–Teller (BET) surface areas values of 1880 , 1800 , and $2110\text{ m}^2\text{ g}^{-1}$ for COF-607, COF-608, and COF-609, respectively (Figure 1C, F, I). This offered excellent ROS exchange and diffusion efficiency, given the high permanent porosity of COFs.

The ROS production performance of molecule-based photosensitizers are dictated by their specific optical properties. In this study, the optical properties of COFs, including bandgap structure, steady-state PL, time-resolved PL, and quantum yield, are systematically tuned to optimize their ROS generation efficiency. (Figure 2A,B) Bandgap structures of COF-607 to COF-609 and COF-909 were investigated by ultraviolet photoelectron spectroscopy and ultraviolet/visible diffuse reflectance spectroscopy. The results showed that these 3D COFs exhibited narrower band gap, from 2.0 to 2.4 eV , capable of generating sufficient superoxide radicals at 660 nm laser irradiation. In contrast, the band gap values of their corresponding monomers were varied from 2.5 to 2.7 eV , too large to be excited under 660 nm laser irradiation (Figures S17 and S18). In comparison to a typical 2D COF, COF-909 with similar building block and bandgap structure, COF-609 with cross-linked pores showed higher quantum yield and longer

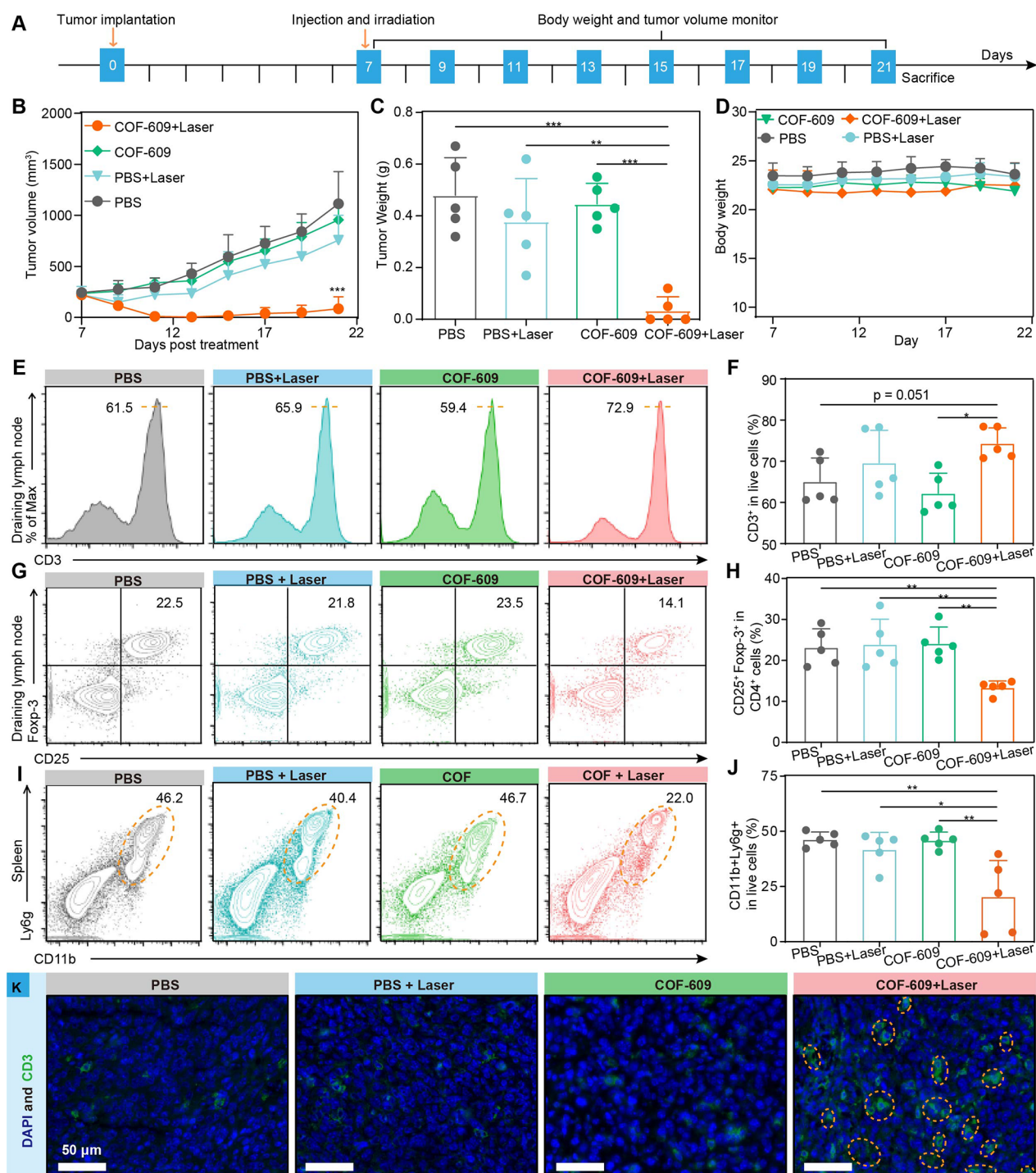


