FUNCTIONAL NANOMATERIALS IN INFLAMMATORY DISEASES: FROM PREVENTION TO DIAGNOSIS AND THERAPY

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FUNCTIONAL NANOMATERIALS IN INFLAMMATORY DISEASES: FROM PREVENTION TO DIAGNOSIS AND THERAPY

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EDITORIAL
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Editorial: Functional Nanomaterials in Inflammatory Diseases: From Prevention to Diagnosis and Therapy

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Keywords: nanomaterials, inflammatory diseases, diagnosis, therapy, anti-inflammation

Editorial on the Research Topic

Functional Nanomaterials in Inflammatory Diseases: From Prevention to Diagnosis and Therapy

Inflammation is a complex process involving multiple immune cell types and the inflammatory response is a key characteristic of many human diseases. Inflammatory diseases include acute disorders such as acute lung injury (ALI) and acute respiratory distress syndrome (ARDS), as well as chronic disorders such as cancer and arteriosclerosis. Clinical outcomes for patients with inflammatory disorders may be improved by the development of novel therapies that specifically and rapidly target the diseased tissue. To this end, novel nanomaterials are being designed to target the inflammatory response for the prevention, diagnosis, and treatment of patients with inflammatory diseases.

A major aim of using nanomaterials in the context of inflammatory diseases is to develop novel diagnostic tests and treatment options for patients with inflammatory diseases, which will help combat these disorders and improve clinical outcomes in these patients. *Frontiers in Pharmacology* recently published a series of articles under the Research Topic, "Functional Nanomaterials in Inflammatory Diseases: From Prevention to Diagnosis and Therapy". This Research Topic contains five Review articles that summarize the use of advanced nanomaterials in distinct types of inflammatory disorders ranging from cancer to prostatitis and rheumatoid arthritis.

Liu et al. describe the challenges associated with treating prostatitis, including inadequate delivery of therapeutic agents to the prostate, which is located deep in the pelvic cavity (Liu et al.). Advantages of nanomaterial-based treatment strategies that are showcased in this review include improvements in controlled drug release by nanoparticles compared with free drug, and the option of loading more than one therapy into nanoparticle-based drug delivery systems (Liu et al.). Another Review summarizes recent literature suggesting that neuro-inflammation could be targeted by nanomedical therapies in diseases such as Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis (Zhu et al.). These authors explain that the blood-brain barrier hinders drug delivery to microglial cells in the central nervous system, and that nanoparticles can act as vehicles for anti-inflammatory drugs to cross the blood-brain barrier, which in turn inhibit over-activation of microglia and excessive neuro-inflammation (Zhu et al.). The Review article by He et al. focuses on the application of gold nanoparticles in cancer immunotherapy (He et al.). Gold nanocarriers can be used for delivery of anti-tumorigenic therapies including drugs and antibodies, for photothermal therapy, or for a combination of both of these treatment strategies (He et al.). In

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another of the Review articles, authors describe how nanomedicines targeting macrophages show potential in the treatment of rheumatoid arthritis (Li et al.). In their paper, Li et al. present studies suggesting that the crucial effector cell in the progression of rheumatoid arthritis—the macrophage—can be specifically targeted by nanomaterial-based treatment strategies (Li et al.). Finally, Lin et al. review the use of selenium-based nanoparticles in infectious diseases to highlight the important role of selenium in anti-oxidation and cell viability, along with the enhanced stability and drug encapsulation capacity of selenium nanoparticles (Lin et al.).

As convincingly shown by this series of Review articles, nanoparticle-based treatment strategies are highly promising for the treatment of several inflammatory diseases. However, large-scale clinical studies will be required to determine whether the benefit of such treatment strategies shown in pre-clinical settings will translate to clinical benefit in humans.

Three Original Research articles are also included in the Research Topic. Yang et al. describe the preparation and administration of carboxymethyl chitosan hydrogels that are cross-linked with a thioketal agent and loaded with the reactive oxygen species scavenger, curcumin, in the scenario of wound healing following burn injury (Yang et al.). Authors showed that the cross-linked and drug-loaded hydrogels increased migration and viability in fibroblasts, reduced inflammatory cytokine expression in macrophages, and accelerated wound healing, hair regrowth, and revascularization following burn injury in rats (Yang et al.). While this study did not compare the treatment efficacy of the drug-loaded hydrogels with free drug alone or verify findings in a second model of inflammatory injury, future studies could aim to determine whether such treatment strategies are effective in human wound healing and also assess whether this treatment is effective in other scenarios. The second Original Research article describes the preparation and testing of itraconazole-loaded poly lactic-co-glycolic acid (PLGA) nanoparticles in cultured macrophages (Mejía et al., 2021). The authors studied how nanoparticle-to-drug ratio, aqueous phase pH, and type and concentration of surfactant altered the formation, drug-loading capacity, and encapsulation efficiency of the nanoparticles (Mejía et al., 2021). Future studies could test whether these itraconazole-loaded nanoparticles can be used to effectively treat inflammatory injury in multiple animal models of infection. The impact of the drug-loaded nanoparticles could also be tested on different cell types following infection with different fungal or bacterial strains. The third Original Research article presents a platelet membrane coated, PLGA drug delivery system, to administer berberine to the lungs of mice with experimental asthma (Jin et al.). These authors also show that coating of nanoparticles with a platelet membrane increases nanoparticle accumulation in the asthmatic lung compared with free drug or uncoated drug-loaded nanoparticles (Jin et al.). They also show that coating of berberine-loaded nanoparticles with a platelet membrane reduces inflammatory cell numbers and cytokine expression in asthmatic lungs compared with free drug or uncoated drug-loaded nanoparticles (Jin et al.). While this study did not verify the mechanism of uptake or action of the platelet-coated nanoparticles, the authors do provide circumstantial evidence that the nanoparticles modulate the expression of Th1 versus Th2 type cytokines (Jin et al.). Future studies could assess the efficacy of this nanoparticle drug delivery system in other experimental models of inflammatory lung injury, such as endotoxemia or polymicrobial sepsis mouse models.

In conclusion, different nanomaterials can be leveraged to improve diagnostic testing and therapeutic treatments for a variety of inflammatory disorders. In future, nanomedical advances will hopefully lead to the development of novel therapies that improve clinical outcome in patients with inflammatory diseases.

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REVIEW
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Nanoparticles: A Hope for the Treatment of Inflammation in CNS

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Neuroinflammation, an inflammatory response within the central nervous system (CNS), is a main hallmark of common neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS), among others. The over-activated microglia release pro-inflammatory cytokines, which induces neuronal death and accelerates neurodegeneration. Therefore, inhibition of microglia over-activation and microglia-mediated neuroinflammation has been a promising strategy for the treatment of neurodegenerative diseases. Many drugs have shown promising therapeutic effects on microglia and inflammation. However, the blood–brain barrier (BBB)—a natural barrier preventing brain tissue from contact with harmful plasma components—seriously hinders drug delivery to the microglial cells in CNS. As an emerging useful therapeutic tool in CNS-related diseases, nanoparticles (NPs) have been widely applied in biomedical fields for use in diagnosis, biosensing and drug delivery. Recently, many NPs have been reported to be useful vehicles for anti-inflammatory drugs across the BBB to inhibit the over-activation of microglia and neuroinflammation. Therefore, NPs with good biodegradability and biocompatibility have the potential to be developed as an effective and minimally invasive carrier to help other drugs cross the BBB or as a therapeutic agent for the treatment of neuroinflammation-mediated neurodegenerative diseases. In this review, we summarized various nanoparticles applied in CNS, and their mechanisms and effects in the modulation of inflammation responses in neurodegenerative diseases, providing insights and suggestions for the use of NPs in the treatment of neuroinflammation-related neurodegenerative diseases.

Keywords: neurodegenerative diseases, central neural system, blood-brain barrier, neuroinflammation, nanoparticles

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INTRODUCTION

Neuroinflammation is characterized by the activation of microglia and astrocytes, as well as the release of cytokines and reactive oxygen species. It may cause synaptic dysfunction, the loss of synapses, and neuron damage. Since neuroinflammation is the common mechanism behind various CNS-related diseases, alleviation and inhibition of neuroinflammation has become a research hotspot over recent years. However, most drugs with anti-inflammatory characteristics cannot cross the blood–brain barrier to the target cells such as microglia and astrocytes. The BBB is formed

by the brain capillary wall, glial cells and the barrier between plasma and cerebrospinal fluid (CSF) that is formed by the choroid plexus. The BBB is an essential defense mechanism of the CNS that restricts the transit of toxins or pathogens and selectively allows individual molecules to pass. However, the BBB also significantly hinders drug delivery to the CNS (Zhou et al., 2018; Tosi et al., 2020).

Nanomaterials can make dramatic changes to the treatment of neuroinflammation. Over recent decades, a rising number of nanomaterials have been developed. There is increasing optimism that nanomedicine will continue to develop and could even change the model of the prevention, diagnosis and treatment of disease (Rodallec et al., 2018; Avasthi et al., 2020). Nanomaterials are made up of engineered materials or devices with the smallest functional organizations in the size range of 1–100 nm (Zielinska et al., 2020). They are mainly classified into two groups: inorganic and organic nanomaterials. Inorganic nanomaterials come in an array of forms, including Au nanoparticles, TiO_2 NPs, IONPs and other metal NPs. Organic nanomaterials mainly include lipid NPs (liposomes and solid lipid NPs), nanoemulsions and polymer NPs (polymeric NPs, dendrimers, nanogels, and micelles) (Kumari et al., 2010; Martinez-Lopez et al., 2020).

NPs can encapsulate drugs with relatively high drug loading (Sim et al., 2020), and the surface of NPs can be easily manipulated to achieve drug targeting (Sun et al., 2014). In addition, NPs can control the release of drugs at the site of target cells or tissues, thereby increasing therapeutic efficacy and reducing the side effects of drugs. Drugs that are insoluble or unstable in aqueous phase could be formulated into nano delivery systems, which improves their solubility and extends their pharmacologic effects. Most importantly, NPs systems could provide a variety of choices for the routes of drug administration, including intravenous, nasal, oral, parenteral, intra-ocular, and dermal topical application (Spuch et al., 2012; Carita et al., 2018; He et al., 2019; Islam et al., 2020). In recent years, a number of NPs have been developed as effective and minimally invasive carriers to help other drugs cross the BBB or as the therapeutic agents for the treatment of neuroinflammation-mediated neurodegenerative diseases (Moura et al., 2019; Tang et al., 2019; Tosi et al., 2020).

In this article, we summarize the current knowledge gained from recent advances in nanomaterials, and their key treatment roles in neuroinflammation-related neurodegenerative diseases, which provides more opportunities and prospects for the therapy of neurodegenerative diseases in the future.

NEUROINFLAMMATION IN CNS-RELATED DISEASES

Neurodegenerative diseases are the main type of CNS-related diseases and include Alzheimer's disease, Parkinson's disease, Huntington disease (HD), frontotemporal dementia (FTD), Lewy body dementia (LBD), etc. The pathologies of neurodegenerative diseases are characterized by neuroinflammation, cerebral protein aggregates, synaptic abnormalities, and progressive loss of neurons (Dugger and Dickson, 2017; Vaquer-Alicea and Diamond, 2019). Gradual cognitive and memory impairments and disorder in movements are common clinical symptoms (Katsnelson et al., 2016; Stephenson et al., 2018).

Neuroinflammation generally refers to an inflammatory response within the CNS or activation of the neuroimmune cells, microglia and astrocytes into the state of pro-inflammatory response (Schain and Kreisl, 2017). Emerging evidence indicates that the resting microglia (M0) is over-activated by various pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs) including particulates, viruses, bacteria, fungi, toxins, lipopolysaccharide (LPS), crystals, silica, and misfolded protein aggregations (Aβ, Tau, α-synuclein, etc.) in neurodegenerative diseases (Agostinho et al., 2010; Allaman et al., 2011; Alcendor et al., 2012; Niranjan, 2014). The transient receptor potential melastatin-related 2 (TRPM2) is a calcium-permeable channel induced by oxidative stress (Alawieyah Syed Mortadza et al., 2018), ultimately causing activation of the NLRP3 inflammasome (Koenigsknecht and Landreth, 2004). Microglia and astrocytes are the primary constituents of a dedicated neuroimmune system in CNS. The moderative activation of microglia (M2) can protect brain by defending against harmful materials by releasing many anti-inflammatory cytokines, including Arg-1, TGF-β, and IL-10. However, amplified, exaggerated, or chronically activated microglia (M1) lead to robust pathological changes and neurobehavioral complications, such as depression and cognitive deficits (Norden and Godbout, 2013). The inflammation process is indicated by the production of pro-inflammatory cytokines, including IL-1β, IL-6, IL-18 and tumor necrosis factor-α (TNF-α), as well as many chemokines, such as C-C motif chemokine ligand 1 (CCL1), CCL5, and C-X-C motif chemokine ligand 1 (CXCL1), and small-molecule messengers, including prostaglandins and nitric oxide (NO), and reactive oxygen species (DiSabato et al., 2016). After treatment with anti-inflammatory drugs, the M1-type microglia are converted into M2-type microglia, which is indicated by the decrease of pro-inflammatory cytokines and increase of anti-inflammatory cytokines (**Figure 1**).

Many researchers have recently reported findings about the mechanism of neuroinflammation associated with neurodegenerative disorders (Schain and Kreisl, 2017). Earlier studies identified amyloid β (Aβ) and hyperphosphorylated tau as playing essential roles in the progress of AD (Eftekharzadeh et al., 2018; Nakamura et al., 2018). Many previous studies found that Aβ oligomers are the most toxic forms among all Aβ species, and the smaller oligomers of Aβ have been proved to be stronger stimuli to activate the microglial cells (Yang et al., 2017). The aggregated tau has been considered to induce microglial changes by activating the NLRP3–ASC axis (Ising et al., 2019; Stancu et al., 2019). Numerous studies have shown that Aβ and hyperphosphorylated tau induce pro-inflammatory conditions *in vitro* and vivo (Maezawa et al., 2011; Morales et al., 2013; Asai et al., 2014; Marlatt et al., 2014; Shi et al., 2019). Moreover, accumulating evidence suggests that soluble α-synuclein aggregates play a significant role in PD and most of them were found within the substantia nigra pars compacta (SNc)

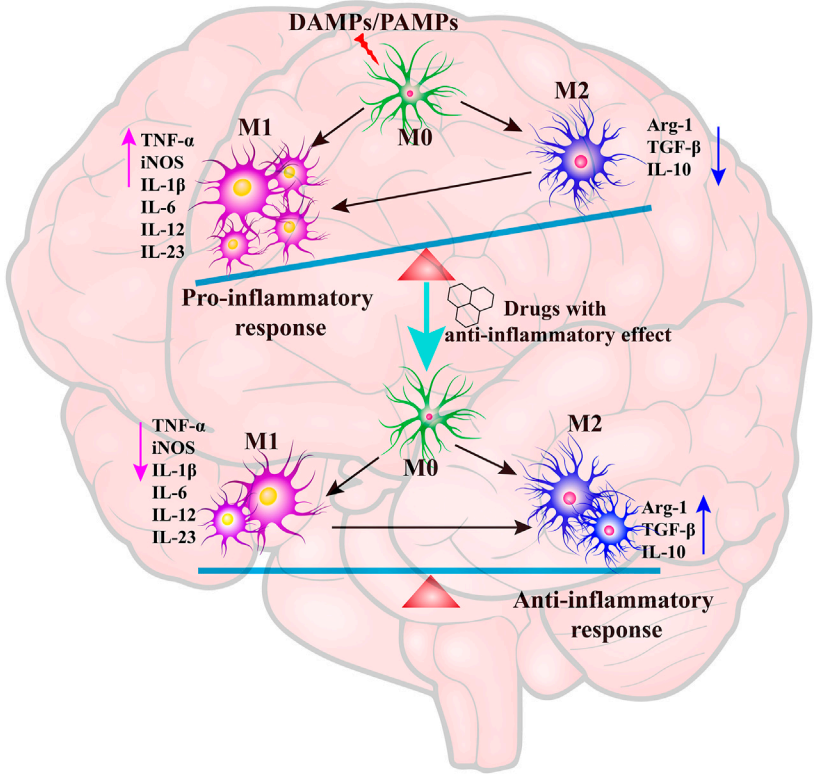

FIGURE 1 | The key role of neuroinflammation in neurodegenerative diseases. The resting microglia (M0) are over-activated by PAMPs/DAMPs into a pro-inflammatory state (M1), which leads to the generation of pro-inflammatory cytokines. The treatment of anti-inflammatory drugs can inhibit the over-activation of microglia and promote the microglia into an anti-inflammatory state to maintain the balance of M1/M2 type microglia.

region of the midbrain (Winner et al., 2011; Choi et al., 2013). Recently, activated microglia were found surrounding Lewy bodies, suggesting that neuroinflammation is a common response to α-synuclein aggregates (Streit and Xue, 2016). In addition, widespread microglial activation was visible by positron emission tomography (PET) in the brain of living ALS patients and $SOD1^{G93A}$ mice, indicating that there is an association between neuroinflammation and ALS (Turner et al., 2004; Corcia et al., 2012; Gargiulo et al., 2016). Therefore, microglia over-activation and the resulting neuroinflammation have been implicated in neurodegenerative diseases, while the inhibition of neuroinflammation has been considered a promising strategy for the treatment of neuroinflammation-mediated neurodegenerative diseases.

