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CRITICAL PERSPECTIVES

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Zinc oxide nanoparticles from drug delivery to immunomodulation: progress and challenges

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ABSTRACT

Introduction: Over the last decades, zinc oxide nanoparticles (ZnO NPs) have emerged as promising nanoplatforms for various biomedical applications. This critical perspective summarizes the main uses of ZnO NPs in cancer therapy, focusing on their roles in advanced drug delivery, stimuli-responsive systems, and immunomodulatory treatments targeting tumor tissues.

Areas covered: Due to their intrinsic physicochemical properties, ZnO NPs can dissolve in the acidic tumor microenvironment and generate radical oxygen species, causing metabolic dysregulations that lead to cancer cell apoptosis. When engineered into multimodal nanoplatforms, the combination of ZnO with standard cancer therapies, such as chemotherapy and immunotherapy, or with energy-activated treatments like photodynamic and sonodynamic therapy, achieves synergistic antitumor effects, overcoming many limitations of current standards of care.

Expert opinion: Crucially, ZnO demonstrates a strong immunomodulatory capability, promoting T-cell activation and dendritic cell maturation necessary to reverse the 'cold' tumor microenvironment often associated with solid and deep-seated tumors. Overall, ZnO NPs offer revolutionary therapeutic prospects for novel anticancer treatments, provided that challenges regarding long-term stability and controlled degradability are addressed in future works for clinical translation.

PLAIN LANGUAGE SUMMARY

Current cancer treatments often feel like a double-edged sword: they can kill the tumor, but they often take a heavy toll on the patient's healthy organs. Our research looks at a more surgical approach using Zinc Oxide Nanoparticles (ZnO NPs), engineered particles so small they can navigate the body's internal landscape. We like to think of these particles as a 'triple threat.' Instead of just being a passive box for medicine, ZnO NPs are active participants. They can be programmed to dissolve only when they hit the acidic environment inside a tumor, acting like a 'smart' carrier that stays closed until it reaches the right target. Once there, they do not just release their cargo; the zinc itself breaks down to create a burst of reactive molecules that disrupt the cancer's internal metabolism.

But the real 'game-changer' is their biodegradability. Most metal-based treatments, like gold or iron, are 'biopersistent,' meaning they can be stuck in the liver or spleen for years. Zinc is different. It is a natural element your body already knows how to handle, so it dissolves, and its excess clears out safely.

Beyond just killing cells, these particles act as a flare for the immune system. They turn 'cold' tumors, which can escape the body's natural defenses, into 'hot' targets that the immune system can actually find. This triggers what we call an 'abscopal effect': a vaccine-like response where the body learns to destroy distant cancer cells that have spread elsewhere. While we are still perfecting how to keep these particles stable in the bloodstream, they represent a massive advancement toward a future where cancer treatment is localized, systemic, and significantly gentler on the patient.

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1. Introduction

Despite significant advancements in cancer treatments, conventional standards of care often face critical limitations such as systemic toxicity, the development of multidrug resistance, and serious collateral effects due to the lack of therapeutic selectivity. Furthermore, solid and deep-seated tumors frequently present a 'cold' tumor immune microenvironment (TME) characterized by immunosuppression and poor infiltration of cytotoxic immune cells, restricting the efficacy of modern immunotherapies. In this context, nanomedicine aims for a paradigm shift by proposing engineered nanoplatforms capable of achieving targeted delivery and spatiotemporal selective therapeutic action. In our experience, among various nanomaterials, zinc oxide nanoparticles (ZnO NPs) represent a unique solution to address these limitations due to their dual role as both therapeutic agents and multifunctional nanocarriers. Indeed, ZnO possesses intrinsic physicochemical properties, such as a wide bandgap and pH-dependent solubility, that allow it to dynamically respond to the acidic TME.

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Article highlights

- ZnO NPs act as a 'triple-threat' in oncology, serving simultaneously as dose-dependent cytotoxic agents, smart drug delivery vehicles, and potent immunomodulators.
- ZnO is a versatile platform for energy-activated therapies, acting as both a photosensitizer for PDT and a sonosensitizer for SDT to achieve synergistic tumor ablation.
- The intrinsic instability of ZnO in acidic environments is exploited for the selective, 'burst' release of chemotherapeutic cargo directly within the tumor microenvironment.
- ZnO-based treatments promote dendritic cell maturation and T-cell activation, leveraging the abscopal effect to target both primary tumors and distant metastases.
- Advanced ZnO nanoplatforms can alleviate tumor hypoxia and reduce fibrosis, effectively remodeling the TME to enhance the efficacy of combined immunotherapy.
- Transitioning toward next-generation zinc-based systems, such as MOFs and nanozymes, is essential to address current challenges in colloidal stability and clinical implementation.

This response triggers the release of Zn^{2+} ions and the production of Radical Oxygen Species (ROS), which disrupt metabolic pathways and trigger apoptosis in malignant cells while sparing healthy tissues. The goal of this critical perspective is to summarize the dynamic and rapid evolution of ZnO-based systems. Reflecting the progression of our own research over the last decade, this opinion is structured around the transition from the study of ZnO as a standalone cytotoxic agent to its development as a drug delivery carrier and stimuli-responsive platform. Building on these foundations, our most recent *in vivo* study has highlighted the transition toward the use of ZnO NPs as an active immunomodulator (Figure 1). These innovative strategies have been widely employed to induce immunogenic cell death (ICD) and to reverse TME immunosuppression, a promising potential for the treatment of deep-seated tumors as well as rare diseases that still lack effective standard-of-care protocols. As discussed in our Critical Perspective, the field is rapidly shifting toward next-generation zinc-based materials to overcome translational hurdles such as colloidal stability and controlled degradability, pivotal to ensure efficient treatment or drug release triggered by either external or endogenous stimulations. This Critical Perspective provides a comprehensive overview of ZnO NPs in oncology, starting from their mechanisms of cytotoxicity and ROS generation, exploring their application as advanced drug delivery vehicles and stimuli-responsive agents, and