Figure 4. In vivo antitumor efficiency experiments and immune profile change of tumor harboring mice after treatment with COF-609. (A) Schematic diagram of tumor implantation and treatment protocol. (B) Tumor volume, (C) tumor weight at day 21. (D) The body weight of tumor-harboring mice at different days. (E,F) Detection and quantification of CD3⁺ T cells in the draining lymph nodes. (G,H) Detection and quantification of CD4⁺CD25⁺Foxp3⁺ Treg cells in the draining lymph nodes. (I,J) Detection and quantification of CD11b⁺Ly6g⁺ MDSCs in the spleen. (K) Images of immunofluorescence staining of CD3 in the tumor slices from different groups; scale bar: 50 μ m. $N = 5$. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

lifetime, indicating better electron–hole separation upon photoexcitation and suppressed recombination, thus favoring ROS generation. (Figure 2C–F). Then, to evaluate the ROS production efficacy of COF-607 to COF-609 and COF-909, 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) was used as a ROS probe.³⁷ The results showed that all of the building blocks were ineffective in generating ROS by

themselves, however, after constructing to 3D COFs, their corresponding COF samples presented obvious ROS production capability, demonstrating that 3D COFs could serve as a favorable platform to turn ROS-inert monomers into ROS-active COFs (Figure 2G,H). Among them, COF-609 exhibited the highest ROS generation efficiency, which could be attributed to their outstanding optical properties, including