THE EFFECT OF NPS IN CNS-RELATED DISEASES

The traditional definition of nanoparticle size is 1–100 nm. Indeed, while most NPs are under 100 nm, the diameter of some composite or drug-loaded NPs are over 100 nm (Suk et al., 2016; Tosi et al., 2020). Furthermore, the generally accepted classification of nanoparticles is based on their organic, inorganic, and carbon-based nature (**Figure 2**). Particle size is the basic attribute of NPs, which determines the biological fate, toxicity, distribution, and targeting ability of NPs to a certain extent. Generally, smaller NPs are prone to aggregate during dispersion, storage, and transport, and exhibit faster drug release due to their larger surface-to-volume ratio. On the contrary, larger NPs lead to faster polymer degradation and slower drug release (Gupta et al., 2019; Zahin et al., 2020). The shape of NPs contributes to biological functions such as drug delivery, half-life period, endothelial intake, and targeting ability (Petros and DeSimone, 2010; Yoo et al., 2010; Zhang et al., 2015). NPs have varied shapes including rod, spherical, triangular, cube, hexagonal, fivefold star shape and monodisperse cubic dendrites, among others (Lacroix et al., 2012; Sun et al., 2014). Surface charge and hydrophobicity are surface properties of NPs, which may influence their biodistribution, circulation time, and toxicity (Arvizo et al., 2011). Positively charged NPs show better efficacy of imaging, gene transfer, and drug delivery, but they are reported to possess higher cytotoxicity. Hydrophobicity is another important surface property, which plays an important role in plasma protein binding and clearance via the reticuloendothelial system (RES) (Frohlich, 2012; Nam et al., 2013).

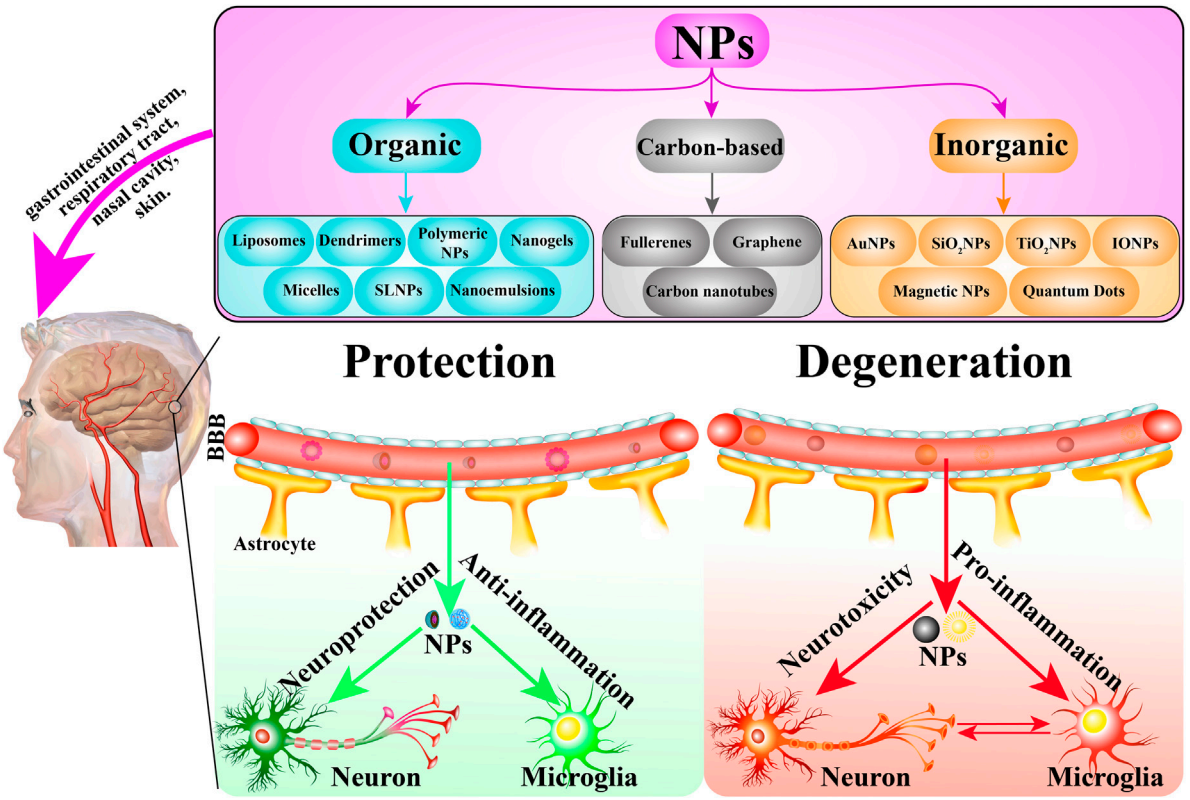

FIGURE 2 | The classification of NPs and the role of NPs in CNS-related diseases. NPs are mainly classified into three groups: organic, carbon-based, and inorganic NPs. In general, these NPs are administrated via the gastrointestinal system, respiratory tract, nasal cavity, and skin, etc. They cross the BBB into the target brain cells including neurons, microglia and astrocyte to exert protective and degenerative effects.

To date, NPs have been widely used in CNS-related diseases including neurodegenerative disease, traumatic brain injury, stroke, and cerebral tumor. As drug carriers or as therapeutic drugs by themselves, NPs show potential for neuroprotective effects by oxidation resistance, anti-apoptosis, and nerve regeneration (**Figure 2**). The initial focus of neuroprotective treatment is the neurons, which are considered the most vulnerable cells to hypoxia and excitotoxicity. However, in recent years, concerns have been extended to astrocytes, pericytes, endothelial cells, and other neural cells, targeting antioxidant enzymes, antiapoptotic pathways, and downstream cytokines (Moretti et al., 2015; Chamorro et al., 2021). Polysorbate 80 (PS80) reduced the secondary spread of neuroinflammation and injury in traumatic brain injuries (TBI) by preventing the spread of reactive oxygen species (ROS) (Yoo et al., 2017a). Poly (lactic-co-glycolic acid) nanoparticles, which encapsulated Lexiscan and Nogo-66, improved stroke survival, suggesting the potential therapeutic effect for stroke (Han et al., 2016). Numerous researchers have demonstrated that organic and inorganic NPs might be helpful in the treatment of neurodegenerative diseases, especially AD and PD. The possible mechanisms include the delivery of a corresponding drug, siRNA transfection, interference with Aβ fibril formation, down-regulating proinflammatory factors, etc. (Tiwari et al., 2014; Karthivashan et al., 2018; Baskin et al., 2020).

NPs can participate in the treatment of neuroinflammation as carriers for therapeutic drugs including curcumin, okadaic acid, quercetin, anthocyanin, and levodopa. With the assistance of NPs, the drugs can cross the BBB to target cells more easily, thereby inhibiting inflammatory pathways and the release of inflammatory cytokines. Besides, magnetic NPs, such as IONPs, have been applied in diagnosis and imaging. Moreover, nanoparticles themselves also have therapeutic effects in neuroinflammation. For example, AuNPs could induce microglia polarization toward the M2 phenotype (Xiao et al., 2020), carbon nanotubes (CNTs) can integrate with neurons and enhance neuronal functions (Matsumoto et al., 2007), and rhubaric acid hydrogel inhibits TLRs signaling pathways (Zheng et al., 2019).

Although NPs exhibit potent neuroprotection and anti-inflammatory effects, many NPs have been reported to exhibit neurotoxicity and pro-inflammatory responses in some cells and animals with CNS-related diseases (**Table 1**; **Figure 2**). For example, copper NPs can cause BBB dysfunction, swelling of astrocytes, and neuronal degeneration once introduced into the bloodstream (Sharma, 2009; Sharma et al., 2009).

TABLE 1 | The role of nanoparticles in neurotoxicity and pro-neuroinflammation.

NPs	Diameter (nm)	Cells/animals	Treatment time	Administration route	Dose	Mechanism and detected markers	References
In vitro							
MWCNTs	5–15 nm	3D brain organoids derived from iPSCs	24 h		0 μg/ml, 16 μg/ml, and 64 μg/ml	NF-κB-KLF4 pathway; nNOS	Jiang et al. (2020)
ZnO NPs	19.61 ± 5.83 nm	PC12 cells	6 h or 12 h		0–20 μg/ml	CAMK2A/CAMK2B pathway Oxidative stress: GSH, MDA, NO, SOD Inflammatory cytokines: IL-1β, TNF-α	Liu et al. (2020a)
IONP, IONP-TPP and IONP-APM	11 nm	Rotenone-induced SH-SY5Y cells	24 h or 48 h		0–200 μg/ml	AMPK pathway	Huang et al. (2019)
Silica NPs	50, 100, and 300 nm	N9, bEnd.3, and BV-2 cells	24 h		25–200 μg/ml	Oxidative stress: ROS, LDH Pyroptosis: N-GSDMD Inflammatory cytokines: IL-1β	Du et al. (2019)
Mn_3O_4 NPs	18.98 ± 4.61 nm	PC12 cells	24 h		5 μg/ml, 10 μg/ml, and 20 μg/ml	Oxidative stress: ROS, Ca^{2+}, LDH Apoptosis: Bax/Bcl-2, caspase-3, caspase-9	Chen et al. (2020)
Co. NPs	Under 100 nm	SH-SY5Y cells	24 h at day 4 and day 12		1–100 μg/ml	Oxytosis:ROS, Ca^{2+}, GSH, GPX4	Gupta et al. (2020)
Ag NPs	20 and 70 nm	Pure cortical neurons from SD rat embryos on embryonic day 18	24 h		0.01–40 μg/ml	Extracellular dopamine, cytoskeleton changes	Zhang et al. (2020)
In vivo							
ZnO NPs	42.31 ± 17.94 nm	Male Wistar rats	30 days	Tongue instillation	134.2 mg/kg and 536.8 mg/kg	NF-κB and MAPK pathways Inflammatory cytokines: TNF-α, IL-1β, IL-6, IL-10, IFNG, NOS2	Liang et al. (2018)
Al_2O_3 NPs	22.63 ± 5.64 nm	Male Wistar rats	15–30 days	Tongue instillation	20 μg/g	Oxidative stress: MDA Inflammatory cytokines: TNF-α, IL-1β	Liu et al. (2020b)
CeO_2-NPs	Under 50 nm	Oncorhynchus mykiss juveniles	28 days	Aquarium's exposure	0.1 μg/L, 0.01 μg/L, and 0.001 μg/L	Oxidative stress: GSTs and catalase	Correia et al. (2019)
f-CNTs	20–30 nm	Female C57/Bl6 mice	Single injection	Stereotactic administration	500 ng/mouse	Inflammatory cytokines: IL-10, TNF-α, and IL-1β	Bardi et al. (2013)

ORGANIC NPS

Lipid-based NPs

Liposomes

Liposomes are vesicular drug-delivery systems containing an aqueous inner core enclosed in multi-lamellar phospholipid bilayers. Hydrophobic and hydrophilic drugs can be loaded in the phospholipid bilayers and aqueous core, respectively (Agrawal et al., 2017; Li et al., 2018).

Liposomes have the characteristics of nanoscale, ideal biocompatibility and relative stability. Due to the structural similarity of phospholipid bilayers to the cell membrane, liposomes can be absorbed by vascular endothelial cells more easily, which makes them promising drug-delivery systems to increase the BBB crossing of therapeutics in CNS diseases associated with neuroinflammation (Li et al., 2017; Patel and Patel, 2017; Li et al., 2019a). However, they can easily be degraded and scavenged by macrophages, and their binding to plasma proteins causes non-specific targeting to other tissues and low targeting to the nervous system. To overcome these drawbacks, long-circulation liposomes, specific active targeting liposomes, and other new types of liposomes have been developed over recent years (Gabizon et al., 2016; Li et al., 2019a).

Dopamine-PEGylated immunoliposomes (DA-PILs)—liposomes modified with polyethylene glycol and conjugated with antibodies—were developed as vehicles for dopamine in PD treatment. In a rat model of PD, the uptake of DA-PIL in the brains increased about 8-fold and 3-fold compared with that of DA and encapsulated DA-PEGylated liposomes (DA-PL), respectively (Kang et al., 2016). The physicochemical properties of liposomes can be modified by altering the phospholipids themselves or their ratio. Since dipalmitoyl phosphatidylcholine (DPPC) was the most pH-stable liposome found, with a sustained drug release at physiological pH (Yaroslavov et al., 2015), DPPC was selected as the carrier of curcumin to explore the therapeutic effect in human fetal astrocyte cell line SVGA model of neuroinflammation and reactive astrogliosis. Compared with free curcumin, LipoCur showed a significant downregulation of glial cell proliferation genes and a lower level of pro-inflammatory cytokines including IL-6, IL-1β, TGF-β, and TNF-α (Schmitt et al., 2020). In addition, Cyclosporine A (CsA) in liposomal formulation (Lipo-CsA)

inhibits the inflammation response, including myeloperoxidase (MPO) activity and TNF-α levels, in the model of ischemia reperfusion injury (I/R) cerebral injuries (Partoazar et al., 2017). Therefore, liposomes serving as drug-delivery systems increase the BBB penetration of drugs to improve the anti-inflammatory effect.

Solid Lipid NPs

Manufactured from synthetic or natural lipids, solid lipid NPs (SLNs) have a lipidic core, which enables them to stay in solid state at room and body temperatures (Cupaioli et al., 2014). SLNs are less toxic than cationic liposomes and are generally recognized as safe in humans. Besides, they have been proved to be physiologically tolerated and have higher drug delivery efficiency compared to other types of lipid-based NPs (Banerjee and Pillai, 2019; Raza et al., 2019).

In LPS-induced BV-2 microglial cells, curcumin-loaded solid lipid nanoparticles (SLCN) dose-dependently inhibited the levels of nitric oxide (NO) and pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6, and this was more effective than curcumin alone (Ganesan et al., 2019). Similarly, SLCN provides a superior effect in anti-Aβ, anti-inflammatory, and neuroprotective outcomes than traditional curcumin in one-year-old 5xFAD AD mouse (Maiti et al., 2018). In addition, sesamol-loaded SLNs were developed and found to significantly alleviate the oxidative stress in intracerebroventricular (ICV)-streptozotocin (STZ)-induced male Wistar rats, suggesting they provide a promising strategy to mitigate neuroinflammation and memory deficits (Sachdeva et al., 2015). SLNs are clearly useful delivery systems to alleviate neuroinflammation and neuronal dysfunction.