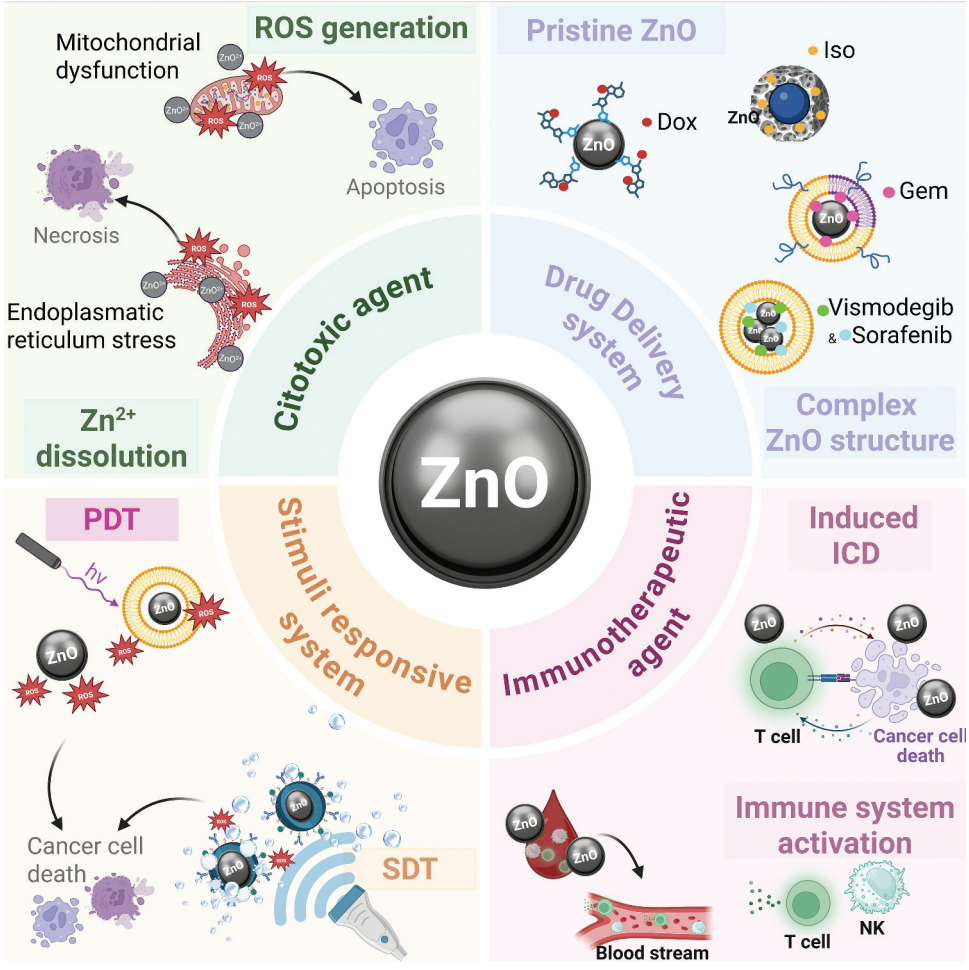

Figure 1. One nanomaterial, multiple anticancer strategies: zinc oxide nanoparticles emerge as a multifunctional and highly effective platform, bridging drug delivery, therapy, stimulus-responsiveness, and tumor-selective mechanisms to tackle cancer from multiple angles. Created with Biorender.com.

finally focusing on their emerging potential to elicit immune modulation.

2. ZnO NPs

2.1. ZnO as a cytotoxic agent

ZnO NPs are among the most widely engineered nanomaterials currently available [1] due to their remarkable properties; their applications span a wide variety of fields, from agriculture [2] to optics [3], with significant emphasis on nanomedicine, especially in cancer treatment. They are considered biocompatible, and their role as anti-bacterial agents [4], as well as promoters for wound healing [5] have been extensively studied in the past.

Nevertheless, ZnO can act as a cytotoxic agent, especially in its pristine state, validating its extensive use in nanomedicine, primarily when ZnO NPs are engineered and functionalized to be selectively delivered to malignant cells. Its mechanism of action as a cytotoxic agent, which is clearly a dose-dependent process, primarily relies on two distinct, but interlinked, phenomena: the dissolution of ZnO into Zn^{2+} ions and the production of ROS.

The intracellular cytosol of cancer cells provides an acidic environment, with a pH of ~6.0–6.5 [6,7], that significantly increases the dissolution rate of ZnO NPs, elevating the intracellular concentration of Zn^{2+}. Zinc cations play indeed a crucial role in cellular homeostasis, regulating cell cycle progression, immune functions, and meiosis, and are gaining increasing attention in cancer research [8]. Under normal physiological circumstances, zinc concentration is able to meet cellular needs, assuring the correct functioning of cellular activity (energy metabolism, gene expression, and genome integrity maintenance [9]). However, upregulation can lead to necrosis via mitochondrial dysfunction and endoplasmatic reticulum stress; apoptosis through mitochondrial electron transport chain damage, caspase cascade reaction, or TNF-α-mediated exogenous apoptotic pathway; or pyroptosis, via inflammatory vesicle signaling [10].

ZnO NPs can also be responsible for an imbalance of intracellular ROS (i.e. O_2^-, H_2O_2, and ·OH) leading to oxidative cellular stress [11]. Optimal physiological functions are ensured by maintaining a crucial balance between ROS production and ROS elimination [12]. ZnO dissolution and the subsequent increase in Zn^{2+} directly upregulate mitochondrial ROS production through two main pathways. Zn^{2+} accumulation into mitochondria can cause the impairment of the electron transport chain leading to mitochondrial O_2^- accumulation, which damages them and causes the release of cytochrome c, which interacts with apoptosis-activating factor 1 to form apoptotic vesicles [10]. Furthermore, Zn^{2+} can also lead to the downregulation of caspase-8 expression, affecting the apoptosis pathway mediated by tumor necrosis factor-α (TNF-α) [10].

The role of ZnO nanoparticles in ROS upregulation is not solely attributed to its dissolution, but also to the intrinsic semiconducting nature of the nanoparticle itself, which confers unique properties. The optical properties of ZnO nanoparticles, characterized by a 3.3 eV band gap, enable them to be excited by UV light to generate photo-excited electrons that reduce oxygen to O_2^-, while holes oxidize water and hydroxide ions, producing ·OH and H_2O_2 [13]. This is the fundamental principle of Photodynamic Therapy (PDT), widely proposed in cancer research, where ZnO NPs act as photosensitizers upon intracellular uptake by tumor cells [14]. Light is not the only external stimulus capable of triggering this activation: ultrasound is another widely investigated energy source [11,15–17], as discussed in subsequent chapters.

Due to their dose-dependent cytotoxic activity, ZnO NPs can act as therapeutic agents themself. Hence, it is fundamental to implement active targeting strategies that allow for selective accumulation in cancer cells while sparing healthy tissues, leveraging the inherent toxicity and selectivity of ZnO nanoparticles against cancer cells [18].

2.2. ZnO for drug delivery

Overcoming drug resistance and achieving improved therapeutic intratumoral drug delivery represent crucial challenges in cancer research [19], which are strategically addressed by nanomedicine. Zinc-oxide-based nanoparticles represent a remarkable and extensively investigated option due to their outstanding physiochemical properties and preferential cytotoxic action toward cancer cells [20]. Consequently, in the past years, they have been widely proposed as carriers for numerous pharmaceutical agents [19,21–23]. In our research group, Percivalle et al. conducted a detailed computational study on the ability of ZnO nanoparticles to act as carriers for carfilzomib (CFZ) [21]. Their innovative works on molecular simulations elucidated the adsorption mechanism of CFZ on the ZnO surface and its thermodynamic interaction, underlining the need to functionalize the ZnO surface by increasing its hydrophobicity prior to drug adsorption. This modification results to be essential to avoid possible drug deactivation and ensure optimal loading efficiency. In addition to CFZ, ZnO nanomaterials have been proposed as delivery carriers for various other drugs, including Curcumin, Paclitaxel, Isotretinoin, Camptothecin, and Daunorubicin [23]. The acidic and inflamed TME can be exploited as a triggering signal for the controlled release of drugs loaded onto the ZnO NP surface. Pristine ZnO nanoparticles are stable under physiological pH conditions but dissolve quickly into Zn^{2+} under acidic conditions, characteristic of the TME. Several drug payloads, such as doxorubicin [24,25] and Isotretinoin [26], among many others, were efficiently delivered through this acidic-mediated dissolution mechanism, obtaining a pH-controlled and efficient drug delivery system, more efficacious than the pristine free drugs. Preventing premature drug release before reaching target cells is a key aspect when ZnO NPs are exploited for drug delivery purposes; therefore, multiple shielding strategies have been also explored [27]. Barui et al. investigated the use of Gadolinium-doped Zinc Oxide (ZnO-Gd) as a Gemcitabine carrier for the treatment of pancreatic cancer [19]. The drug was secured onto the ZnO-Gd surface and shielded by a biomimetic hybrid shell composed of artificial lipids and extracellular vesicles derived from healthy B-lymphocytes, engineered with a targeting agent. This sophisticated nanoconstruct demonstrated superior capability as a drug delivery