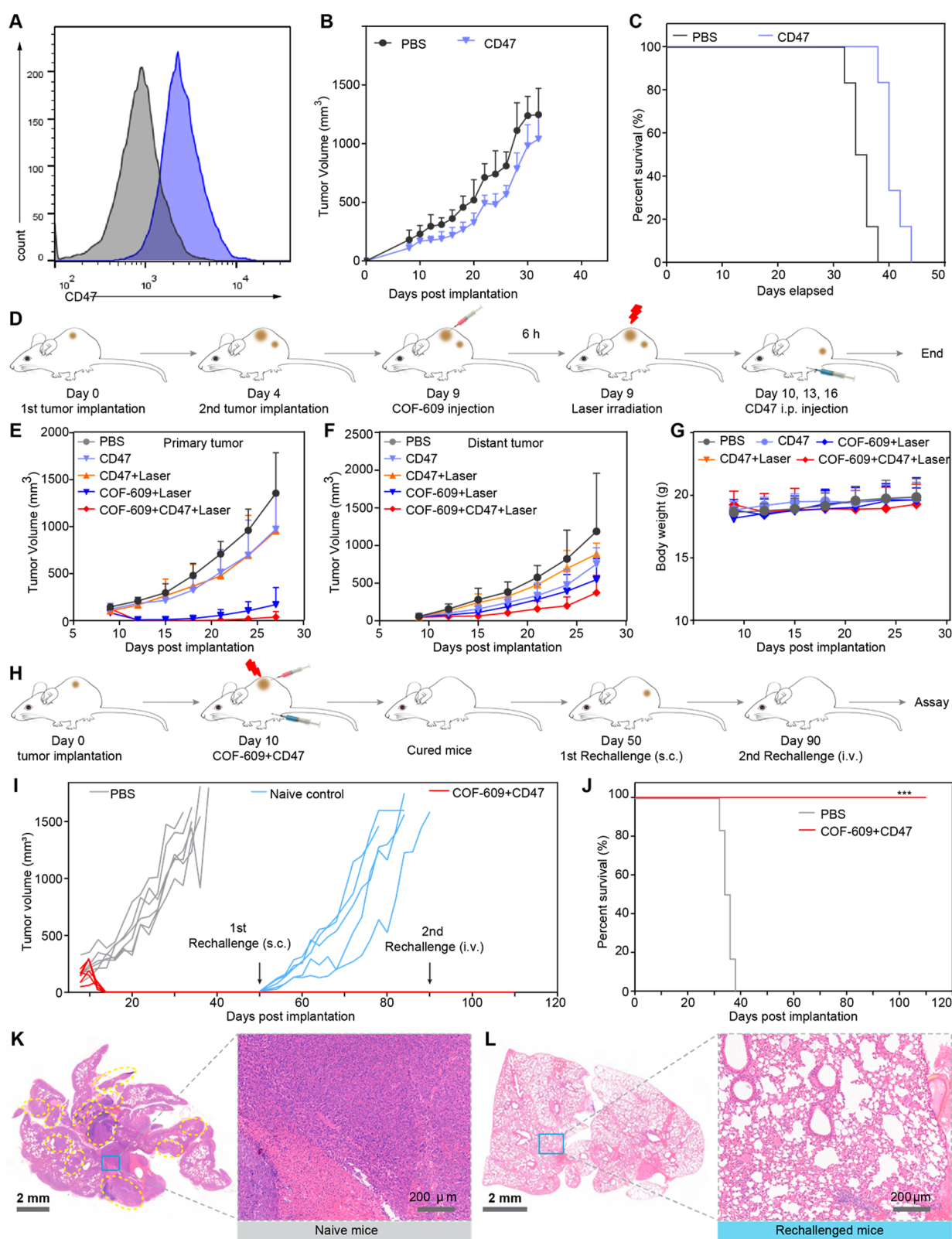


Figure 5. Metastasis inhibition efficacy and immune memory effect of COF-609 in combination with CD47 blockade therapy. (A) The expression of CD47 in 4T1 tumor cells. (B) Tumor growth curves of single CD47 blockade therapy. (C) Percent survival of tumor harboring mice after CD47 treatment. (D) Time schedule of the construction of bilateral tumor mouse model and treatments. (E,F) Growth curves of primary and distant tumors of bilateral 4T1 tumor-bearing mice. (G) Body weight of 4T1 tumor-bearing mice with various treatments. (H) Time schedule of 4T1 tumor rechallenge assay. s.c., subcutaneous injection; i.v., intravenous injection. (I) Tumor volume of 4T1 tumor-bearing mice with various treatments. (J) Percent survival of tumor harboring mice with various treatments. (K,L) Hematoxylin and eosin (H&E) staining of lung tissue from naive mice and COF-609+ α CD47 group. The yellow circles indicate metastatic foci.

bandgap structure, steady-state PL, lifetime, and quantum yield value. More importantly, such excellent ROS generation efficiency induced by COF-609 could further trigger endoplasmic reticulum (ER) stress, which are critical for eliciting robust ICD (Figure S29).