Nanoemulsions

Nanoemulsions (NEs) are a colloidal dispersion consisting of two immiscible liquids stabilized by surfactants. A typical NE usually contains water, oil, and an emulsifier at appropriate ratios. NEs show some excellent properties including good biocompatibility, kinetical stability, cell transport by paracellular and transcellular pathways, and prevention of hydrolysis and enzymatic degradation of residues (Nirale et al., 2020). NEs can be administrated through nasal and ocular delivery in addition to the oral and intravenous administrations (Karami et al., 2019a; Karami et al., 2019b; Nirale et al., 2020).

Chitosan-coated rosmarinic acid nanoemulsions (RA CNE) have been shown to offer protection by inhibiting cellular death and repairing the astrocyte redox state in LPS-induced neuroinflammation and oxidative stress in astrocyte cells (Fachel et al., 2020a). Based on these *in vitro* results, researchers further illustrated the neuroprotective effects of RA CNE on the alleviation of neuroinflammation, oxidative stress, and memory deficit in Wistar rats (Fachel et al., 2020b). In LPS-induced rat neuroinflammation models, the brain uptake of siRNA delivered by cationic nanoemulsions was almost five times higher than non-encapsulated siRNA. More importantly, siRNA nanoemulsions significantly reduced the level of TNF-α, a signaling molecule which aggravates inflammation. Therefore, nanoemulsions encapsulated with TNF-α siRNA were suggested to be potential candidates in the treatment of neuroinflammation (Yadav et al., 2016). Ropinirole, a dopamine agonist as combination therapy with levodopa, is widely used in the treatment of PD. However, its efficiency was limited by its low bioavailability and short half-life. After modification, the transdermal delivery of ropinirole NE gel exhibited better drug absorption and less irritation and toxicity for the skin compared to ropinirole alone (Azeem et al., 2012).

Polymer-Based NPs

Polymeric NPs

Polymeric NPs consist of amphiphilic block copolymers with varying hydrophobicities. They can be categorized into two groups: natural and synthetic polymeric NPs. Synthetic polymeric NPs can be manufactured via nanoprecipitation or the double emulsion method. Owning to the core–shell structure, polymeric NPs are able to encapsulate slow-release hydrophobic drugs and prolong circulation time. The surface of polymeric NPs can be decorated with ligands for targeted drug delivery. Therefore, polymeric NPs are considered drug carriers with high biological activity and bioavailability and have a high therapeutic index (Chen et al., 2015; Zielinska et al., 2020).

Natural polymeric macromolecules mainly include chitosan, alginates, dextrane, gelatin, collagen and their derivatives. Chitosan often derives from exoskeletons of crustaceans and cell walls of fungi and is a cationic polymer. As the second most abundant natural polysaccharide, chitosan, together with chitosan oligosaccharide and its derivatives, have been widely applied as the material of nano-carriers for the treatment of neuroinflammation. Besides, chitosan have neuroprotective effects in AD by inhibiting Aβ, acetylcholinesterase (AchE), oxidative stress, and neuroinflammation (Ouyang et al., 2017). Chitosan-coated synergistically engineered nanoemulsion of Ropinirole and nigella oil was suggested as a potential therapeutic strategy for PD by downregulating the NF-κB signaling pathway and inhibiting lipid peroxidation (Nehal et al., 2021). Alginate is an acidic polysaccharide from various marine brown algae. Alginate-derived oligosaccharide (AdO) was reported to significantly reduce the level of nitric oxide (NO) and prostaglandin E2 (PGE2), as well as the secretion of other proinflammatory cytokines. Furthermore, AdO significantly attenuated the overexpression of toll-like receptor 4 (TLR4) and NF-κB induced by LPS in BV2 cells (Zhou et al., 2015). In addition, alginate micro-encapsulation of mesenchymal stromal cells could modulate the neuroinflammatory response by decreasing the production of PGE2 in LPS induced astrocytes and microglia (Stucky et al., 2015; Stucky et al., 2017).

Synthetic polymers include polyesters and their copolymers, polyacrylates and polycaprolactones. Compared with natural molecules, their synthesis conditions can be controlled to regulate chain length, composition, and degradation to perform multiple functions (Colmenares and Kuna, 2017). In addition, synthetic polymers have been proved to possess relatively low toxicity profiles. Polymeric surface modification has been used to minimize the uptake by the reticuloendothelial system, thus increasing blood circulation half-life, which is a promising strategy to improve controlled drug release for long periods (Modi et al., 2010). Currently, poly-lactic-co-glycolic acid

(PLGA), which is approved by United States Food and Drug Administration (FDA) for human application, is the most commonly studied polymer with good biocompatibility and biodegradability (Pavot et al., 2014; Younas et al., 2019). A novel brain-target nanoparticle, poly (lactide-co-glycolide)-block-poly (ethylene glycol) (PLGA-PEG) conjugated with B6 peptide and loaded with curcumin (PLGA-PEG-B6/Cur) was designed (Fan et al., 2018). Compared with native Cur, PLGA-PEG-B6/Cur significantly improved the spatial learning and memory ability of APP/PS1 mice by increasing the average half-life, decreasing metabolism, and maintaining the release of Cur, which showed potential for use in the treatment of AD. In addition, PEGylated-PLGA nanoparticles of epigallocatechin-3-gallate (EGCG) were developed to improve drug stability and increase the brain delivery in the treatment of temporal lobe epilepsy. Indeed, immunohistochemistry and neurotoxicity studies confirmed reduced neuronal death and neuroinflammation (Cano et al., 2018). NPs also showed a better effect on the reduction of the frequency and intensity of epileptic episodes than EGCG. In some other studies, PLGA NPs were synthesized to transfer superoxide dismutase (SOD) in cerebral ischemic reperfusion injury (IR) injury mouse models, and the results showed that PLGA NPs were effective in reducing apoptosis, inflammatory markers (TNF-a, IL-1β, and TGF-β), and infarct volume (Yun et al., 2013). In addition, Foxp3 plasmid-encapsulated PLGA NPs was found to significantly reduce microglial activity and decrease the generation of pro-inflammatory cytokines including TNF-α, I L-1β, IL-6, cyclooxygenase (COX)-2, and inducible nitric oxide synthase (iNOS) (Shin et al., 2019). The cl PGP-PEG-DGL/CAT-Aco system (cross-linked dendrigraft poly-L-lysine nanoparticles modified with Pro-Gly-Pro (PGP)peptide and catalase (CAT), a neuroprotective enzyme) was developed (Zhang et al., 2017). In this system, leukocytes serve as 'Trojan horses' and freight the CAT penetrate across the BBB more effectively. In the middle cerebral artery occlusion (MCAO) model, the cl PGP-PEG-DGL/CAT-Aco system significantly enhanced the delivery of catalase to ischemic subregions and reduced the volume of brain infarct. Therefore, the studies reviewed suggest the effectiveness, drug protection, and long cycle life of synthetic polymers.

Dendrimers

Dendrimers consist of a group of highly ordered macromolecules synthesized through repetitive chemical reactions from a core with a structure (Araujo et al., 2018; Dias et al., 2020). They were first discovered in 1985 and have been extensively studied. Through covalent bonds and ion interactions or adsorption, dendrimers deliver drugs, genes and proteins with molecules loaded inside or bound to their surface to bring them across the BBB (Chauhan, 2018; Sherje et al., 2018).

The advantages of dendrimers include the following: 1) controlled biodistribution and pharmacokinetics; 2) high structural and chemical homogeneity, which facilitates pharmacokinetic reproducibility; 3) the ability to associate with various compounds and/or ligands, improving their solubility and specificity; 4) and numerous surface groups of dendrimers contribute to multifunctionality and/or high drug loads (Lyu et al., 2020; Sandoval-Yanez and Castro Rodriguez, 2020; Yousefi et al., 2020). However, their higher cost of production is a limitation compared to linear polymers. Moreover, the toxicity of dendrimers was reported by some studies. A temporary increase of liver aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels was observed in a macaque model accepting anionic AzaBisPhosphonate groups (ABP dendrimer). Injections of G0-G3 amine-terminated PAMAM dendrimers in a mouse air pouch model caused a significant increase in leukocyte infiltration (Durocher and Girard, 2016).

However, other researchers found that dendrimers were beneficial to human health. Dendrimer-based N-acetyl-L-cysteine (NAC) could be a therapy for neuroinflammation and cerebral palsy (CP) using a CP rabbit model induced by maternal intrauterine endotoxin by increasing the concentration of GSH in astrocytes and inhibiting neuroinflammation as indicated in GSH, 4-HNE, NT-3, 8-OHG, NF-kB, and TNF-a. Further study found that dendrimers are nontoxic, nonimmunogenic, and can be cleared completely through the kidneys (Kannan et al., 2012). Dendritic polyglycerol sulfates (dPGS) have been shown to be multivalent inhibitors of inflammation (Dernedde et al., 2010) and potent complement inhibitors (Silberreis et al., 2019). It was reported that dPGS interfered with Aβ fibril formation and reduced the production of the neuroinflammagen lipocalin-2 (LCN2) in astrocytes through its direct binding to $A\beta_{42}$ and interaction with $A\beta_{42}$. In addition, dPGS could normalize the impaired neuroglia cell and prevent the loss of dendritic spines at excitatory synapses in the hippocampus (Maysinger et al., 2018). Therefore, dPGS might be helpful in the treatment of neuroinflammation and neurotoxicity in AD and other neurodegenerative diseases. Moreover, fourth-generation poly amidoamine (PAMAM) dendrimers were synthesized by Li et al. (Li et al., 2012). Sino, a potent anti-inflammatory and antioxidant drug was combined with hydroxyl terminated generation-4 PAMAM dendrimer by Sharma et al. (2020b). D-Sino was demonstrated to be a potential therapy for attenuating inflammation in TBI at early stage through inhibiting the pro-inflammatory cytokines, including TNF-α, IL-1β, CCL-3, and IL-6, reducing the level of iNOS and NO, and inhibiting NF-κB activation and its nuclear translocation (Sharma et al., 2020b). Researchers also demonstrated that NAC, based on G4-OH PAMAM dendrimers (D-NAC), could increase intracellular GSH levels and prevent extracellular glutamate release and excitotoxicity in microglia and astrocytes, compared with NAC alone (Nance et al., 2017). Therefore, dendrimers, especially PAMAM, are considered a promising drug delivery system for CNS disease associated with neuroinflammation (**Table 2**; **Figure 3**).

Nanogels Solid Lipid NPs

Aqueous-based liquids can be used as supporting media for polymer gels by physical/chemical intercrossing. Nanogels are three-dimensional hydrogel particles composed of hydrophilic or amphiphilic polymer chains. Based on their structure, nanogels can be divided into four groups: hollow, multi-layered, core cross-linked, and hairy nanogels (Soni et al., 2016; Li et al., 2017; Hajebi

TABLE 2 | Dendrimers for the inhibition of neuroinflammation and their mechanisms within *in vitro* and *in vivo* models.

Dendrimers	Diameter (nm)	Biological model	Treatment	Dose	Mechanism and detected inflammatory cytokines	Toxicity	References
In vitro							
D-mino	~8.4 nm	LPS-induced BV2 cells	24 h co-culture	Concentration range of 50–500 μM	NO, TNF-a	50–500 μM did not show cytotoxicity	Sharma et al. (2017)
PEGOL-60	Not Given	LPS-induced BV2 cells	24 h co-culture	500 μg/ml	TNF-a, IL-4, IL-6, IL-10, and iNOS	>1,000 μg/ml did not show cytotoxicity for 24 h	Sharma et al. (2020a)
dPGS	13.55 ± 0.14 nm	Primary neuroglia and organotypic hippocampal slice cultures exposed to Aβ-42 peptide	Pre-treated for 1 h	1 M	Interfered with Aβ fibril formation and downregulation of LCN2	Not Given	Maysinger et al. (2018)
D-Sino	4.9 nm	LPS-induced RAW 264.7 cells	8 h co-culture	50 μg/ml, 100 μg/ml and 300 μg/ml	NF-κB pathway; TNF-α, IL-1β, CCL-3, IL-6, iNOS, and NO	>300 μg/ml did not show cytotoxicity, 500 μg/ml decreased cell viability to 82.7 ± 7.4%	Sharma et al. (2020b)
PAMAM-(COOH)46-(NAC)18	Not Given	LPS-induced BV2 cells	Pre-treated for 3 h	0.5 mM 2 mM, and 8 mM	ROS, NO, and TNF-α	0.04–0.59 mM did not show cytotoxicity for 24 h	Wang et al. (2009)
PAMAM	~4 nm	Brain slice culture model from newborn rabbits exposed by endotoxin	4 h co-culture	5 ng in 10 μL of DPBS solution	More rapid diffusion and ability to "find" the less mobile activated microglia, increasing microglial uptake	Not Given	Zhang et al. (2016)
In vivo							
ABP Dendrimer	Not Given	Mouse model of MOG35–55-induced autoimmune encephalomyelitis	Intravenous injection in different time in prophylactic and therapeutic groups	10 mg/kg	IFN-γ, IL-17, and IL-10	Did not induce immunosuppression or systemic toxicity in nonhuman primates	Hayder et al. (2015)
D-NAC	5.4 nm	A rabbit model of cerebral palsy induced by maternal intrauterine endotoxin	Intravenous injection to newborn	1 mg/kg, 10 mg/kg	NF-κB pathway; GSH and TNF-α	Nontoxic, nonimmunogenic, and are cleared intact through the kidneys	Kannan et al. (2012)
TPP-D-NAC	7.5 ± 0.2 nm	A rabbit model of TBI induced by surgery	Intravenous injection at 6 h post-injury	0.5 μg/ml, 5 μg/ml, and 50 μg/ml	Targeted delivery to mitochondria	Did not exhibit any reduction in cell viability at the doses tested	Sharma et al. (2018)
shCCL20-CCR6	100 nm	Mouse model of rTBI induced by surgery	Intranasal and intravenous administration after 3rd, 4th and 5th TBI	Not Given	IL-6 and CCL20	Low doses did not show cytotoxicity	Mayilsamy et al. (2020)

et al., 2019). Since nanogels have many advantages over other delivery materials including adjustable size, swelling, biocompatibility, hydrophilicity, ease of preparation, and stimulus responsiveness. Thus, they offer a promising prospect for drug, gene, or imaging agents transport. It was found that activin B-loaded hydrogels (ABLH) could provide lasting release of activin B for over five weeks in an MPTP-induced male C57BL/6J mice model of PD. Additionally, ABLH significantly increased the density of tyrosine hydroxylase (TH) positive nerve fibers and induced a noticeable reduction in neuroinflammatory responses, suggesting that ABLH may be a promising drug candidate for PD (Li et al., 2016). Self-assembling hydrogels possess superior characteristics without any structural modifications, as they are self-releasing, stable, soluble, injectable, stimuli responsive, and almost nontoxic. As a result, they are considered optimal therapeutic materials. Zheng et al. (2019) reported that rhein hydrogels—natural herbal drug hydrogels—enter the LPS-induced BV2 microglia and bind to TLR4 easily to inhibit the nuclear translocation of p65 in the NF-κB signaling pathway, thus reducing neuroinflammation with a sustained effect. Besides, it showed minimal cytotoxicity compared to rhein alone (Zheng et al., 2019). Therefore, nanogels have been developed in new ways, and their potential as a treatment for neuroinflammation needs to be explored further.