system, without altering its mechanism of action. Drawing on the expertise in ZnO nanoparticle synthesis, Barui et al. proposed gadolinium-doped ZnO as a dual drug carrier for Vismodegib and Sorafenib, designed to specifically address pancreatic cancer [22]. The dual drug strategy was adopted to target both the pancreatic cancer cells and the tumor microenvironment, specifically the angiogenic vessels, an approach facilitated by ZnO surface reactivity. Their study demonstrated the synergistic action of the two drugs when administered simultaneously by means of these nanovehicles, offering an innovative and efficient approach that avoids covalent bonding and the use of toxic solvents.

3. ZnO as a stimuli-responsive material

Achieving efficient and targeted drug delivery in the tumor area remains one of the biggest challenges of drug delivery systems [28–30]. For this reason, designing nanoplatforms able to accumulate in specific target organs by means of active targeting while allowing a precise and controlled release of their cargo in response to both external or endogenous stimuli is of pivotal importance [31].

As introduced above, PDT and SDT are minimally invasive treatments that utilize either a photo- or sonosensitizer, respectively, and an energy source – namely light at a specific wavelength, or ultrasound waves at non-thermal powers and doses, thus harmless to healthy cells. These waves act to trigger the sonosensitizer, generating ROS for the selective destruction of diseased tissues, such as tumors [32,33]. Due to their intrinsic physicochemical properties, ZnO NPs are often incorporated as a stimuli-responsive material in both PDT and SDT. Indeed, ZnO NPs can act as both a photosensitizer [34,35] and a sonosensitizer [36–38] and can react to these external stimulations (light and ultrasound) to generate ROS, producing measurable effects through various action mechanisms in biological tissues. The introduction of defects in the crystalline structure of ZnO can be pivotal to increase the reactive oxygen species generation, establishing these ZnO NPs as sonosensitizers. Over the years, our research group has produced various works focused on the synthesis and anticancer applications of ZnO NPs. Indeed, Carofiglio et al. demonstrated the superior performance of iron-doped ZnO (Fe:ZnO) NPs with respect to pristine ones, claiming increased colloidal stability, magnetic properties, and cytocompatibility [39]. These biodegradable and biocompatible Fe:ZnO NPs were then tested in combination with acoustic shock waves (SW) in an *in vitro* model of pancreatic cancer and its healthy counterpart, obtaining promising synergistic effects resulting in cancer cell death via apoptosis and necrosis [17]. The same external stimulus was employed in combination with Fe:ZnO for the treatment of colorectal cancer, following either a single or a repeated and sequenced SW treatment (three times/day, 3SW) after Fe:ZnO exposure. This combined therapy resulted in a synergistic antitumor activity, not observable when the single treatments (SW or Fe:NPs) were administered separately [16]. Moving toward more complex *in vitro* models, Rosso and colleagues implemented 3D colorectal cancer cell (CRC) spheroids which were treated with SW and lipid coated Fe:ZnO NPs, employing a targeting peptide selective toward CRC cells (YSA) [40]. Synergistic effects of the combined treatment were observed in these spheroids, leading to a complete ablation of the CRC mass. Building on these results, Carofiglio and coworkers further corroborated the stimuli-responsiveness of targeted, lipid-coated Fe:ZnO (safe and biocompatible when administered alone) employing both a different excitation source and a different 3D spheroid model [41]. The administration of Fe:ZnO and the following ultrasound stimulation (1 MHz, 1 or 2 W/cm^2, 1 min) on 3D spheroids of bone osteosarcoma led to ROS generation and tumor growth suppression. Similarly, in a more advanced and complex 3D co-axially bioprinted model, lipid-coated ZnO NPs selectively localized within colorectal cancer masses growing in the lumen of a healthy tissue conduit. Synergistic treatment with low-intensity US or SW achieved precise tumor ablation while sparing surrounding healthy cells, confirming the potential for highly targeted therapeutic interventions [42].

Advancing to preclinical animal models, the limitations of both PDT and SDT, such as the low penetration of light through tissues, the accuracy of the tumor irradiation, and the tissue oxygenation, which is often very poor in deep-seated tissues, must be carefully considered [43]. Indeed, in the context of PDT, the use of ZnO can be crucial for the upconversion-like effect, allowing for the use of deep-penetrating near-infrared light, which is absorbed by ZnO and then re-emitted at much lower wavelength, thus exciting nearby photosensitizers directly deep inside the body [44]. Moreover, by trapping gas bubbles on their surfaces, ZnO NPs can partially help overcome tumor hypoxia, while simultaneously catalyzing the decomposition of H_2O_2, abundant in tumors, into water and O_2, boosting ROS yield [42]. Major limitations of SDT are the low efficiency of sonosensitizers, their low biocompatibility, and their inability to reach the target tumor. ZnO NPs have intrinsic piezoelectric properties and can therefore enhance ROS production with respect to other sonosensitizers, while remaining completely biocompatible [11]. Thanks to their large surface area, ZnO NPs can carry gas pockets on their structures, which in turn increase the effect of acoustic cavitation triggered by ultrasound stimulation [45]. Moreover, thanks to surface functionalization strategies, biomimetic coatings, and active targeting, ZnO NPs can increase their circulation time, efficiently reach the region of interest, and accumulate in tumors once systemically injected, dramatically increasing the effect of SDT [46].

Hu's work addressed the low yield of ROS typically generated during SDT treatment by employing iron (Fe) and manganese (Mn) doped ZnO nanosonosensitizers, producing both ferroptosis and apoptosis of tumor cells. The ZnO core had a crucial dual role: augmenting SDT efficiency through the Fe/Mn co-doping strategy and actively triggering ferroptosis via Fenton-like reaction with H_2O_2 overexpressed by the TME. The resulting synergistic effect, which involved both ferroptosis and apoptosis pathways, was identified by RNA sequencing [47]. Wu and colleagues designed a core-shell nanoconstruct consisting of ZnO NPs as the core, an interlayer of photosensitizer chlorin e6 (Ce6), and an outer layer of polydopamine (PDA) for synergistic photodynamic and photothermal therapy. In this system, the direct photo-responsive therapeutic action is provided by the Ce6 and PDA components. However,

the ZnO core, which serves as a structural backbone and drug delivery vehicle for Ce6, possesses intrinsic pH-responsiveness, a crucial mechanism that could transform the dual-modal system into a triple-responsive platform, a potential which is, however, overlooked by the authors [35]. Liu et al. engineered a defect-rich gadolinium-doped ZnO_x nanobullet with superior ROS production capacity compared to defect-free ZnO and other benchmark sonosensitizers. The nanoplatform also provides MRI (Magnetic Resonance Imaging) guidance, thanks to the presence of Gd, and offers good optical absorption in the near-infrared region, enabling a synergistic SDT/PTT (photothermal therapy) treatment for *in vivo* tumor eradication [38].