As it is known, ICD plays an important role in triggering antitumor immune responses, which could further stimulate an abscopal effect to achieve long-term antineoplastic activity. Here, the expression of three representative ICD parameters, including ATP, CRT, and HMGB1, was investigated to assess the ICD eliciting capability of COF-609 and COF-909 (Figure 3D). The ATP released by 4T1 cells was found to be obviously increased after being treated with COF-609, where other groups, including phosphate buffered saline (PBS), monomer, and COF-909, displayed almost no distinctly ATP release (Figure 3E). A similar tendency could also be found in their CRT expression experiment, where a clear red fluorescent signal was only observed at the surface of 4T1 cells after being treated with COF-609; their corresponding monomer and COF-909 group exhibited no obvious red fluorescent signal (Figure 3G). These, combined with the increased release of HMGB1 (Figures 3F and S26), demonstrated high ICD eliciting efficiency of COF-609. The intracellular uptake of COF-609 was proven by flow cytometry in a mouse breast cancer (4T1) cell line where over 60% of COF particles could be internalized into 4T1 cells after incubation for 12 h, suitable for *in vivo* experiments (Figure 3B). To further evaluate the PDT efficiency of COF-609, a cell counting kit (CCK8) assay was carried out. The results showed that more than 90% cell viability in the dark was observed, even treated with COF-609 at different concentration, confirming the good biocompatibility of COF-609 (Figure 3C). However, after irradiated under 660 nm laser for 5 min, less than 30% of tumor cells could be survived when treated with COF-609 at a concentration of 100 $\mu\text{g/mL}$, demonstrating the high power of COF-609 mediated PDT. The excellent antitumor performance of COF-609 was also confirmed by using a 4T1 tumor-bearing mice animal model (Figure 4A). In comparison to PBS group, the COF-609+Laser group showed a much smaller tumor volume and weight, indicating effective tumor suppression by PDT primed ICD (Figures 4B,C and S20). Furthermore, no obvious body weight change was observed in any group during treatment with COF-609, indicating the minimal systemic toxicity of COF-609 (Figure 4D). The hematoxylin and eosin (H&E) staining results also proved the good antitumor performance of COF-609, where few tumor cell deaths were observed in the control groups, including PBS, PBS+Laser, and COF-609 without laser irradiation groups (Figure S21). In contrast, grave damage could be observed in COF-609+Laser group (Figure S21). Similar results could also be found in the immuno-histochemical staining studies, where a drastic decrease in Ki67 expression was only found in the COF-609+Laser group, which indicated that the cancer cell proliferation rate of this group was much lower than those of the other groups, and revealed the favorable antitumor efficiency of COF-609 (Figure S21C).

To assess the antitumor efficiency of COF-609, antitumor immune profile changes were studied in both the tumor macroenvironment (tumor draining lymph nodes and spleens) and tumor microenvironment *in vivo*. The gating strategy is shown in Figure S22. Flow cytometry analysis was used to assess the ratio of T cells in draining lymph nodes (Figures 4E,F and S28),³⁸ which indicated that the ratio T cells (CD3^+

cells) were obviously upregulated after treatment with COF-609. Furthermore, regulatory T cells, which play a vital role in impeding the antitumor effective T cell response, were also systematically detected^{39,40} where a sharp decrease in the proportion of Treg cells ($\text{CD4}^+\text{CD25}^+\text{Foxp3}^+$ T cells) was observed and unveiled that the immune suppressive status in 4T1 tumor-bearing mice was partially normalized after treatment with COF-609 mediated antitumor immunity (Figure 4G,H). Moreover, the spleen of tumor-harboring mice, representing a distinct niche for generating MDSCs, a kind of cell that could potentially fuel the immune suppressive status, was also tested.^{41–43} Clear splenomegaly was observed in the PBS and COF-609 without laser irradiation groups. In contrast, a sharp decrease in spleen size was observed in the COF-609+Laser group (Figure S23). This, combined with a drastic decrease in $\text{CD11b}^+\text{Ly6g}^+$ MDSCs and monocytic $\text{CD11b}^+\text{Ly6g}^-\text{Ly6c}^+$ MDSCs, unambiguously revealed that COF-609 mediated ICD could effectively normalize the antitumor immune response in tumor microenvironment (TME) (Figures 4I,J and S24 and 25). Next, an immuno-fluorescence staining strategy was used to further investigate the immune profile change in the TME. Obvious green fluorescence could be observed in the COF-609 group, while no apparent fluorescence was observed in cells treated with PBS and COF-609 without laser irradiation groups (Figure 4K), revealing CD3^+ positive T cells were highly infiltrated in the TME and transformed immunologically cold tumors into hot tumors after treatment with COF-609 under laser irradiation.