Polymeric Micelles

Micelles are colloidally made from amphiphilic block copolymers which aggregate in aqueous solutions and consist of a hydrophobic core and a hydrophilic surface (Zhang et al., 2012; Li et al., 2017). The mechanism of acute ischemic stroke includes oxidative stress, neuroinflammation, and cerebrovascular injury, which might lead

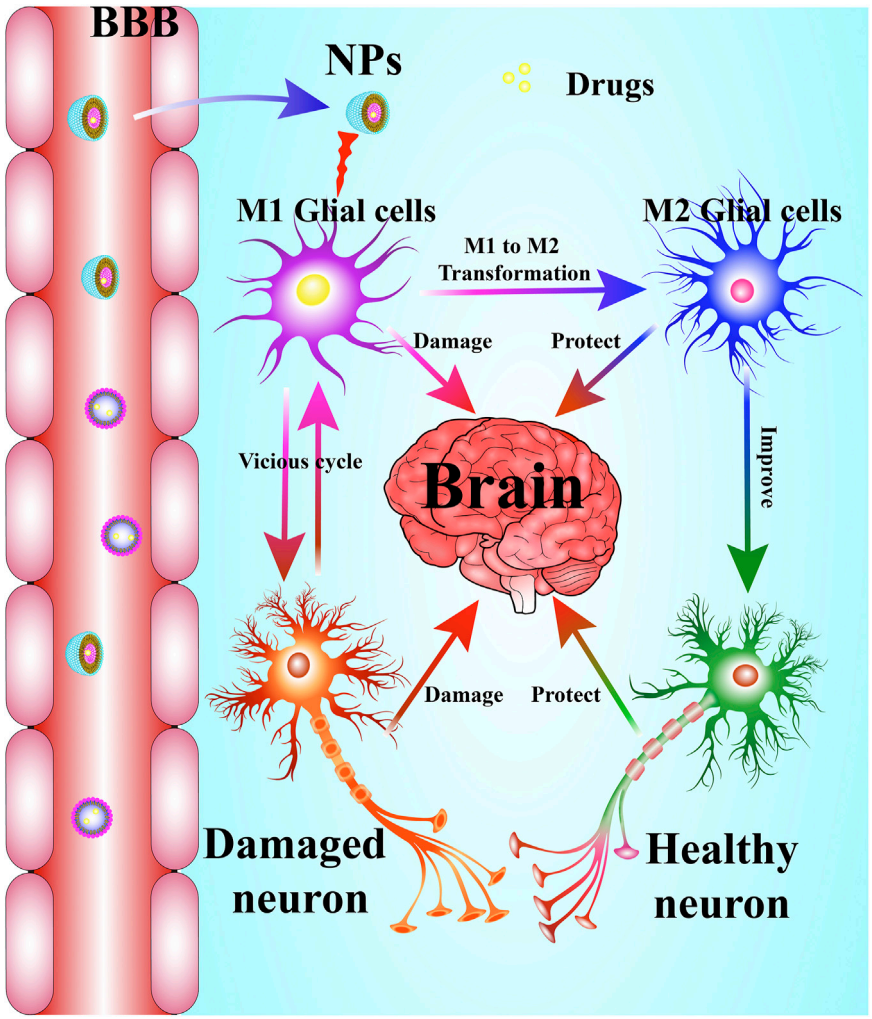

FIGURE 3 | NPs serve as a drug delivery system in neuroinflammation-mediated CNS-related diseases. NPs delivery systems help drugs cross the BBB to inhibit over-activated microglia and its resultant neuroinflammatory response, which promotes the transformation of M1-type microglia into M2-type microglia and improves neuronal viability.

to neuronal death. Lu et al. (2019) encapsulated rapamycin in self-assembled micelles consisting of ROS-responsive and fibrin-binding polymers. They found that the microthrombus-targeting micelles eliminated ROS generation and contributed to micelle polarized M2 microglia repair, thereby enhancing neuroprotection and blood perfusion.

INORGANIC NPS

AuNPs

AuNPs are a type of inorganic nanoparticle which play an important role in pharmacology, sensing (Uehara, 2010), and bio-imaging (Kim et al., 2009; Duncan et al., 2010; Hutter and Maysinger, 2011; Yoo et al., 2017b) with a suitable size and shape. Although AuNPs are widely considered to be safe and have low phototoxicity (Li et al., 2019b), they still induce gold toxicity and the hepatobiliary elimination of AuNPs has attracted considerable attention (Bahamonde et al., 2018; Park et al., 2019). In neurodegenerative disease, AuNPs are reported to suppress the pro-inflammatory responses in a microglial cell line by inducing polarization toward the M2 phenotype, which is beneficial for CNS repair and regeneration (Xiao et al., 2020).

Emerging evidence showed that AuNPs regulated inflammatory signaling by inhibiting the TNF-α pathway and downregulating the NF-κB signaling pathway (Xiao et al., 2020). The mice injected intracerebroventricularly with streptozotocin (STZ) exhibited sporadic AD symptoms, activation of the NF-κB signaling pathway, and increased secretion of IL-1β, while the treatment of AuNPs significantly inhibited the pro-inflammatory response via the NF-κB pathway (Muller et al., 2017).

Furthermore, there are many AuNPs-modified drugs which are more effective as anti-inflammatories than AuNPs or drugs alone. IL-4 is an anti-inflammatory cytokine that can decrease pro-inflammatory cytokines (TNF-α and IL-6) and ameliorate the chronic inflammatory process (Casella et al., 2016).

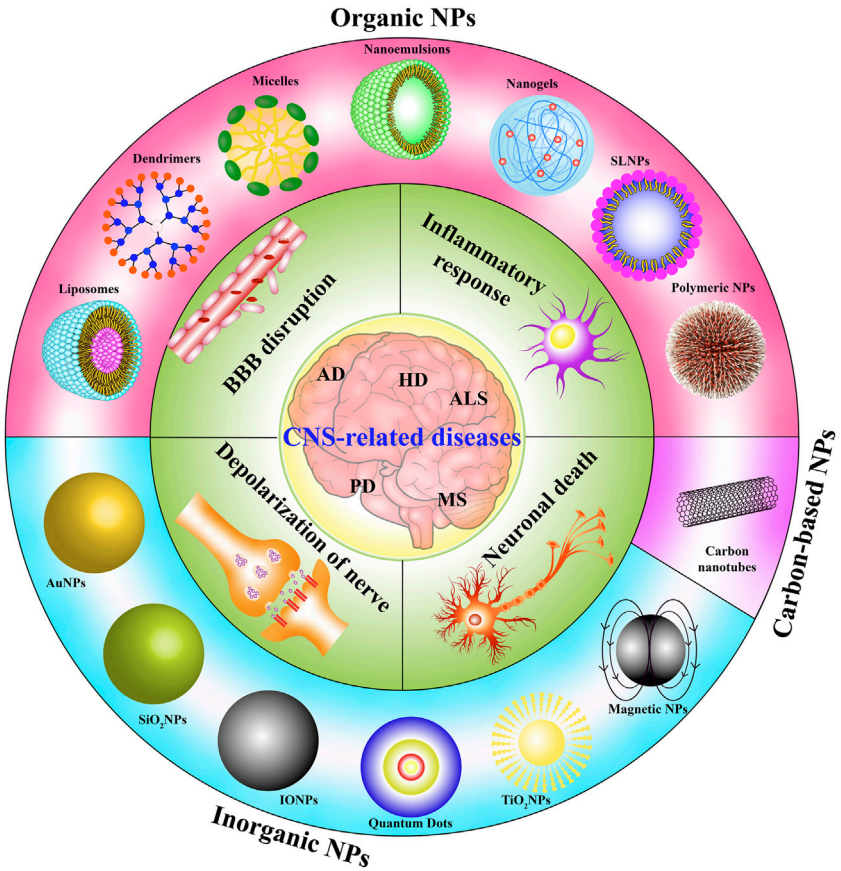

FIGURE 4 | The potential therapeutic effect of NPs on the inflammatory response, neuronal death, depolarization of the nerve, and BBB disruption in CNS-diseases including AD, PD, HD, ALS, and MS.

Compared to the AuNPs treatment alone group, the combination of okadaic acid and AuNPs significantly increased the level of IL-4 both in the hippocampus and cortex regions, suggesting that AuNPs together with okadaic acid exert a synergistic anti-inflammatory effect (Dos Santos Tramontin et al., 2020). In BV-2 cells, gold-quercetin NPs were demonstrated to have stronger anti-inflammatory effects than quercetin or AuNPs alone by decreasing the expression of inflammation-producing enzymes (COX-2 and iNOS) at both the transcriptional and translational levels (Ozdal et al., 2019). *Ephedra sinica* Stapf-AuNPs reduced pro-inflammatory cytokine levels and ROS production by downregulating the IKK-α/β, NF-κB, JAK/STAT, ERK-1/2, p38 MAPK, and JNK signaling pathways, upregulating the expression of HO-1 and NQO1, and by activating Nrf2 and AMPK in BV-2 microglial cells. In addition, the combination of AuNPs and n-acetylcysteine (NAC) significantly attenuated sepsis-induced neuroinflammation by decreasing myeloperoxidase activity and proinflammatory cytokines production, as compared with NAC or AuNPs treatment alone (Petronilho et al., 2020). Anthocyanins administered either alone or loaded with PEG-AuNPs reduced $A\beta_{1\text{-}42}$-induced neuroinflammation and inhibited neuronal apoptosis by constraining the p-JNK/NF-κB/p-GSK3β pathway in BV2 cells and $A\beta_{1\text{-}42}$-injected mice; anthocyanins loaded with PEG-AuNPs exhibited a stronger effect than anthocyanins alone (Kim et al., 2017). Moreover, L-DOPA-AuNF, a multi-branched nanoflower-like gold nanoparticles based on L-DOPA, efficiently improved the penetration of L-DOPA across the BBB (Gonzalez-Carter et al., 2019), which provides evidence for the further development of drugs with potent anti-inflammatory effects that cannot cross the BBB. Therefore, AuNPs, and especially AuNP-modified drugs, exhibit powerful anti-inflammatory effects against neurodegenerative disease (**Table 3**).

Iron Oxide Nanoparticles (IONPs)

IONPs belong to the ferrimagnetic class of magnetic materials, which are widely used in biomedical and bioengineering applications (Figuerola et al., 2010). Magnetic NPs have shown great promise in many fields (Dinali et al., 2017). Superparamagnetic iron oxide nanoparticles (SPIONPs) are applied in magnetic resonance imaging (MRI), magnetic particle imaging (MPI) and targeted drug delivery (Xu et al., 2011; Du et al., 2013; Khandhar et al., 2013; Jin et al., 2014; Schleich et al., 2015). SPIONPs have been extensively used for diagnosis to visualize tumors and metastases in liver (Choi et al., 2006), and for angiography as a blood pool agent to visualize

TABLE 3 | AuNPs for neuroinflammation and their mechanisms as part of *in vitro* and *in vivo* models.

Cells/animals	Diameter (nm)	Treatment	Dose	Mechanism and detected inflammatory cytokines	References
In vitro					
BV2 cells	27 nm	24 h co-culture	100 μg/ml	iNOS and COX-2 mRNA	Ozdal et al. (2019)
BV2 cells	100 nm	24 h co-culture	20 μg/ml	NO, PGE2, IL-6, and IL-1β	Xue et al. (2019)
BV2 cells	35.04 ± 4.02 nm	24 h co-culture	>20 μg/ml	NF-κB, JAK/STAT, MAPK, and PLD pathways NO, PGE2, TNF-α, IL-1β, and IL-6	Park et al. (2019)
BV2 cells, N2a cells	1.87 ± 0.14 nm	24 h co-culture	<5 μg/ml	NF-κB pathway IL-1β, IL-6, TNF-α, IL-10, and iNOS	Xiao et al. (2020)
Mouse microglia N9 cell line	Not Given	24 h co-culture	10 μg/ml	NO	Gonzalez-Carter et al. (2019)
In vivo					
Wistar male rats	20 nm	The injection was given every 48 h over 21 days, beginning 24 h after AD model induction	2.5 mg/kg	IL-1β, IL-4, and TNF-α	Dos Santos Tramontin et al. (2020)
C57BL/6 mice	100 nm	C57BL/6 mice were induced with Parkinsonism for 5 consecutive days and treated only with 20 mg/kg body wt. of *Paeonia moutan*–AuNPs for 14 days	20 mg/kg	NO, PGE2, IL-6, IL-1β, and TNF-α	Xue et al. (2019)
C57BL/6 mice	1.87 ± 0.14 nm	The OGD-challenged brain slices were treated with AuNCs (0, 2 or 5 ug/mL, 0 ug/mL served as OGD controls). After 48 h treatment, the samples were fixed using 4% PFA.	5 μg/ml	NF-κB pathway IL-1β, IL-6, TNF-α, IL-10, iNOS, and ROS	Xiao et al. (2020)
Male Wistar rats	20 nm	Rats received 50 mg/kg of AuNP and/or NAC (20 mg/kg) s.c. immediately after surgery and 12 h after surgery	50 mg/kg	TNF-α, IL-1β, and IL-6	Petronilho et al. (2020)
Wistar male rats	20 nm	The intraperitoneal GNPs treatment was initiated 48 h after administration of streptozotocin. GNPs administration frequency was every 48 h until the 21st after stereotactic surgery	2.5 mg/kg	NF-kB pathway IL-1β	Muller et al. (2017)

inflammatory lesions such as atherosclerotic plaques (Neuwelt et al., 2015). In addition, IONPs also suppress the production of IL-1β in LPS-stimulated microglia (Wu et al., 2013). Therefore, IONPs are mainly employed to diagnose and suppress inflammatory lesions in neurodegenerative diseases.

Silica Nanoparticles (SiO_2NPs)

SiO_2NPs, one of the most broadly exploited nanomaterials, have been utilized in a variety of industries (Vance et al., 2015). SiO_2 NPs have been widely applied in the pharmaceutical industry to encapsulate water-insoluble therapeutic agents to improve their dispersal in aqueous media (Durfee et al., 2016; Echazu et al., 2016). Small-sized SiO_2 has potential applications in the delivery of diagnostic and therapeutic agents across the BBB and brain imaging (Liu et al., 2014). Importantly, SiO_2 exposure does not affect cell viability on different neural cells and does not induce neuroinflammation (Murali et al., 2015; Ducray et al., 2017). However, long-term NPs exposure leads to mood dysfunction and cognitive impairment and alters the synapse by activating MAPKs (You et al., 2018). Therefore, SiO_2NPs can pass through the BBB, and their potential in the treatment of neuroinflammation needs to be explored.

Nanocarbon Lipid-Based NPs

Carbon Nanotubes (CNTs)

CNTs are tubular structures made of a layer of graphene rolled into a cylinder (Eatemadi et al., 2014). These NPs are classified as single-walled carbon nanotubes (SWCNTs) or multi-walled carbon nanotubes (MWCNTs) according to the number of wall sheets in their structure (Salvador-Morales et al., 2006). The potential advantage of CNTs is their capacity to integrate with neurons and enhance neuronal functions as substrates for neuronal growth in different neuron cells (Hu et al., 2004; Matsumoto et al., 2007; Cellot et al., 2009).

After modification by polymers, CNTs can offer additional sites for conjugation of other molecules (Wong et al., 2013). There are some contradictions concerning the effect of CNTs on the nervous system. It was reported that SWCNTs exert an anti-inflammatory effect and protect neurons from ischemic damage in a rat stroke model (Nunes et al., 2012). The release of dexamethasone by polypyrrole/CNTs led to the attenuation of lipopolysaccharide (LPS)-induced microglia activation (Luo et al., 2011). Kermanizadeh et al. (2014) demonstrated downregulation of IL-1β after MWCNT exposure, indicating the inhibition of neuroinflammation. Meanwhile, others found an increase in the expression of IL-1β in mice following exposure to MWCNTs (Hamilton et al., 2013; Kido et al., 2014; HelmyAbdou et al., 2019). It has been reported that oxidation-shortened amino-functionalized MWNT and amino-functionalized MWNT induced a transient increase in almost all pro-inflammatory cytokines (Bardi et al., 2013). Rats exposed to MWCNTs showed an increase in the expression of IL-1β compared with a control group, while the rate of TNF-α expression in male

albino rats was significantly increased after MWCNT exposure (Kido et al., 2014; HelmyAbdou et al., 2019). However, another study revealed a decrease in the rate of TNF-α expression after MWCNT exposure (Kermanizadeh et al., 2014). In addition, MWCNT exposure resulted in neuroinflammatory responses via BBB impairment in cerebrovascular endothelial cells treated with serum from MWCNT-exposed mice (Aragon et al., 2017). Therefore, most CNTs can be used as an effective drug delivery system for neuroinflammation.