As previously stated, ZnO NPs can react not only to exogenous stimuli (such as light or ultrasound) but also to endogenous ones (acidic pH of tumors, enzymes of the TME) [48], and a precise design of the carrier can ensure a timely and spatially selective cargo delivery.

Cai and coworkers developed pH-responsive nanocarriers based on ZnO quantum dots (QDs) cross-linked by poly(ethylene glycol) (PEG) for doxorubicin (DOX) delivery to tumors [49]. By leveraging ZnO rapid dissolution below pH 5.5, the stable nanocarrier was able to efficiently release its cargo, which was previously anchored via a Zn^{2+}-DOX chelate complex. Crucially, the subsequent release of Zn^{2+} ions contributed to the system's enhanced and selective cytotoxicity toward cancer cells. Similarly, Liu et al. bypassed multidrug resistance (MDR) by using DOX-loaded ZnO NPs to circumvent *P*-glycoprotein efflux pumps. Internalized via endocytosis, the nanoplatform achieved high drug retention compared to free DOX and a pH-triggered cytoplasmic burst release. Furthermore, the quenching and subsequent restoration of DOX fluorescence upon carrier dissolution enabled real-time theranostic monitoring of drug delivery [50].

Sadhukhan and coworkers synthetized ZnO NPs functionalized with phenylboronic acid (PBA) and loaded with quercetin (Q) for breast cancer drug delivery [51]. The combinatorial effect of quercetin and Zn^{2+}, both released under acidic conditions, enhanced ROS generation inducing apoptosis, which resulted in tumor growth inhibition *in vivo* without systemic toxicity. Collectively, the intrinsic multifunctionality and stimuli responsiveness of ZnO NPs establish them as nanoplatforms well suited for selective tumor accumulation, pH-responsive drug release, and potent response for external and endogenous stimulations, leveraging their potential as highly efficient

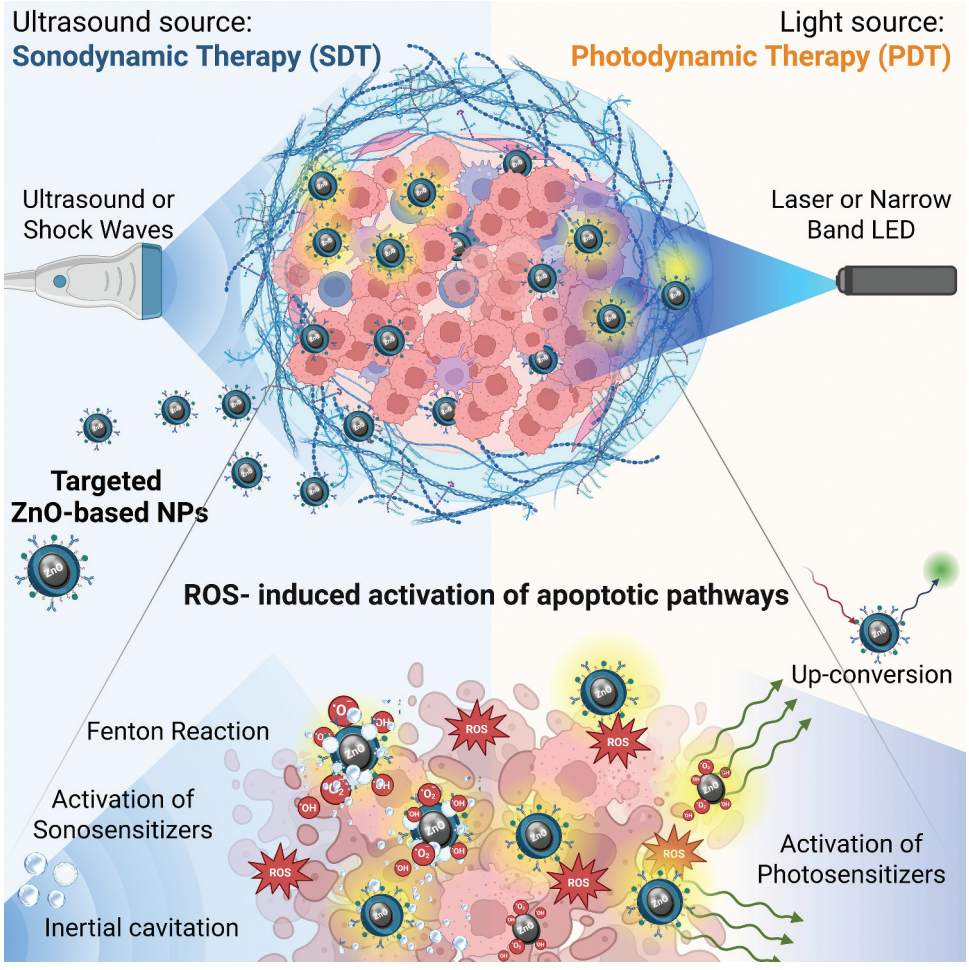

Figure 2. Schematic representation of anticancer activity of stimuli-responsive ZnO based nanoparticles. Stimuli-responsive ZnO NPs produce ROS and are able to induce activation of apoptotic pathways in cancerous cells. This interesting phenomenon can be activated by lasers or narrow-band LEDs (PDT), and by ultrasound exposure. Created with Biorender.com.

carriers capable of overcoming critical barriers in targeted drug delivery and SDT/PDT applications (Figure 2).