CD47, which serves as a “don’t eat me” signal for preventing the engulfment of cancer cells by antigen presenting cells, could inhibit the initiation of antitumor immune response.⁴⁴ CD47 blockade not only enhances the phagocytosis process but also triggers the cytotoxic T cell function for killing tumor cells, which make it effective in multiple clinical trials.^{45,46} However, single CD47 blockade therapy mode is inefficient in treating breast cancer. Here, we revealed that the CD47 blockade efficacy could be potentiated by COF-609 induced ICD. The CD47 expression behavior was studied first, which showed that CD47 was highly expressed in 4T1 tumor cells (Figure 5A). However, the single CD47 blockade therapy mode is inefficient in treating 4T1 tumor-bearing mice (Figure 5B,C). To explore the potential of potentiating CD47 blockade therapy by COF-609 mediated antitumor immunity, 4T1 tumor cells were injected into the both sides of Balb/c mice to establish a bilateral breast cancer mouse model (Figure 5D). Then, after combined CD47 blockade therapy with COF-609 mediated antitumor immunity, the growth of primary tumors could be sharply regressed in both COF-609 and COF-609+ αCD47 treatment groups. More importantly, the distant tumors were effectively delayed in the COF-609+ αCD47 treatment group, where only partially tumor inhibition could be achieved in both CD47 and COF-609 treated groups, uncovering the high efficiency of such synergistic combination therapy in regression of both primary and distant tumors (Figure 5E,F). In addition, T cells in the draining lymph nodes were also upregulated after this combination therapy (Figure S28).

Immune memory effect plays a crucial role for inhibiting tumor metastasis and recurrence. To evaluate the immune memory effect of COF-609 mediated antitumor immunity, we established a rechallenged tumor mouse model through reinoculating 4T1 tumor cells into the mice, which were

previously cured by COF-609 (Figure 5H). In order to assess the immune memory effect of COF-609 systematically, 4T1 tumor cells were reinoculated into the cured mice two times, via subcutaneous and intravenous injection, respectively. The results showed that almost all of the rechallenged 4T1 tumors could rapidly disappear in the cured mice group even after inoculated with 4T1 tumor cells twice, where the naïve control group displayed a drastic tumor volume increase (Figure 5I), suggesting that COF-609+ α CD47 could be effectively inhibiting tumor recurrence. Meanwhile, the excellent tumor metastases and recurrence inhibiting performance of COF-609+ α CD47 could also be manifested in the H&E staining results of lung tissues, which is a common 4T1 tumor metastasis organ. The H&E-staining images showed that no apparent metastatic lesions were observed in lung tissues of mice cured by COF-609+ α CD47 (Figure 5L), where massive metastases could be observed in the lung tissues of the naïve mice groups (Figure 5K). The survival time of COF-609+ α CD47 group could be drastically prolonged, yielding >95% mice surviving a treatment period of 110 days, where all of the mice that were dead within 40 days were observed in the control group (Figures 5J and S27), revealing that the immune memory protection system of 4T1 tumor-bearing mice was successfully elicited after being treated with COF-609+ α CD47. Furthermore, no obvious body weight change could be observed in both COF-609 and COF-609+ α CD47 treatment groups, revealing the good biosafety of COF-609 (Figure 5G).

In summary, this work was not just a first example of synergistic PDT with cancer immunotherapy by using 3D COFs for inhibition of cancer metastasis and recurrence, but more importantly, to demonstrate a new way to design ICD inducers, where a large number of ICD inactive organic monomers can be used for the construction of new COF-based ICD inducers. Such strong ICD could successfully trigger antitumor immune responses and stimulate an abscopal effect to achieve long-term antineoplastic activity. To the best of our knowledge, this is the first example of COF-based ICD inducers, which was constructed by ICD inert monomers, and could elicit durable antitumor immune response effectively, pave a new avenue for COF primed cancer immunotherapy, and encourage more studies on exploring the biomedical applications in COFs.

■ ASSOCIATED CONTENT

SI Supporting Information

(PDF) The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.nanolett.1c02050>.

Additional experimental details, including instrumentations; cell culture; tumor model; rechallenger assay; flow cytometry; tissue slices staining and scanning; phagocytosis; cytotoxicity; zeta potential analysis; FT-IR spectra for ligands and COFs; X-ray photoelectron spectrometer (XPS) measurement; UV-vis diffuse reflectance spectra; N₂ adsorptions analysis; scanning electron microscopy (SEM) images; crystal structure of these COFs; additional results (Figures S1–S29) (PDF)

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Notes

The authors declare no competing financial interest.

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