Graphene Quantum Dots (GQDs)

GQDs exhibit similar physical and chemical properties to graphene. GQDs are novel 2D nanomaterials composed of graphene nanosheets with a lateral size below 10 nm and ten graphene layers forming the final particle (Ponomarenko et al., 2008; Zhou et al., 2012; Li et al., 2013). GQDs have been considered to alleviate immune-mediated neuroinflammation in a dark agouti (DA) rat model of chronic relapsing experimental autoimmune encephalomyelitis (EAE) via the activation of MAPK/Akt signaling and the suppression of the encephalitogenic Th1 immune response, as well as inflammatory cytokine IL-10, IL-17, and IFN-γ (Tosic et al., 2019). In addition, GQDs inhibit fibrillization of α-synuclein, trigger their disaggregation, and rescue neuronal death against PD (Kim et al., 2018b). GQDs have a potential therapeutic effect for AD by inhibiting the aggregation of $A\beta_{1\text{-}42}$ peptides (Mahmoudi et al., 2012; Liu et al., 2015; Wang et al., 2018). Besides this, curcumin-GQDs can be used as a platform to sense APO e4 DNA, which is responsible for AD (Mars et al., 2018). Therefore, GQDs are considered a promising therapeutic strategy for neuroinflammation and neurodegenerative disease.

NEUROTOXICITY OF NANOMATERIALS

Nanomaterials have wide applications in neutral inflammation therapy with exciting therapeutic effects. However, some researchers have raised questions about the toxicity of nanoparticles, because at nanoscale many atoms may become very active. Therefore, toxicity, and especially neurotoxicity, of nanoparticles for neuroinflammation therapy must be taken into account.

Neurotoxicity refers to any adverse effect on the structure, function, or chemistry of the nervous system produced by physical or chemical causes (Teleanu et al., 2019). The main mechanisms for neurotoxicity involve the excessive production of reactive oxygen species leading to oxidative stress; the release of cytokines causing neuroinflammation; and dysregulations of apoptosis leading to neuronal death (Teleanu et al., 2018). Neurotoxicity of nanoparticles is closely connected with different parameters of nanoparticles like their shape, dosage, size, surface area, and so on. Among the parameters, the size and surface area are the key determinants of toxicity (Saifi et al., 2018). NPs that are commonly used have been studied for potential neurotoxic effects. For example, AuNPs might cause astrogliosis, which is defined as an increase in the number and size of astrocytes and cognition defects including attention and memory impairment. Astrogliosis is closely connected with hypoxia, ischemia, and seizures in brain diseases, and is commonly observed in AD patients (Saijo et al., 2010; Flora, 2017). A high dose of anatase TiO_2NPs significantly increased the IL-6 level in plasma and brain, suggesting that oral intake of anatase TiO_2NPs could induce neuroinflammation and neurotoxic effects (Grissa et al., 2016). IONPs exposure may affect synaptic transmission and nerve conduction (Kumari et al., 2012), causing immune cell infiltration and neural inflammation apoptosis (Wu et al., 2010), inducing oxidative damage in the striatum but not in the hippocampus (Kim et al., 2013). A study showed the drug-free liposomes induced neuropathologic changes, specifically neuroinflammation and necrosis (Yuan et al., 2015). Another study showed that the accumulation of Polysorbate 80-modified chitosan nanoparticles induced neuronal apoptosis, a slight inflammatory response and increased oxidative stress (Huo et al., 2012). Generally, inorganic NPs show more frequent and severe toxicity than organic nanoparticles (Mohammadpour et al., 2019). Most NPs exhibit anti-neuroinflammatory effects either alone or by carrying anti-inflammatory drugs; however, some NPs induce neuroinflammation.

CONCLUSION AND FUTURE PERSPECTIVES

Neuroinflammation is an inflammatory response within the CNS that is marked by the activation of microglia and astrocytes and the production of pro-inflammatory cytokines. Neuroinflammation is the common mechanism behind CNS-related diseases including acute brain injury, stroke, and neurodegenerative diseases. Neurodegenerative diseases are characterized by gradual cognitive or memory impairment and movement disorder. They pose a severe threat to people's health and lower their quality-of-life, especially affecting the elderly population. The treatment of neuroinflammation is faced with many difficulties owing to the poor BBB penetration of drugs. Nanomaterials, an emerging therapeutic tool, may help overcome this obstacle and improve the effect of drugs on anti-neuroinflammation. NPs are a promising delivery system that can combine with drugs by dissolution, adsorption, encapsulation or covalent bonding and be used in the treatment of CNS disorders. The superiorities of NPs enable them to reduce enzymatic degradation, clearance by endothelial cells, and peripheral side effects, while increasing targeting and bioavailability and helping overcome the obstacle of the BBB.

To date, neurodegenerative diseases have affected millions of people worldwide, placing a serious financial and spiritual burden on societies and families. AD and PD are the most common neurodegenerative diseases. Although some drugs can alleviate their symptoms, there are still no drugs approved for the treatment of AD and PD. The BBB penetration of various drugs with potent neuroprotective effects, such as anti-inflammatory drugs in microglia and anti-apoptosis in neurons, is limited. The development of NPs represents a promising strategy for the improvement of the BBB

penetration and neuroprotective effect of these drugs (**Figure 4**). For example, L-DOPA-AuNF improved the penetration of L-DOPA across the BBB (Gonzalez-Carter et al., 2019). ApoE3 polymeric nanoparticles loaded with donepezil showed an enhanced brain uptake of the drug, binding to amyloid beta with high affinity and accelerating its clearance (Krishna et al., 2019). Therefore, it is important to further develop the NPs with high BBB penetration capacity to encapsulate drugs with potent anti-inflammatory and anti-apoptosis effects such as galantamine, noferriamine, rivarasmine, risperidone, curcumin, quercetin, and ropinirole (Cao et al., 2016; Agrawal et al., 2018; Dudhipala and Gorre, 2020) for the treatment of AD and PD in the future.

In addition to the inherent characteristics of NPs, a variety of artificial designs have been developed to further improve performance by altering their size, surface area, surface charge, hydrophilicity and lipophilicity for the treatment of neuroinflammation. For example, binding with polyethylene glycol and polysaccharides prolongs the residence time of NPs. Transferrin-conjugated NPs exhibit higher permeability of the BBB. Prednisolone-loaded liposomes, nanoemulsions with oils rich in omega3 PUFA, polyclonal antibodies against brain-specific antigen and insulin-attached micelles, apolipoprotein E attached SLNs, G4HisMal, and D-mino dendrimers have all exhibited increased targeting of brain tissue (Naqvi et al., 2020).

The synthesis of multifunctional NPs is a hot research topic. Each NP has its own merits and drawbacks. In some cases, the properties of NPs are not compatible with drug binding, drug delivery, crossing the BBB, localization, and drug release (Kim et al., 2018a; Habibi et al., 2020). Therefore, by integrating NPs of different sizes, structures, and functions, multicomponent and multifunctional NPs are designed and their superior characteristics, (e.g. specific-targeting and long-circulation time) can be maximized. In recent years, some researchers have reported the application of PEG-cationic bovine serum albumin (Liu et al., 2013a), PEG-PLA NPs (Liu et al., 2013b), PEG–PLGA NPs (Zhang et al., 2014), chitosan-coated nanoemulsions (Fachel et al., 2020a), mSPAM (Rajendrakumar et al., 2018), and CeNC/IONC/MSN-T807 (Chen et al., 2018) as therapeutic strategies for neuroinflammation and neurodegenerative disease. Multifunctional NPs have extensive application prospects and warrant further exploration.

However, the drawbacks of NPs cannot be ignored. In the last decade, many studies reported that nanomaterials induce pro-inflammatory responses, apoptosis, and excessive oxidative stress of neurons in the brain. In addition, NPs were also demonstrated to accumulate in the liver, kidney and spleen, which may pose a threat to long-term health after administration. Considering these issues, the application of only those organic and degradable NPs with relatively minimal toxicity could be a possible solution. Furthermore, investigations of these nanomaterials in pharmacodynamics and pharmacokinetics are still limited, and their side effects remain to be explored.

NPs are still making their way from bench to clinical application, and many more studies are needed to solve the outstanding problems regarding the treatment of neuroinflammation.

AUTHOR CONTRIBUTIONS

A-GW and Q-ZF conceived the idea. F-DZ, Y-JH, and LY, wrote the original manuscript. X-GZ, J-MW, and D-LQ revised the manuscript. A-GW and YT draw the figures. F-DZ and Y-JH summarized the tables. All authors approved the final version of the manuscript.

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MINI REVIEW
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Therapeutic Applications of Functional Nanomaterials for Prostatitis

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Prostatitis is a common disease in adult males, with characteristics of a poor treatment response and easy recurrence, which seriously affects the patient's quality of life. The prostate is located deep in the pelvic cavity, and thus a traditional infusion or other treatment methods are unable to easily act directly on the prostate, leading to poor therapeutic effects. Therefore, the development of new diagnostic and treatment strategies has become a research hotspot in the field of prostatitis treatment. In recent years, nanomaterials have been widely used in the diagnosis and treatment of various infectious diseases. Nanotechnology is a promising tool for 1) the accurate diagnosis of diseases; 2) improving the targeting of drug delivery systems; 3) intelligent, controlled drug release; and 4) multimode collaborative treatment, which is expected to be applied in the diagnosis and treatment of prostatitis. Nanotechnology is attracting attention in the diagnosis, prevention and treatment of prostatitis. However, as a new research area, systematic reviews on the application of nanomaterials in the diagnosis and treatment of prostatitis are still lacking. In this mini-review, we will highlight the treatment approaches for and challenges associated with prostatitis and describe the advantages of functional nanoparticles in improving treatment effectiveness and overcoming side effects.

Keywords: prostatitis, functional nanoparticle, inflammatory microenvironment, engineering strategy, reactive oxygen species

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BACKGROUND

Prostatitis is one of the most common urogenital diseases and mainly manifests as hypogastrium, perineum, scrotum, urethra and penis pain, and even bladder irritation, seriously affecting the patient's quality of life (Krieger et al., 2008; Brede and Shoskes, 2011; Kogan et al., 2018). According to statistics, approximately half of males have ever suffered from prostatitis, and prostatitis outpatient services account for approximately 25% of services provided by urology clinics. Prostate cancer and benign prostatic hyperplasia mainly occur in older males, while prostatitis occurs in males of all ages, especially in young and middle-aged males (Drake et al., 2021). It is the third most common urinary disease in males (Khan et al., 2017).

Prostatitis is mainly divided into 2 class I acute bacterial prostatitis, class II chronic bacterial prostatitis, class III chronic prostatitis/chronic pelvic pain syndrome, and class IV asymptomatic

inflammatory prostatitis (Krieger et al., 1999). In addition, chronic prostatitis/chronic pelvic pain syndrome accounts for 90-95% of prostatitis cases (Sharma and Kumar, 2021). In 2008, the National Institutes of Health (NIH)-affiliated National Institute of Diabetes, Digestive and Kidney Disease established the Map Research Network to guide researchers in more disciplines to participate in collaborative research on chronic pelvic pain and to update and improve its definition and treatment standards. Currently, the pathogenic factors causing chronic prostatitis in the clinic are controversial. Traditional treatments for prostatitis include antibiotics, antioxidants, and surgery (Vahlensieck et al., 2013; Ihsan et al., 2018). Many patients have turned to alternative therapies because of the limited effectiveness of traditional therapies and recurrence. In recent years, physical therapies for prostatitis have included biofeedback, hyperthermia, and magnetic therapy, but the efficacy and side effects are controversial (Hu et al., 2019; Birowo et al., 2020). Therefore, studies exploring the etiology and pathogenesis of prostatitis and identifying new strategies to improve its therapeutic effectiveness are needed. This review highlights the treatment approaches for and challenges associated prostatitis and describes the advantages of functional nanoparticles in improving treatment effectiveness and overcoming side effects.

MAIN FACTORS CAUSING PROSTATITIS

The pathogenesis of prostate disease is complex with numerous influencing psychological factors, including pathogen infection, sex hormone imbalance, urination dysfunction, inflammation, and abnormal immune response (Sharp et al., 2010), among which the inflammatory response is the key pathological mechanism of prostatitis, and the inflammatory microenvironment determines the process of prostatitis, (Crocetto et al., 2020; Huang et al., 2020) as schematically depicted in **Figure 1**.

Pathogen Infection

Viruses, fungi, bacteria and other pathogenic microorganisms can cause prostatitis, and bacterial infection is an important pathogenic factor causing prostatitis (Delcaru et al., 2016; Khan et al., 2017). Most of the pathogens detected in patients with prostatitis are gram-negative bacteria, and 60% of the bacteria are *Escherichia coli*

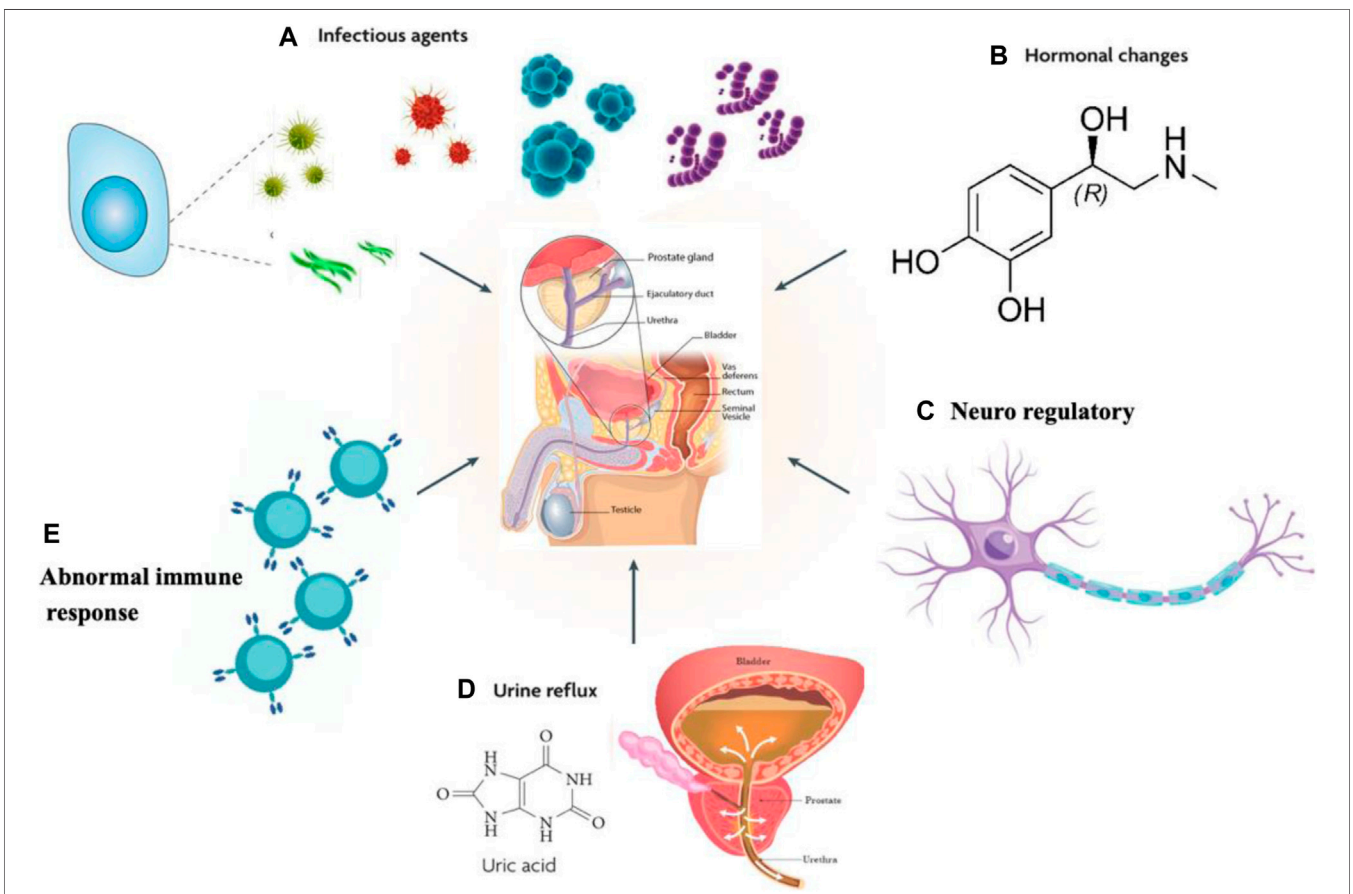

FIGURE 1 | Possible causes of prostate inflammation. (**A**) Pathogen infection. (**B**) Sex hormone imbalance. (**C**) Urination dysfunction. (**D**) Neuroregulatory mechanisms. (**E**) Abnormal immune response.