4. ZnO as immunomodulatory agent

In cancer therapy, considerable attention has been recently given to the importance of improving the penetration of immune cells such as cytotoxic T lymphocytes (CTLs) in the tumor site, since their scarce infiltration is nowadays among the main causes of resistance to immunotherapy [52]. Immunogenic cell death (ICD) inducers are therapeutics that can elicit a specific type of cell death that facilitates the recognition of dying cancer cells by the immune system. Indeed, this type of cancer cell death is characterized by the release or cell-surface expression of highly immunostimulatory damage-associates molecular patterns (DAMPs), triggering innate and adaptive immune responses [53]. Being able to incorporate tumor-specific ICD inducers in standard cancer therapies would highly impact their outcome and the overall response of cancer patients. As previously highlighted, ZnO dissolution in Zn^{2+} ions can disrupt many molecular pathways, inducing cell death via necrosis, apoptosis or pyroptosis, and can therefore be combined with immunotherapy to enhance the production of tumor-associated antigens [10]. Some research groups have also proved that, even without incorporating immunotherapy moieties in the ZnO-based nanoconstructs, ICD could be induced by applying exogenous stimuli both *in vitro* and *in vivo* after their administration. Zhang and colleagues demonstrated that DOX-loaded multifunctional nanocomposites made of a gold core and ZnO QDs could elicit ICD *in vivo*. The nanocomposites combined photothermal and pH-responsive properties, while showing preferential tumor accumulation in unilateral and bilateral subcutaneous melanoma and lung metastasis. ICD promoted maturation of dendritic cells (DCs), stimulated the infiltration of effector T cells into the tumor site and prevented both tumor growth and lung metastasis [50]. J. Wang and colleagues obtained multifunctional ZnO-Ce6 NPs, able to overcome the two major limitations of traditional PDT, namely poor light penetration and tumor hypoxia. Indeed, ZnO NPs exhibited an intrinsic upconversion-like property, absorbing deep-penetrating 808 nm NIR light and re-emitting 401 nm visible light, thus exciting Ce6 and allowing PDT to be triggered deep within the tissue. Moreover, ZnO acted as a catalase-like nanozyme, exploiting the high presence of H_2O_2 within the TME and converting it in O_2 in situ, while also inducing ferroptosis and ICD. Results *in vivo* showed tumor growth inhibition, hypoxia alleviation, and tumor eradication in some mice [54]. Interestingly, they also assessed the ability of the combined therapy to promote DC maturation and T cell activation *in vivo*: increased mature DCs in tumor draining lymph nodes and spleen were observed, together with enhanced $CD4^+$ and $CD8^+$ T cell proliferation, NK cell upregulation and Treg downregulation. Hira et al. prepared pectin-guar gum-ZnO nanocomposites as immunomodulator to improve cancer cells killing capabilities of human peripheral-blood lymphocytes (PBLs) [55]. They demonstrated activation of the overall T-cell population, especially cytotoxic T cells (CD8+), and activation of natural killer (NK) cells, proving that the ZnO component was responsible for such activation; pro-inflammatory cytokines such as interferon gamma (IFN-γ), interleukin-2 (IL-2) and tumor necrosis factor alpha (TNF- α) were also produced by PBLs after nanocomposite administration. This translated into a better ability to kill cancer cells, observed through co-cultures of pre-treated PBLs with lung and breast cancer cells. To further investigate the immune modulatory potential of ZnO NPs in human lymphocyte, Moratin and coworkers pre-treated primary human pPBL of nine donors with sub-cytotoxic concentrations of ZnO NPs and then analyzed their changes in the activation profile and the proportion of T cell subpopulations [56]. Surprisingly, in this study ZnO did not induce significant alterations in the examined markers and therefore no immune modulation was detected at non-cytotoxic concentrations. The authors concluded highlighting the need for more complex *in vitro* settings, such as the addition of stimulating factors like cytokine-rich cell culture medium, the presence of antigen-presenting cells and longer incubation times. Conversely, X. Wang and colleagues demonstrated that hollow ZnO nanospheres (HZnO) could enhance the immune system anticancer response by boosting the activation of T cell populations [57]. Actually, the hollow structure of such HZnO provided a large cavity and superior surface area compared to dense ZnO, allowing for the loading of antigens like ovalbumin (OVA) and immune stimulants like poly(I:C). This strategy further promoted antigen uptake by bone marrow-derived dendritic cells (BMDCs) and the secretion of the cytokine granulocyte macrophage colony-stimulating factor (GM-CSF) by macrophage-like cells *in vitro*. To prove the anticancer immunity *in vivo*, mice were immunized with HZnO-OVA-Poly(I:C) before cancer cell challenge: HZnO significantly slowed cancer growth, prevented metastases formation to the inguinal lymph nodes and improved the population of both $CD4^+$ and $CD8^+$ cells in spleens, demonstrating a systemic immune response. Further confirmation was obtained using autologous tumor fragments as the antigen loaded in HZnO, which resulted in re-challenge inhibition and increased $CD8^+$ T cell population. A work by J. Wang et al. explored the role of DOX-loaded ZnO NPs as drug nanocarriers, cytotoxic agents, drug sensitizers and immunological adjuvants against MDR cancer [24]. pH-responsive release at acidic pH in tumors and remarkably higher cellular uptake in MDR cells compared to free DOX were achieved. Downregulation of the expression of CD44 (a key cancer stem cell surface marker and receptor) in cancer cells was observed, leading to inhibition of cancer cell adhesion and migration *in vitro* and spheroid disintegration. Intracellular ROS production and ROS-mediated apoptosis pathways were observed; pro-inflammatory cytokines production, M1 co-stimulatory markers increase on macrophages and the production of pro-inflammatory cytokines in the medium conditioned by ZnO/DOS-activated macrophages all contributed to cancer cells killing efficacy. Similarly, Gomaa and coworkers investigated the potential of ZnO nanocomposites loaded with DOX and folic acid (ZnONPs/DOX/FA) *in vitro* and *in vivo* against Ehrlich ascites carcinoma (EAC)