(Barzelai and Whittem, 2017; Zhao et al., 2019; Su et al., 2020). In anti-infection treatment, because the pathogenic bacteria increase or exert an inhibitory effect on the defense function of the patient, pathogenic bacteria exist for a long time and cannot be eradicated (Benway and Moon, 2008). Bacterial infection may be the trigger rather than the cause of the clinical syndrome.

Sex Hormone Imbalance

The prostate is a sex accessory organ, and pathological changes in the prostate and the progression of prostatitis are closely related to sex hormones and their receptors (Letkiewicz et al., 2020). In addition, prostate gland lesions and the occurrence and development of prostatitis are closely related to sex hormones and their receptors, and a sex hormone imbalance is the main reason for class IIIB prostatitis (Lan et al., 2017).

Urination Dysfunction

Uric acid is filtered through the glomerulus and is the product of nucleic acid decomposition, cell metabolism and purine metabolism in the body. Most urate is reabsorbed through the proximal convolution tubule, but an accumulation of urate crystals in the tissue leads to an inflammatory reaction that produces high-frequency contraction and spasm of the urethral sphincter. These changes cause an imbalance in bladder detrusor and sphincter synergism or bladder outlet obstruction and urine reflux.

Neuroregulatory Mechanisms

Neuroregulatory mechanisms are closely related to prostatitis. In patients with prostatitis (Park et al., 2015; Shulyak et al., 2019), inflammation is stimulated and may cause long-term nervous system damage that result in, clinical symptoms with a spinal nerve segmental dominance. Prostate pain may be the cause of spinal nerve segmental secondary lesions. Some prostatitis pain may be caused by constant pain in spinal nerve segmental nerves, but scholars have also indicated that prostatitis pain may be due to the abnormal state of the chronic neuroregulatory mechanism caused by multiple factors or a single cause, which may be related to spinal cord glial cells or spinal cord nerve cells (Shih et al., 2020).

Abnormal Immune Response

Relevant studies have suggested that prostatitis is likely an autoimmune disease (Motrich et al., 2007). People with normal immune function generally do not experience inflammation after an infection, while those with low immune function are prone to inflammation. Some scholars also proposed that the prostate is an immune organ with more than 90% T lymphocytes, which exist in the epithelial stromal area of the gland, along with a small number of other inflammatory cells (Motrich et al., 2020). T lymphocytes produce IFN-γ and stimulate the production of IL-15 in the prostate, and this paracrine signaling is the cause of chronic inflammation (Handisurya et al., 2001). Both prostatic epithelial cells and stromal cells express cytokine receptors, participate in local immune regulation as anti-inflammatory presenting cells (Carlo et al., 2007; Penna et al., 2009; Fibbi et al., 2010; De Nunzio et al., 2011), and secrete pro-inflammatory cytokines such as IL-1α, IL-1β and IL-6 (Kramer et al., 2003; Beadling and Slifka, 2006; Magri et al., 2019). Prostatitis is considered an autoimmune disease (Li et al., 2019).

CHALLENGES IN THE CLINICAL TREATMENT AND DIAGNOSIS OF PROSTATITIS

Currently, ideal treatment and diagnostic methods for prostatitis are still lacking, and thus new drug delivery systems and diagnostic strategies for prostatitis are urgently needed.

Challenges in the Treatment of Prostatitis

The etiology of prostatitis is unclear due to the numerous symptoms with no specificity (Verze et al., 2016). In recent years, some experts have proposed the concept of prostatitis syndrome, a clinical syndrome with different etiologies, clinical manifestations, disease processes and responses to treatment (Ramakrishnan and Salinas, 2010). Antibiotics, nonsteroidal anti-inflammatory analgesics and alpha-blockers are used in the traditional clinical treatment of prostatitis (Xiong et al., 2021). In addition, pharmacological treatments remain largely ineffective due to the difficulty in penetrating the prostatitis microenvironment. Prostatitis is characterized by inflammatory hyperplasia, a high pH, bacterial accumulation and a disruption of the blood-prostate barrier (El Meliegy and Torky, 2015). These four characteristics and properties are analyzed in the remainder of the article.

Inflammatory Hyperplasia

Prostatitis is accompanied by inflammatory hyperplasia, leading to prostatic hyperplasia and edema, prostate duct stenosis or obstruction caused by pressure in the gland, and the blood circulation barrier obstructs the entry of drugs (Ravindran et al., 2020). At the same time, inflammatory exudates extravasate around the prostate due to high pressure, causing or exacerbating symptoms of pelvic and urinary tract irritation.

High pH

In addition, prostatitis increases the pH of the prostate and decreases drug dispersion, and the concentration of drug that penetrates the prostatic canal, acini and prostatic fluid is insufficient. Recurring episodes of prostatitis lead to the formation of calcified plaques in the prostate.

Bacterial Accumulation

Bacteria accumulate inside or on the surface of calcified plaques (Dibb et al., 2001), exist and multiply sustainably under protective biofilms. Calcified spots can develop into stones, which may block the prostate gland duct and induce infection. Therefore, calcified spots and stones are important factors affecting the effectiveness of prostatitis treatment, leading to repeated attacks.

Blood-Prostate Barrier

Rectal administration is one of the most common methods used in prostate treatment, but some drugs are unable to pass through the blood-prostate barrier and do not reach effective therapeutic concentrations in the prostate tissue and acinus. (El Meliegy and Torky, 2015) Direct injection into the prostate solves the problem of the prostate anatomical barrier, but invasive treatment easily causes damage to the nerve and vascular tissues of the perineum and aggravates local inflammation.

Challenges in the Diagnosis of Prostatitis

The NIH classifies prostatitis into four subtypes (Sharma and Kumar, 2021), and the main cause of type I and II prostatitis is pathogen infection. According to the type of pathogen, the choice of appropriate antibiotics results in a better treatment effect. Type IV prostatitis is difficult to detect due to a lack of clinical symptoms, relevant pathogenesis and treatment studies. Among the CP/CPPS, is the most common, accounting for more than 90% of chronic prostatitis cases. (Holt et al., 2016) The diagnostic criteria are that the patient has persistent or recurrent pain in the pelvic area for at least 3 of the past 6°months. However, the definition of prostatitis is still relatively vague, the classification is complex, the diagnostic method is also quite controversial, and reliable physical and chemical indicators are lacking. No unified standard for the clinical diagnosis and evaluations of the curative effect are available, and the curative is generally difficult to evaluate and analyze (Coker and Dierfeldt, 2016).

APPLICATION OF FUNCTIONALIZED NANOMATERIALS IN PROSTATITIS

Many pathogenic factors contribute to prostatitis (Delcaru et al., 2016), and lesions induced by different factors require different detection and treatment methods, which undoubtedly increases the difficulty of diagnosing and treating prostatitis. At the same time, urethral inflammation has a long disease course, and traditional diagnostic and treatment methods are invasive, which will exert a certain effect on the patient's body and mind (Mangir and Chapple, 2020). For example, the most commonly used mode in clinical practice, rectal administration, may damage the intestinal mucosa due to unstable drug absorption. Therefore, the treatment of prostatitis requires good imaging performance, strong compatibility and high universality of imaging technology, and a high bioutilization of pharmaceutical preparations.

The inflammatory response is the core pathological mechanism of prostatitis and the key link affecting the disease process (Motrich et al., 2018). Methods to effectively alleviate the inflammatory microenvironment are the key to improving the clinical efficacy of prostatitis treatments. In addition, prostatitis is often accompanied by a microbial infection. For prostatitis caused by a microbial infection (Kogan et al., 2018), treatment with anti-infectious agents is the most direct and effective method. Inflammation and infection are also major diagnostic indicators of urethral inflammation, including prostatitis.

Nanotechnology refers to the study and application of materials at the nanoscale, and its application in the medical field is called nanomedicine (Richardson and Caruso, 2020). Advances in nanotechnology have facilitated the development of delivery systems to treat prostate-related disorders. Advantages of nanocarrier preparations include the combination of a variety of drugs, including biomacromolecule drugs; reduced degradation of unstable drugs for slow and controlled release; and increased residence time of relevant drugs to avoid frequent injections and meet the needs of prostatitis treatment (Thakur and Agrawal, 2015; Liu et al., 2020; Liu et al., 2020; Wang et al., 2020; Lin et al., 2021; Liu et al., 2021). More importantly, the modular design and preparation characteristics of nanotechnology endow nanomaterials with intelligent characteristics (van der Meel et al., 2019). Smart NPs are designed to respond to environmental or external stimuli that trigger drug release after passive or active accumulation, as schematically depicted in **Figure 2**.

Nanotechnology is a powerful tool for developing new treatments and diagnoses for prostatitis and is expected to continue to grow in the future. In recent years, a number of nanomaterials with anti-inflammatory and antimicrobial properties have emerged,including $CuFeO_2$ and Fe_3O_4 NPs, nanohydrogels, photosensitive H_2-generated nanosystems, and polydopamine nanoparticles (Salari et al., 2018; Yu et al., 2018; Zhao et al., 2018; Antonoglou et al., 2019; Zhang et al., 2019). Nanomaterials with anti-inflammatory and anti-infective properties show good application prospects in the treatment of prostatitis. We will summarize the applications of functionalized nanomaterials in prostatitis and evaluate the advantages and disadvantages (**Table 1**).

The Application of Functionalized Nanomaterials in Prostatitis

Inorganic Nanomaterials

Inorganic nanomaterials have been widely used in biomedical fields because of their easy availability and stable properties. Inorganic nanomaterials generally refer to the incorporation of metal and nonmetal elements, metal oxides, salts and other components into nanoparticles alone or in combination (Rao et al., 2007). These nanomaterials have different physical and chemical properties due to their different compositions and structures. Thus, different inorganic nanomaterials have different applications (Liang et al., 2014). In UTI (including prostatitis), inorganic nanomaterials are mainly used in the scenarios described below.

Inflammation is associated with oxidative stress and can be alleviated by antioxidants (Czarny et al., 2018). A variety of inorganic nanomaterials have been found to possess antioxidant activity. Iron nanoparticles, such as Fe_3O_4 nanoparticles, reduce oxidative pressure by catalyzing the degradation of H_2O_2 (Alavi and Karimi, 2019). Fe_3O_4 is also considered a magnetic nanoparticle with good biocompatibility and anti-inflammatory activity (Xie et al., 2019). Fe_3O_4

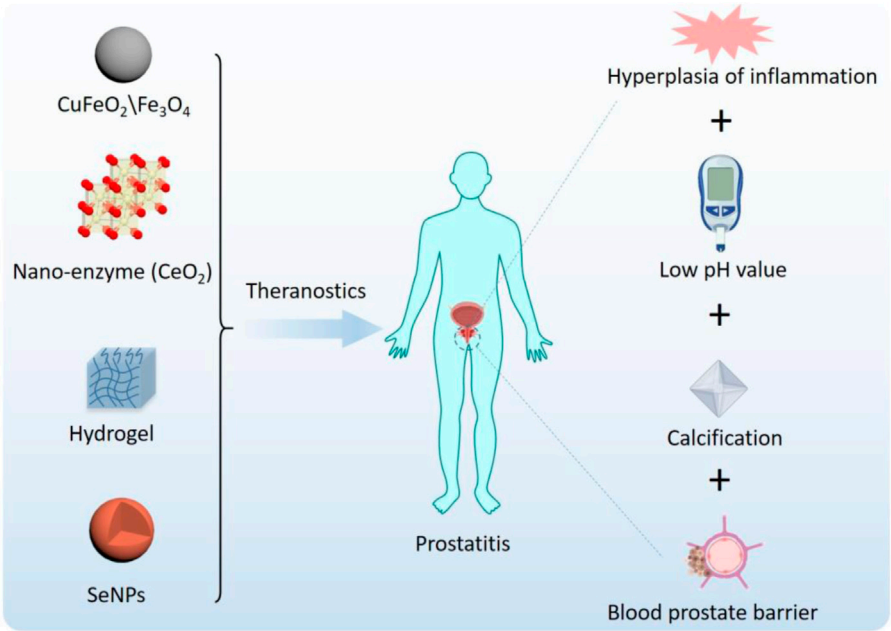

FIGURE 2 | Smart nanoparticles for prostatitis.

nanoparticles have been combined with various anti-inflammatory drugs as a new strategy for the treatment of prostatitis (Kojima et al., 2018). Nanoparticles composed of another metal oxide, zinc oxide, are also widely used to treat urethral inflammation (Ihsan et al., 2018; Hosseini et al., 2019; Abd Elkodous et al., 2020). Zinc oxide nanoparticles have a good antioxidant function in combination with other components (García-López et al., 2018). In recent years, the development of enzymology has provided an effective tool for the removal of reactive oxygen species, and some inorganic nanoenzymes with an inherent antioxidant capacity have also been developed as neuroprotective therapeutic drugs, among which CeO_2 is the most promising (Kwon et al., 2018; He et al., 2020). Hirst et al. documented the anti-inflammatory properties of CeO_2 nanoparticles for the first time in 2009, as these nanoparticles inhibited the expression of iNOS in LPS-induced macrophages (Hirst et al., 2009). Soh et al. prepared a cerium oxide-zirconia compound nanoenzyme and found that it eliminated ROS production to inhibit sepsis (Soh et al., 2017). Therefore, inorganic nanomaterials with enzyme-like effects scavenge free radicals and exert anti-inflammatory effects by producing enzymatic reactions.

In addition, inorganic nanoparticles have good performance in fighting microbial infections. Gold nanoparticles improve the antibacterial activity of antibiotics through the targeted delivery of antibiotics (Patil and Kim, 2017). Meanwhile, the photothermal effect of gold nanoparticles irreversibly destroys the bacterial membrane structure and then kills bacteria (Hu et al., 2017). Silver, magnesium and iron particles, when reduced to nanosize, were suggested to exhibit antibacterial activity against *E. coli* and *S. aureus* (Yousefshahi et al., 2018; Videira-Quintela et al., 2020). Nanosilver is widely used in the medical field because of its strong antibacterial activity, lack of drug resistance and safety. Silver NPs target the respiratory system and cell division of microorganisms that eventually result in cell death (Panáček et al., 2018). Copper nanoparticles have a similar antibacterial mechanism in urinary tract infection (Al-Enizi et al., 2018). Titanium dioxide nanoparticles have also been used to destroy bacterial cells (Zheng et al., 2018). The antimicrobial activity is based on the photocatalytic property of TiO_2 NPs (Guo et al., 2019). The production of reactive oxygen species (ROS) by TiO_2 was also reported (Yousefshahi et al., 2018). In addition to their direct anti-infective effects, nanoparticles can also be loaded with antibiotics. Biocompatible Fe_3O_4 nanoparticles increase the efficacy of amoxicillin against gram-positive and gram-negative bacteria through magnetic targeting (Lu et al., 2017). Sulfur nanoparticles enhance the killing of urethral pathogens by delivering antibiotics (Paralikar et al., 2019). Notably, the metabolism of inorganic nanoparticles remains controversial, especially those containing heavy metals, which have the risk of metabolic toxicity (De Matteis, 2017). These nanomaterials have important application prospects in the treatment of urethral infectious inflammation.

Organic Nanomaterials

In the field of medicine, hydrogels have great potential for development. The structure determines the properties, and the biocompatibility, biodegradability and nanometer compound properties of hydrogels are commonly used in the medical field (Fuchs et al., 2020). Therefore, hydrogels are widely used in the medical field as drug release carriers and corneal contact lenses, in bone tissue and soft tissue regeneration, and in reconstruction and burn treatment (Chen et al., 2019). Sun

TABLE 1 | The application of functionalized nanomaterials in prostatitis.

Nanocarrier	Therapeutic strategy	Model	Effective constituent	Advantages	Disadvantages	Refs.
silver NPs	Antimicrobial for multidrug-resistant bacteria	Urinary tract infections	Silver	Inhibits biofilm formation, inhibits the growth of UTI-causing pathogens. Inhibits multidrug-resistant bacteria	Metabolic toxicity	(Divya et al., 2019) (Lopez-Carrizales et al., 2018) (Maharubin et al., 2019), (Al-Ansari et al., 2020)(El-Batal et al., 2019)
sulfur NPs	Antimicrobial	Urinary tract infections	Sulfur	Use as an antibacterial agent alone or in combination with antibiotics to exert synergistic effects	Metabolic toxicity	(Paralikar et al., 2019)
Zinc oxide NPs	antioxidant activity and antibacterial activity	Urinary tract infections	Zinc oxide	ZnO NPs displayed antibacterial activities and moderate antioxidant potential.	none	(Santhoshkumar et al., 2017; Chandra et al., 2019; Hosseini et al., 2019; Abd Elkodous et al., 2020)
Extracellular vesicles	Anti-inflammatory	chronic prostatitis	Extracellular vesicles	Ameliorates chronic pelvic pain, improves voiding dysfunction, suppresses inflammatory reactions, and facilitates prostatic tissue repair.	preparation is relatively complicated and the active ingredients are complex	(Peng et al., 2021)
Nanoparticle-conjugated Autoantigen Peptide T2	Anti-inflammatory	Autoimmune prostatitis	Autoantigen Peptide T2	Ameliorates the manifestations of CP/CPPS that will improve the effectiveness of therapeutic approaches.	autoimmune risk	(Cheng et al., 2019)
Selenium NPs	Antimicrobial	Urinary tract infection	Selenium	Increased percentage of biofilm. Efficient inhibition of *S. aureus, P. aeruginosa,* and *E. coli*.	none	(El-Sayyad et al., 2020)
copper NPs	Antioxidant and antibacterial	Urinary tract infection-causing pathogens	Copper	Proved to effectively kill or significantly inhibit the activity of urinary tract infection-causing pathogens and exhibits excellent antioxidant activity.	Metabolic toxicity	(Malarkodi and Rajeshkumar, 2017; Al-Enizi et al., 2018)
PLGA nanoparticles	Antimicrobial	Urinary tract infections	Trimethoprim	No effects on metabolism and good histocompatibility	One function of the carrier	(Brauner et al., 2020)

Xiaoyong conducted clinical trials, and patients with prostatitis were divided into two groups, a group treated with terazosin hydrochloride combined with levofloxacin, another group treated with nanosilver hydrogels and silver nanoparticles through the anal route, and premature ejaculation grading and erectile function index were evaluated in the two groups of patients before and after treatment to assess sexual function and quality of life (Sun et al., 2019). He observed improvements in these indicators in patients treated with the nanosilver hydrogel (Sun et al., 2019). Although hydrogels have good sustained release and anti-inflammatory effects, chemical cross-linking reagents are often needed.

In addition to hydrogels, organic nanoemulsions are also commonly used as drug carriers. PLGA nanoparticles show excellent antibacterial properties against urethral pathogens by delivering trimethoprim (Brauner et al., 2020). Liposomes are widely used in biomedical research, especially in nucleic acid delivery research. Zhao et al. reported that the in vivo delivery and expression of hBD-2 via liposomes reduced mucosal damage, interstitial edema and inflammatory cell infiltration in animal models of UTI (Zhao et al., 2011). Active peptide nanoparticles have also been used in prostate therapy. According to Cao et al., nanoparticles coupled with the autoantigen peptide T2 display improved efficacy against CP/CPPS, which would improve the treatment approach (Cao et al., 2019).

In recent years, biologically derived nanomaterials, including extracellular vesicles, have been widely used in the field of biomedicine (Fan et al., 2019; Fan et al., 2019; Jiang et al., 2020; Wang et al., 2020). Extracellular vesicles are phospholipid bilayer membrane vesicles that are released by cells and transmit information between cells. They also play an important role in regulating inflammation in the body. Extracellular vesicles derived from neutrophils exert an anti-inflammatory effect because they express inflammatory cytokine receptors that bind to and clear inflammatory cytokines (Gao et al., 2017; Li et al., 2020). Researchers found that extracellular vesicles from other cellular origins also exert anti-inflammatory effects (Liu et al., 2020; Gao et al., 2021). Extracellular vesicles derived from mesenchymal stem cells inhibit inflammatory phenotypes by regulating immune cell signal transduction in individuals with chronic prostatitis (Peng et al., 2021). In addition, bionic extracellular vesicles are widely used in anti-inflammatory and anti-infection research. Jiang et al. achieved endotoxin and exotoxin cleanup and

antimicrobial effects by constructing hybrid bionic extracellular vesicles targeting bacteria (Jiang et al., 2021). These studies provide new insights into the treatment of urethral inflammation.

Nanomaterials for the Diagnosis of Prostatitis

Nanomaterials have been widely used in biosensors, molecular diagnosis, medical imaging and other research fields (Liu et al., 2014; Deng et al., 2017; Liao et al., 2018; Liu et al., 2019; Liu et al., 2020) and have wide application prospects in the diagnosis of prostatitis (Qindeel et al., 2021). In particular, in medical imaging, nanomaterials have been used as contrast agents to guide the treatment of prostatitis. Contrast agents enter the body through surface coupling or encapsulation in nanoparticles, which increase the acoustic reflectivity and form clearer images with increased brightness (Wu et al., 2019; Fan et al., 2021). Magnetic resonance (MR) produces an image of resonance signals caused by radioexcited external magnetic fields based on the spin of protons. MNPs have been used as contrast agents to modulate the undulation of T2 of water molecules to form the "target-MNP" polymer. At this point, MNPs and target molecules form a magnetic cluster through the specific binding of high-affinity ligands, resulting in faster attenuation of the NMR signal or a shorter transverse relaxation time (Polackwich and Shoskes, 2016). Compared with GMP, MTJ and μHall sensors effectively shorten the time required to complete immunoassays [58]. Computed tomography (CT) uses X-rays to create cross-sectional and three-dimensional images of different tissue decay states. The CT contrast medium plays a key role in distinguishing similar attenuation coefficients. Two types of CT contrast agents are composed of nanoparticles. One is an iodine-based nanosynthetic drug in which nanoparticles act as carriers of iodine (Xu et al., 2019), such as liposomal iodine [59]. The second category is metal-based contrast agents, which are composed of nanoparticles derived from various metals with high X-ray attenuation factors, including gold and zirconia. Nano-CT contrast agents are widely used in biomedical imaging. For example, gold nanoparticles are engulfed by red blood cells to form blood flow images (Han et al., 2019). CT pulmonary angiography is a minimally invasive angiography technique that rapidly infuses an iodine contrast agent into the pulmonary artery through the superior vena cava, the right atrium and the right ventricle through the superior vena cava and then to the pulmonary artery. Scanning using spiral CT or electron beam CT has been used as a first-line clinical screening method for acute pulmonary embolism.

With the rapid development of biomedical imaging technology in the 21st century, this technology has become an important method for the clinical diagnosis and detection of prostatitis. The field of biomedical imaging expanded from the initial X-ray imaging to magnetic resonance imaging (MRI), computed tomography (CT), and ultrasound used today after a long period of exploration and growth. Although these imaging techniques have different imaging principles, they all observe tiny lesions in a noninvasive manner, providing excellent images of humans due to their unique advantages. However, they have some inherent limitations. For example, magnetic resonance imaging has an insufficient spatial resolution, leading to low sensitivity [46]. Therefore, many contrast agents have been developed to improve the contrast between normal tissue and prostate lesions and thus improve the diagnostic accuracy.

The cause of prostatitis is multifactorial, and the disease course is long. The most commonly used mode in clinical practice, rectal administration, may cause damage to intestinal mucosa due to unstable drug absorption. Therefore, the treatment of prostatitis requires good imaging performance, strong compatibility, a high universality of imaging technology, and a high bioutilization of pharmaceutical preparations. Advances in nanotechnology have facilitated the development delivery systems to overcome prostate-related disorders. Advantages of nanocarrier preparations include the combination of a variety of drugs, including biomacromolecule drugs; reduced degradation of unstable drugs and slow and controlled release; and increased residence time of relevant drugs to avoid frequent injections to meet the needs of prostatitis treatment (Thakur and Agrawal, 2015). In addition, an increasing number of nanomaterials have attracted attention due to their excellent imaging performance. Currently, many nanomaterials have been successfully developed as contrast agents for clinical use (Lu et al., 2017; Hu et al., 2018; Song et al., 2018). For example, iron oxide nanoparticles and manganese oxide nanoparticles are used as MRI contrast agents because of their unique magnetic properties (Waddington et al., 2020). Gold nanorods have been used in photoacoustic imaging (PAI) due to their unique surface plasmon resonance properties (Huang et al., 2019). Surface engineering modification is often performed to maintain or improve their biocompatibility, colloidal stability and disease targeting and to achieve the more effective use of nanocontrast agents (Fan et al., 2021). Zhao Meng prepared a series of inorganic nanoparticles with a uniform morphology and imaging performance using polyglycol for ligand exchange to improve the colloidal stability and biocompatibility of the nanoparticles (Zhao et al., 2020). Then, the inappropriate groups were modified with spermidine, and finally, the targeted nanocontrast agent based on supramolecular chemical surface modification was obtained. Various methods were used to measure its properties, and the prepared contrast agents displayed good dispersiveness, colloid stability, and targeting, and the surface modification method was universal [51]. Surface engineering modifications based on supramolecular chemistry provide a new design idea and experimental basis for the future design and development of targeted prostatitis-related nanoagents. MR has the advantages of a high soft tissue resolution and no ionizing radiation, and thus it could be used in the diagnosis of prostatitis.

Nanomaterials for the Prevention of Prostatitis

The key to preventing and controlling infectious diseases is to control the source of infection, cut off the transmission route and protect vulnerable groups (Nii-Trebi, 2017). Nanoantibodies can eliminate pathogenic microorganisms in animals, control the

source of infection or cut off the transmission route to prevent diseases and protect people. *Campylobacter* infection is one of the most common foodborne infections in humans, and broilers are the main source of *Campylobacter* infection (Kelly et al., 2017; O'Brien, 2017). Nanoantibodies specifically target the outer membrane proteins of *Campylobacter jejuni* and *Campylobacter coli* in broilers, inhibit the fixed value of *Campylobacter jejuni* and block bacterial transmission (Vanmarsenille et al., 2017).

Passive immunity refers to the provision of pathogen-specific foreign antibodies to susceptible populations to achieve rapid protection in the short term. Traditional monoclonal antibodies are derived from the serum of humans or immunized animals, the manufacturing process is complicated, and the cost is high. Some animal-derived monoclonal antibodies easily cause adverse reactions. Nanoantibodies have become an alternative to existing passive immune antibodies. Many pathogens and external harmful substances enter the human body through the gastrointestinal mucosa. Vaccines targeting the mucosal surface can induce a mucosal immune response and prevent gastrointestinal infection. Oral vaccines are the most attractive route of treatment. However, vaccine antigens in the intestine often fail to reach potential immune-inducing sites, leading to a poor immune response. Aminopeptidase N (APN) is a receptor expressed on small intestinal cells and antigen-presenting cells (APCs). The combination of APN-specific targeting drugs with vaccine antigens significantly stimulates the immune response in the intestinal mucosa. Bakshi [53] constructed an anti-porcine APN nanoantibody with the Fc domain of conventional antibodies to form a bivalent fusion protein that triggered the intestinal IgA response after oral administration and confirmed the potential of vaccine antigen carriers (Wu et al., 2020). Modern bioengineering technology can help construct a variety of expression systems for nanoantibodies, improve the biosafety of nanoantibodies and promote their popularization and application. Rotavirus is the main cause of severe diarrhea in infants and young children, and specific therapeutic drugs are still lacking. Researchers have constructed expression systems in yeast, lactobacillus and transgenic rice to produce anti-rotavirus nanoantibodies that prevent rotavirus-induced diarrhea (Vandervaart et al., 2006; Martín et al., 2011). Transgenic rice were consumed by mice to absorb the nanoantibody expressed and stored in rice and to subsequently prevent diarrhea. These measures all suggest that nanoantibodies can be used as a complement to current vaccine-based infectious disease prevention measures (Tokuhara et al., 2013).

Nanomaterials for the Treatment of Prostatitis

Anti-inflammatory and antimicrobial agents are the two main strategies for the treatment of urethral inflammation. We will summarize the application of nanomaterials in the treatment of prostatitis from antibacterial and anti-inflammatory aspects.

Application of Antimicrobial Nanomaterials in Prostatitis

Pathogenic microorganisms such as viruses, fungi and bacteria cause prostatitis, among which bacterial infection is the main pathogenic factor causing prostatitis. The number of antimicrobials used to eradicate type II chronic bacterial prostatitis is very limited. Treatment of CBP is hampered and challenging because most antimicrobial agents have a poor ability to penetrate infected prostate fluids and tissue (Charalabopoulos et al., 2003). Another reason is the lack of an active transport mechanism. Some drugs reach the prostate and achieve a minimum inhibitory concentration, but they also run the risk of bacterial resistance (Yu et al., 2018). Nanomaterials or nanoparticles may exhibit antimicrobial properties alone or enhance the efficiency of antibiotic administration. Antimicrobial NPs consist of metals and metal oxides, antimicrobial compounds, surfactant-based nanoemulsions and carbon-based nanomaterials. These nanoantibiotics may damage pathogens through several mechanisms: a) they may produce reactive oxygen species, damaging microbial cell components; b) they may degrade the cell walls of pathogens; c) they may interfere with energy transduction mechanisms; and d) they may slow or hinder DNA synthesis (Yoon et al., 2011; Kumar and Das, 2017; Fernando et al., 2018; Raza et al., 2019). Nanoantibiotics would be more useful in eradicating intracellular infections. While conventional antibiotics are effective at suppressing bacterial growth, they are least effective against bacteria that remain in quiescent cells. Urinary tract pathogens often take advantage of this limitation and cause urinary tract infections to recur after antibiotics have failed. Nanoantibiotics target residual bacteria in cells to avoid recurrence mechanisms.

Bacterial biofilms are an important barrier that promote bacterial self-protection and an important mechanism of therapeutic tolerance. Nanomaterials have shown unprecedented advantages in destroying bacterial biofilms. Li et al. realized the antimicrobial effect of the biofilm microenvironment response by designing antibiotic quantum dots (Li et al., 2020). In urethral infections, well-designed nanoparticles inhibited the production of biofilms, thereby inhibiting infection. For instance, Hosseini et al. reported that ZnO nanoparticles exert inhibitory effects on the biofilms of both isolates (Hosseini et al., 2018). These findings confirm the potential of zinc oxide as a treatment for catheter-associated urinary tract infections. In comparison, research into the antibiofilm effects of silver nanoparticles is more extensive and mature (Martinez-Gutierrez et al., 2013). Silver nanoparticles inhibit the formation of biofilms in organisms, including the natural marine environment (Fabrega et al., 2011), wastewater (Sheng and Liu, 2011) and mammals (Qin et al., 2014). The oxidation of silver ions is widely recognized as an antimicrobial mechanism. However, recent studies have shown that other mechanisms may exist. Saleh et al. found that Ag nanoparticles downregulated the expression of *Proteus novelis* and *Proteus vulgaris* fliL genes, which are clinically useful for urinary tract infections, thus exerting an anti-infection effect (Saleh et al., 2019). As in-depth research is conducted, the

antibacterial mechanism of nanomaterials will be expanded, which will provide a more detailed basis for the antibacterial application of nanomaterials.