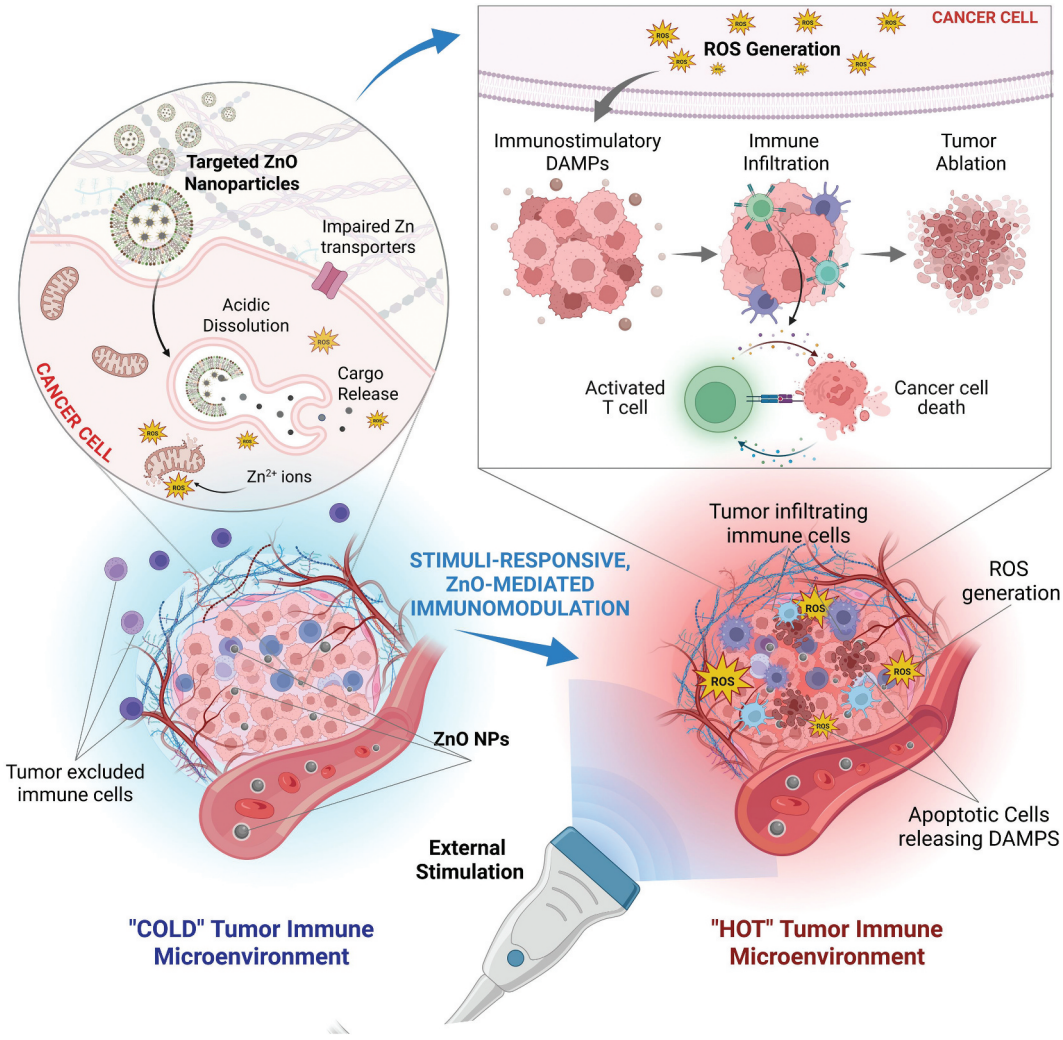

Figure 3. Mechanism of ZnO-mediated immunomodulation: stimuli-responsive ZnO NPs produce a systemic immune response via immunogenic cell death (ICD). External stimulation triggers localized ROS generation and DAMP release, catalyzing the recruitment and activation of cytotoxic T cells for targeted tumor destruction. This mechanism effectively shifts an intrinsically 'cold' immunosuppressive tumor microenvironment (TME) into a 'hot' one, bridging the gap between localized physical therapy and systemic anticancer immunomodulation. Created with Biorender.com.

tumor cell line [58]. ZnONPs/DOX/FA showed targeted efficacy *in vitro*, mainly relying on necrosis and late-apoptosis of EAC tumor cells due to ROS generation. Moreover, they exhibited a suppressive effect on pro-inflammatory cytokines (IL-6 and TNF-α) in the tumor environment *in vivo*, a significant increase in splenocyte count, suggesting reduced overall toxicity to immune cells compared to free DOX, and protection against liver and kidney toxicity typically associated with cancer growth and conventional chemotherapy. Finally, we developed iron-doped, lipid-coated zinc oxide nanoparticles (Lipid-ZnO NPs) enhanced with a fluorescent sonosensitizer (IR780) and local ultrasound stimulation for the treatment of pancreatic ductal adenocarcinoma [59]. ZnO acted as the core immunogenic agent, triggering ROS production *in vitro* and inducing apoptosis *in vivo*. A reduction of fibrosis was observed, demonstrating a remodeling of the TME. Furthermore, enhanced immune cell infiltration in the tumor area was quantitively demonstrated through flow cytometry. Indeed, the infiltration of helper T cell ($CD4^+$) and cytotoxic T cell ($CD8^+$) in tumors and proximal lymph nodes demonstrated an immune response to the treatment, which was dependent on the presence of ZnO NPs and did not rely on the ultrasound stimulation alone. This enhanced immune response correlated with a positive therapeutic outcome, including tumor shrinkage and prolonged survival in mice receiving the combined treatment. Overall, research confirms that ZnO NPs can act as potent immunomodulators capable of enhancing T cell and NK cell activation, DC maturation, and systemic anticancer immune responses for improved therapeutic outcomes (Figure 3).

5. Conclusions

Taken together, these works demonstrate the intrinsic potential of ZnO NPs as effective adjuvants to conventional cancer therapy. The release of zinc ions can disrupt a plethora of cancer cells molecular pathways, leading to the induction of oxidative stress, apoptosis, and immune response. By synergizing with other therapies, ZnO NPs can

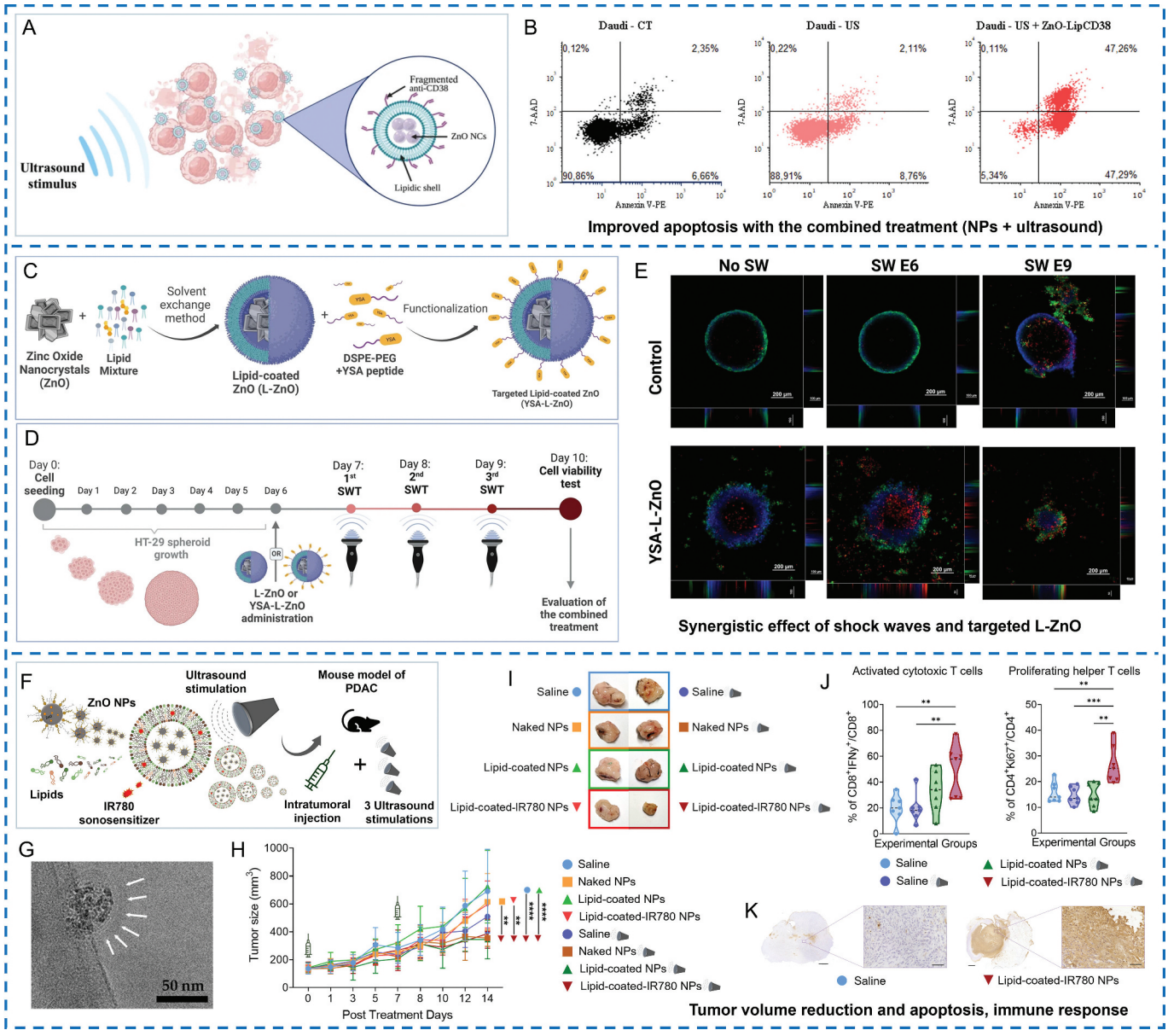

Figure 4. Representative and most promising results achieved by our research group employing ZnO NPs as a stimuli-responsive nanoplatform. (A) Schematic illustration of lipid-coated, AntiCD-38 targeted ZnO NPs administered to Daudi cells and their activation via ultrasound stimulation. (B) Apoptosis/necrosis analysis highlighting the synergistic therapeutic effect of combining ZnO NPs with ultrasound, which increases the percentage of early and apoptotic Daudi cells Reproduced with permission from [15]. (C) Fabrication scheme of the YSA-targeted, lipid-coated NPs. (D) Treatment plan for CRC organoids. (E) Fluorescence microscopy images of organoids after lipid-coated NPs treatment, with and without the YSA peptide, and SW stimulation; staining identifies live cells (Calcein, green), dead cells (PI, red) and cell nuclei (Hoechst, blue). The synergistic effect of shock waves and targeted L-ZnO produces spheroid destruction. Reproduced with permission from [40]. (F) Schematic of lipid-coated NPs loaded with the sonosensitizer IR780. (G) Cryo-elecron microscopy image of the nanoconstruct. (H) *In vivo* treatment protocol. (I) Tumor volume progression *in vivo*. (J) Weights of the explanted tumors. (K) Flow cytometry analysis showing activation of cytotoxic T cells in tumors (left) and proliferating helper T cells in tumor-draining lymph nodes (right). (L) Representative tumor sections, stained with an apoptosis assay, showing expanded apoptotic regions (brown) in treated groups. Reproduced with permission from [59].

help overcome some limitations of SDT, PDT, and immunotherapy, by providing a highly customizable platform able to encapsulate sonosensitizers, photosensitizers, chemotherapeutic and immunotherapeutic drugs, providing targeted and selective delivery in tumors and avoiding systemic toxicity. Figure 4 reports some of the most promising results obtained by our research group when employing ZnO NPs as a stimuli responsive platform: an increased apoptosis of Daudi leukemic cells was observed when using both ZnO NPs and ultrasound stimulation (Figure 4(B)); colon cancer spheroids were ablated when ZnO NPs and shock waves were employed (Figure 4(E)); finally, the combination of ZnO NPs and ultrasound, with the addition of a sonosensitizer, was even able to elicit an immune response in mice bearing subcutaneous pancreatic ductal adenocarcinoma tumor models (Figure 4(I–K)). Some technical

challenges, such as the overall toxicity, the long-term stability, and the controlled degradability of the nanoplatforms, as well as the effective integration of multimodal synergistic therapies must be effectively addressed in view of clinical translation. Nevertheless, ZnO NPs hold the promise of revolutionary therapeutic prospects in the field of novel, nanomedicine-based anticancer strategies.

6. Expert opinion on role of ZnO NPs in immunomodulation and applications on rare diseases

Previous sections have highlighted the enormous multifunctional potential of ZnO NPs as cytotoxic agents, smart drug carriers, and immunomodulators. However, translating these promising *in vitro* and preclinical results into clinical practice remains challenging. A first significant hurdle lies in the complexity of the currently developed ZnO-based nanoplatforms: many successful works have achieved synergistic effects by using more than one external stimulus (combining various lasers, different light wavelengths, or specific ultrasound frequencies). The resulting treatments are therefore very complex and require a fine-tuning of precise dosing, delivery coordination, and device integration, which leads to a complicated regulatory approval process. Indeed, the difficulty in defining a standardized treatment procedure and the implementation into clinical practice would require multiple specialized pieces of equipment and extensive training of clinicians and hospital staff, turning the high potential for personalized treatments into a translational hurdle in clinical settings.

We have recently demonstrated, though, that ZnO NPs could potentially be employed for both diagnosis and treatment by using the same ultrasound stimulation [45]. This aspect would simplify the amount of necessary equipment to obtain a multimodal therapy, leveraging the piezoelectricity, sonoluminescence, and ROS generation ability of ZnO NPs in future clinical applications. We have observed that, in some complex nanoconstructs, the ZnO contribution is regrettably minimized, and the NPs are used predominantly as a structural core or an acidic pH trigger, instead of fully leveraging their intrinsic piezoelectric or nanozyme properties. This approach reduces the unique therapeutic advantage of the ZnO component with respect to other metal oxide NPs.

The unique advantage of ZnO with respect to other well-established drug carriers, which currently outperform ZnO in terms of loading capacity and controlled release, is its intrinsic biodegradability. Gold, silica, iron oxide NPs, and zeolites are biopersistent, meaning that they remain stuck in filtration organs, such as liver and spleen indefinitely, without being able to degrade into harmless byproducts. On the contrary, ZnO are fully biodegradable and are digested in cell lysosomes, zinc being a natural trace element easily handled in specific cellular pathways. This ensures the complete release of their cargo, the dissolution of potential biomimetic coatings, such as lipidic bilayers, and an enhanced cytotoxic action due to the presence of Zn^{2+} ions that are consequently generated. Crucially, healthy tissues possess an intrinsic ability to maintain zinc homeostasis, meaning that the excess of zinc ions can be easily transported out of the cells in normal conditions, while cancer cells suffer from a dysregulation of zinc metabolism [60]. Therefore, we postulate that ZnO NPs would only be detrimental in compromised tissues and not at systemic level [61].

Another major advantage of the use of ZnO NPs with respect to other metal-based NPs relies on the fact that their toxicity has been extensively studied, being ZnO GRAS (generally recognized as safe by the FDA) and employed in the food industry as a nutrient supplement, for food packaging and as a color additive [62,63]. Several studies have already investigated the acute and long-term effects of ZnO NPs over the years, and we believe that their results pave the way to a streamlined regulatory path for future clinical applications [64–66]. Recent evidence highlights that ZnO can be considered by all means as a cytotoxic drug itself, not only due to its dissolution in zinc ions able to disrupt cell metabolism, but more specifically as a sonosensitizer for stimuli-responsive therapy. In fact, ZnO is classified as an inorganic sonosensitizer, such as titania and manganese-based ones [67], with the pivotal difference of being fully biodegradable once injected into the organism. We firmly believe that a future translation of SDT from bench to bedside, which inextricably requires significant attention to the biosafety, excretion, and systemic toxicity of these inorganic nanoparticles, would hugely benefit from the use of ZnO NPs as a biodegradable sonosensitizer.

Many recent works have employed various other Zn-based nanomaterials to maximize Zn^{2+} release and leverage unique mechanisms of action against cancer cells: ZnO_2 is able to degrade producing both Zn^{2+} and H_2O_2, triggering the Fenton reaction [68,69]; ZnS release H_2S, which inhibits the mitochondrial respiratory chain and synergizes with Zn^{2+} to cause energy deprivation in cancer cells; ZIF-8 (Zeolitic Imidazolate Framework-8), a type of metal-organic framework (MOF) material, was proved to induce focal death and ICD, while also serving as a porous drug carrier, providing controlled release, and offering the opportunity to expand the photo-response through metal doping [70,71]; other Zn^{2+} materials, such as zinc copper oxide, can provide metal ion synergy [72]; single-atom nanozymes could dramatically increase catalytic efficiency while potentially reducing overall toxicity by minimizing the total dose required [73].

Overall, Zn-based nanomaterials alternative to ZnO could represent the next-generation systems: they are highly customizable and more efficient in zinc delivery, and they have proved their efficacy in robustly eliciting an anticancer immune response [74]. With respect to ZnO NPs, however, we consider these other Zn-based nanomaterials less prone to advance toward clinical translation due to their lack of chemical simplicity and the generation of potentially toxic byproducts of sulfides, which could constitute a hurdle in the regulatory path.

On the other hand, the simple chemistry of ZnO NPs allows them to be an efficient piezoelectric and semiconductive material, without adding any double-edged complexity. To further exploit the piezoelectric potential of ZnO, while keeping its intrinsic chemical simplicity intact, we advance the notion that other versions of ZnO NPs, such as Desert Roses (DRs), MultiPods (MPs), MicroWires (MWs), and Nanowires (NWs), which we have fully produced and characterized in

our laboratory [75], would possess a shape that is better suited for ROS generations and piezoelectric responses to ultrasound treatments [76]. Indeed, we believe that the application of ZnO-based nanomaterials as anticancer agents could be further expanded by exploiting their intrinsic physical properties, such as piezoelectricity, potential magnetic behavior, and ability to entrap gas bubbles on their surface, hereby extending their use into the diagnostic field and making them promising candidates for theranostic applications. Combining imaging and therapy using a highly responsive core material, such as ZnO, represents both a significant research challenge and a unique opportunity, which can be addressed in future research studies, with the aim of optimizing not only the ZnO-based nanomaterials design but also the external parameters that enable their functionalities.

Additionally, advancing ZnO research requires addressing current technical limitations, particularly in modeling the immune response in a more accurate and rigorous way. Due to the high variability and complexity of the TME in different cancer types, it is very hard to translate promising multimodal therapies involving ZnO NPs without having to recalibrate important parameters such as dosages, external stimuli, and administration routes or times. For instance, deep-seated tumors suffer from poor accessibility and would require external stimulations able to penetrate various centimeters into the abdomen cavity, while more superficial tumors such as melanoma could benefit more from PDT rather than from SDT; tumor accumulation also varies considerably among different organs, and a timely external stimulation after proper tumor targeting can greatly impact the outcome of the multimodal therapy. *In vitro* models lack the cellular heterogeneity, dense desmoplastic stroma, and complex immune cell interactions observed *in vivo*, and therefore they often fail to properly recapitulate the cascade of metabolic and cellular events leading to the immune response.

Despite these significant challenges, we strongly support the view that the versatility of ZnO NPs could offer a transformative path forward. The high sonosensitivity of ZnO enables SDT with a penetration depth of up to 100 mm, effectively reaching the deep-seated tumors that currently evade conventional treatments. Furthermore, by narrowing the bandgap through strategic metal doping and surface functionalization, as we extensively studied in previous works on the topic [39,77,78], ZnO can be transitioned into the visible/NIR window for PDT as well. This way, by slightly changing some crucial aspects during its synthesis or functionalization, the same nanoplatform can be employed for both superficial and deep-seated tumor treatment, ensuring a comprehensive range of anticancer applications.

An essential aspect of future ZnO NPs research concerns the synergy between physical tumor destruction and systemic immunity. In fact, contrary to the prevailing focus on drug loading and, more recently, on stimuli-responsive applications alone, we argue that the true potential of ZnO lies in its ability to elicit an immune response once externally stimulated. The abscopal effect of SDT mediated by ZnO NPs should be further confirmed and demonstrated, not only to recruit immune cells in the tumor while causing its shrinkage but also to allow for the regression of distant, untreated metastatic lesions, which are currently responsible for the majority of treatment inefficacy in the standards of care.

To address this issue, ZnO NPs could be employed as an adjuvant in cancer vaccines and immunotherapy, exploiting the pH-responsive cargo release and the induced local inflammation to deliver moieties whose effect relies on the spatiotemporal selectivity of their delivery at the site of interest [79]. The potential for ZnO-mediated immune modulation is further supported by emerging evidence in regenerative medicine, where functionalized ZnO NPs have been shown to successfully recalibrate systemic inflammation in liver disease treatment. Although these results stem from non-oncological applications, we consider this evidence a highly encouraging precedent, suggesting that ZnO is not merely a passive carrier, but a bioactive agent capable of re-programming suppressed microenvironments toward a more favorable therapeutic outcome [80].

We believe this would have impactful implications in all those tumors that possess and intrinsically 'cold' microenvironment, promoting immunomodulation and a shift toward a more 'hot' and active one.

Finally, it is the authors' belief that ZnO NPs are highly promising for application in rare diseases, in which standard systemic treatments have too many side effects and have not proved their efficacy yet. Our guess is that Zn-based nanoplatforms will be used for precision sonodynamic immunotherapy, exploiting their targeting abilities, their cargo loading efficiency, and their intrinsic ability to locally generate ROS, minimizing off-target effects. We view ZnO NPs in the future as active participants in multimodal therapies due to their ability to smartly respond to both environmental and external stimuli, leveraging their potential at inducing systemic immunomodulation in response to stimuli-responsive treatments.

Abbreviations

ZnO	Zinc Oxide
NPs	Nanoparticles
ROS	Radical Oxygen Species
UV	Ultraviolet
PDT	Photodynamic Therapy
CFZ	Carfilzomib
SDT	Sonodynamic Therapy
SW	Shock Waves
TME	Tumor Microenvironment
Ce6	Chlorin e6
PDA	polydopamine
MRI	Magnetic Resonance Imaging
PTT	Photothermal Therapy
QDs	Quantum Dots
PEG	Poly(ethylene glycol)
DOX	Doxorubicin
MDR	Multidrug Resistance/Resistant
CTCLs	Cytotoxic T lymphocytes
ICD	Immunogenic Cell Death
DAMPs	Damage-associates Molecular Patterns
DCs	Dendritic Cells
NIR	Near Infrared
NK	Natural Killer
IFN- γ	interferon gamma
IL-2	interleukin-2
TNF-α	Tumor Necrosis Factor Alpha
PBLs	Peripheral-blood Lymphocytes

BMDCs	Bone Marrow-derived Dendritic Cells
GM-CSF	Granulocyte Macrophage Colony-stimulating Factor
OVA	Ovalbumin
FA	Folic Acid
EAC	Ehrlich ascites carcinoma
IL-6	Interleukin-6
MAF	Metal Organic Framework

Declarations of interest

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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•• **This paper demonstrates that the use of ZnO NPs can impact the immune regulation in the context of liver regeneration, with crucial implications translatable to cancer therapy as well.**