Induction of the Immune Response by Nanomaterials in Prostatitis

A large number of experiments have proven that the pathogenesis of prostatitis is closely related to the inflammatory microenvironment (Rees et al., 2015). However, the pathogenesis of prostatitis is complex, and the efficacy of monotherapy is limited. A treatment combining immunotherapy, antioxidant therapy and functional nanomaterials shows advantages. T2 is a specific peptide sequence isolated from the TRPM8 protein, which is encoded by prostate-specific genes (Miller et al., 2007), and has the ability to induce antigen-specific immune tolerance to antigenic peptides (Cheng et al., 2019). A prostatitis model was established in male C57 mice by intravenously injecting 0.2°mL of normal saline and 0.2°mL of a mixture of PLGA, PLGA-OVA and PLGA-T2. The PLGA-T2 group had a higher pain threshold, a lower frequency of urination and a significantly lower level of CPR than the other groups. Novel peptide T2-binding functional nanoparticles with autoantigens have been suggested to successfully alleviate or even cure prostatitis (Shandilya et al., 2020). Cao used antigen T2 combined with polyethylene-maleic anhydride-modified biodegradable PLGA nanotherapy, including the synthesis of biodegradable nanoparticles and conjugation to antigen T2 peptide, to induce immune tolerance in CP/CPPS mouse models (Cao et al., 2019). Mice treated with PLGA-PEMA-T2 showed increased pain thresholds, and reduced urination and prostate pathology. Compared with the other groups, serum levels of inflammatory mediators (TNF-α and CRP) were decreased and the level of the anti-inflammatory cytokine IL-10 was increased in the PLGA-PEMA-T2 group. PLGA-PEMA-T2 nanoparticles improved disease manifestations and upregulated IL-10 in mouse CP/CPPS models. The experiment confirmed the feasibility of using biodegradable nanoparticles combined with T2 antigen to treat prostatitis.

In addition, oxidative stress and inflammation are closely related to the immune responses that maintain homeostasis in the body. Oxidative stress is not only an important feature of inflammation but also a cause of inflammation (Czarny et al., 2018), (Liu et al., 2020). Selenium, a trace element in the human body, is a component of glutathione peroxidase and has the ability to inhibit the production of reactive oxygen species (Rao et al., 2019). In recent years, selenium nanoparticles bound to functional nanocomposites have developed rapidly. Yang, B-Y et al. eliminated oxidative stress after wound healing in the prostatic urethra following transurethral prostatectomy (TURP) using a multivoid Se@SiO2 nanosphere. A randomized beagle dog TUPR model was used to observe the level of oxidative stress during wound healing. Porous Se@SiO_2 nanoballs promoted prostate urethral epithelial changes, enhanced the antioxidant capacity by inducing Ikk expression in macrophages, where I kappa B predominates, and p65 phosphorylation to inhibit oxidative stress and induce macrophages to differentiate into M2 phenotypes, reducing inflammatory reactions (Yang et al., 2019). Nanoselenium has been studied in combination with antibiotics for the treatment of urinary tract infections. El-Sayyad et al. synthesized gentamicin-assisted fungal-derived selenium nanoparticles under γ-ray irradiation to inhibit the resistance of urinary tract infection-causing pathogens (El-Sayyad et al., 2020).

SUMMARY AND OUTLOOK

The causes of prostatitis are complex and include pathogen infections, inflammation, free radicals, an abnormal immune response, sex hormone imbalance and so on. The treatment and diagnosis of prostatitis is facing great challenges. Nanomaterials with anti-inflammatory effects, such as CeO_2, Fe_3O_4 and nano silver hydrosol, have been experimentally proven to be useful in the prevention, diagnosis and combined treatment of prostatitis. Although nanomaterials have achieved impressive results in experimental studies, their clinical conversion still faces significant obstacles. First of all, the metabolic pathway of some inorganic nanomaterials *in vivo* is not clear, and the cumulative toxicity is high. For example, silver nanoparticles, commonly used in urinary tract infections, accumulate in the body and cause liver and kidney toxicity. Secondly, traditional nanomaterials, as exogenous substances, are easy to trigger the immune response of the body, and are easily cleared by the immune system. In addition, nanomaterials as contrast agents also have the defects of low resolution and limited imaging depth. These problems greatly limit the application of nanomaterials in the clinical diagnosis and treatment of prostatitis. How to overcome the above obstacles has become the current research focus of nanomedicine.

The latest research progress summarized in this review, and it is not hard to find out the future research direction in this field. First, nanotechnology will promote the development of clinical diagnosis of prostatitis, especially molecular imaging research based on multimodal imaging technology will further improve the sensitivity and specificity of diagnosis. At the same time, nanotechnology will also facilitate the development of liquid biopsies, which are called upon to combine body fluid detection with medical imaging. Secondly, nanomedicine will break away from the traditional nanomaterials to the clinical application, which mainly depends on the development of new organic or biological sources of nanomedicines. The emergence of natural nanocarriers, such as exosomes, eliminates the immunogenicity and metabolic risks of traditional nanomaterials, making their clinical applications possible. Third, nanotechnology combined with machine learning can help achieve multifunctional integration and personalized diagnosis and treatment. Nanomedicine will undoubtedly revolutionize prostatitis diagnosis and treatment.

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All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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REVIEW
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The Application of and Strategy for Gold Nanoparticles in *Cancer* Immunotherapy

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Immunotherapy of malignant tumor is a verified and crucial anti-tumor strategy to help patients with cancer for prolonging prognostic survival. It is a novel anticancer tactics that activates the immune system to discern and damage cancer cells, thereby prevent them from proliferating. However, immunotherapy still faces many challenges in view of clinical efficacy and safety issues. Various nanomaterials, especially gold nanoparticles (AuNPs), have been developed not only for anticancer treatment but also for delivering antitumor drugs or combining other treatment strategies. Recently, some studies have focused on AuNPs for enhancing cancer immunotherapy. In this review, we summarized how AuNPs applicated as immune agents, drug carriers or combinations with other immunotherapies for anticancer treatment. AuNPs can not only act as immune regulators but also deliver immune drugs for cancer. Therefore, AuNPs are candidates for enhancing the efficiency and safety of cancer immunotherapy.

Keywords: nanomaterials, gold nanoparticles, cancer immunotherapy, nanocarriers, drug delivery system

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INTRODUCTION OF IMMUNOTHERAPY AND NANOPARTICLES

Cancer immunotherapy has rapidly emerged in the past few years (Hegde and Chen, 2020). *Cancer* immunotherapy has been applied in sorts of cancers such as melanoma, nonsmall cell lung cancer and colorectal cancer. Most cancer patients who received immunotherapy gained a great breakthrough therapeutic effect and prolonged prognosis compared with those who underwent traditional chemotherapy and radiotherapy. *Cancer* immunotherapy at present mainly includes monoclonal antibodies, immune checkpoint inhibitors, tumor vaccination, and adoptive T lymphocytes. These factors all show substantial efficacy for treating cancer in the clinic (Khalil et al., 2016). These immunotherapies awaken the human body's immune system to attack abnormal tumor cells with powerful cytokines, tumor vaccines, antibodies, and immune-stimulating adjuvants (Banstola et al., 2020). Compared with traditional methods of treating malignant tumors, especially chemoradiotherapy, immunotherapy is an innovative antitumor approach that dynamically regulates the immune system to assault cancer cells with multiple targets and directions (Tan et al., 2020).

The number of approved immunotherapy drugs has increased in recent years, and more combined treatment strategies, such as radiotherapy, chemotherapy or antitumor angiogenesis therapy, have been developed for the treatment of different types of cancer (Yu et al., 2019). However, a pivotal challenge we are facing in the widespread implementation of cancer immunotherapy is the controlled regulation of the immune system. On the one hand, these therapies have serious side effects, for instance, autoimmune and nonspecific inflammation are common (Riley et al., 2019); on

the other hand, traditional immune stimulants lack the ability to target solid tumor tissue that results from many factors, including the tumor microenvironment (TME), immune evasion processes, and pharmacokinetics (Connor and Broome, 2018; Tan et al., 2020; Yang et al., 2021). In addition, because solid tumors face transmission barriers (such as complex TME), the main immunotherapy was initially evaluated in hematological malignancies. Given this factor, a fraction of immunotherapies, such as activated cytokines and immune checkpoint inhibitors (ICIs), have been licensed by the FDA as investments in the pharmaceutical market for solid tumors (Menon et al., 2016).

Even though many immunotherapies face challenges, surprisingly, the implementation of nanotechnology can effectively improve the efficiency of targeted delivery and the therapeutic efficacy of immune drugs (Li et al., 2020). Nanotechnology has become a trend in the field of medical science and has made great progress with the development of functional, engineered nanoparticles. Among them, gold nanoparticles (AuNPs) have been widely reported to guide an impressive resurgence and are highly remarkable (Hu et al., 2020). AuNPs can pass through the "EPR agent" (enhanced and retention effect) and specifically accumulate in tumor tissues and cells, which is highly beneficial for the targeted delivery of tumor vaccines and immune adjuvants (Riley et al., 2019). Recent studies have shown that nanoscience and technology continue to develop in the fields of tumor immunotherapy (Ou et al., 2020). AuNPs can not only be used for the targeted delivery of traditional antitumor immune adjuvants such as tumor-associated antigens and immune cytokines but also have become a research hotspot, such as in adoptive immune cell therapy and immune checkpoint inhibition therapy, which shows excellent potential clinical value (Savitsky and Yu, 2019). In this review, we summarized the application of AuNPs as immune agents, drug carriers or combinations with other immunotherapies for anticancer treatment.

THE EMERGENCE OF NANOTECHNOLOGY FOR *CANCER* IMMUNOTHERAPY

The conventional treatments for primary tumors are surgery, chemotherapy and radiotherapy. However, tumor recurrence and final treatment failure are still daunting challenges in the clinic. Indirect evidence from preclinical studies shows that the long-term success of cancer treatment lies in immunotherapy. Therefore, cancer immunotherapy is considered to be an effective treatment for the elimination of primary and metastatic tumors and the establishment of immune memory. Nanotechnology can simultaneously deliver various immunological reagents to the desired target site (tumor or lymph node). The ultimate application of nanomedicine is to reprogram or to regulate immune responses by accurately targeting biological pathways (Yu and De Geest, 2020). Hence, the emergence of nanotechnology provides a variety of materials and targeted properties to overcome many difficulties in immunotherapy. Nanoparticle systems have been widely used in the medical field and have many advantages compared with traditional methods (Hagan et al., 2018). Nanoparticles are used either as a protective delivery vehicle for a variety of cargo, improving the stability and solubility of their cargo, extending their half-life, or being used to target cancer cells (Qiu et al., 2017). There are many different applications of nanoparticle systems that can be used for immunotherapy in cancer. From the previously published literature, these methods include the delivery of vaccines and antibodies, and even more specifically, the targeting of specific cells such as antigen presenting cells (APCs) or dendritic cells and the modification of the tumor microenvironment to counteract many immunosuppressive effects of tumors (Qiu et al., 2017). It has been reported that polylactic-coglycolic acid (PLGA) nanoparticles (Surendran et al., 2018; Riley et al., 2019), liposomes, gold nanoparticles, and artificial exosomes are widely used for studies on the delivery of tumor immunotherapy drugs. First, this class of materials has the capacity to improve the synthesis process and to increase the modification of some molecules such as polyethylene glycol (PEG) and to develop its properties in biological distribution, pharmacokinetics, biological safety and other aspects. Second, these materials can not only utilize the modification of arginine-glycine-aspartic acid tripeptide (RGD) and other active targeting molecules to further improve its tumor targeting ability but can also deliver immune agonists to tumor tissues specifically and enhance the body's antitumor immune response while reducing the probability of systemic inflammatory reactions. Therefore, nanotechnology is a candidate approach for enhancing cancer immunotherapy.

THE APPLICATION OF AUNPS IN *CANCER* IMMUNOTHERAPY

AuNPs as Nanocarriers for Immunotherapy

Gold nanoparticles (AuNPs) have attracted much attention due to their unique advantages among nanoparticles (Singh et al., 2018). In addition to their excellent targeting of tumor tissues and the immune system, AuNPs also have advantages compared with other metal nanoparticles. With the continuous development of nanotechnology, AuNPs are easily synthesizable in various shapes and sizes through chemical, physical or eco-friendly biological methods. AuNPs play many roles as multifunctional therapeutic agents, such as targeted delivery systems (vaccines, nucleic acids, and immune antibodies), theranostics and agents in photothermal therapy. They have also made great contributions in the field of biological imaging, such as radiotherapy, magnetic resonance angiography and photoacoustic imaging (Mioc et al., 2019).

First, gold nanoparticles are a kind of biologically inert material suitable for medical applications with strong plasticity (Boisselier and Astruc, 2009). Even delicate adjustment of the size and shape of AuNPs can lead to changes in the distribution, metabolism, cytotoxicity, immunogenicity and other properties of AuNPs. Moreover, AuNPs are highly modifiable, and the molecular density on their surface is higher than that of most other nanomaterials. AuNPs can conjugate molecules of different types and functions in a variety of ways while avoiding

interference between these molecules. By simultaneously modifying molecules with different functions, such as PEG, RGD and immune adjuvants, researchers can improve many aspects of the performance of AuNPs and comprehensively enhance the efficacy of the targeted delivery of immune drugs and activation of the immune system (Lopez-Campos et al., 2019). More importantly, AuNPs can generate heat under a specific wavelength of laser irradiation due to their unique photodynamic properties. On the one hand, heat-related signaling stimulates immune factors in tumor tissues, inflammation, and transmitter secretion. On the other hand, it collaborats with the immune response for cancer cells and releases immune-activated drugs in cancer tissues, which achieves efficient and low toxicity of antitumor immune effects (Liu et al., 2018).

The Nanocarrier Role of AuNPs for Drug Delivery

The application of nanotechnology is mainly based on the early detection and diagnosis of tumors by nanodevices that can selectively target chemotherapeutic drugs and deliver them to specific tumor sites. The special properties of AuNPs have long been regarded as potential tools for the diagnosis of various cancers and drug delivery applications. These properties include a high surface area to volume ratio, surface plasmon resonance, surface chemistry and multifunctionalization, facile synthesis, and a stable nature. Various types of drugs can be immobilized on the surface of AuNPs, most notably by direct -S or -N binding, ligand bonding, and adsorption by electrostatic interaction, van der Waals forces, and hydrogen bonding. In general, because of the stronger interaction between Au and S, -N binding holds more promise for delivering drugs in cancer cells than -S binding (Cheng et al., 2010). Moreover, the nontoxic and nonimmunogenic characteristics of AuNPs and their high permeability and retention provide additional benefits by enabling them to penetrate and to accumulate drugs easily at tumor sites (Lee and Choi, 2018). Various innovative approaches with AuNPs are under development. Of note, novel strategies, especially improved delivery strategies, can not only target tumors and immune cells more effectively but also increase the abundance of immunotherapeutics within lesions (Singh et al., 2018). Some materials, such as lipids, polymers, and metals, have been used to exploit delivery strategies (Lakshminarayanan et al., 2018). Currently, new delivery strategies for immunotherapy, including nanoparticles, scaffolds and hydrogels, are being researched and developed (Zhao et al., 2019). Among them, gold nanoparticles are particularly prominent.

Nanocarrier of Tumor Vaccines

Tumor-associated antigen (TAA) reactivates the body's immune response to tumor cells and plays a vital role in the early prevention and treatment of cancer. In general, tumor vaccines may mainly consist of TAAs and adjuvants (Aly, 2012). Compared with direct injection of TAAs, AuNPs linked with vaccines are more suitable for protecting antigens from degradation and can be targeted for delivery to dendritic cells (DCs) or T lymphocytes. In addition, they are able to penetrate blood vessels and barriers and to be targeted to a specific cell by means of specifically functionalized molecules (**Figure 1**) AuNPs can also cross-present antigens to more effectively stimulate cytotoxic T lymphocytes and to promote antitumor immunity (Sehgal et al., 2014; Popescu and Grumezescu, 2015).

An excellent carrier is born at the right moment, and AuNPs have proven to be competent for the job because of the properties of AuNPs (such as the biocompatibility and nontoxicity of gold) (Zhou et al., 2016a). They show better performance than other nanoparticulate-based carriers; for example, their size is easily controlled for different applications, and the majority of antigens and adjuvants can be easily connected to and displayed on their surface. At the same time, AuNPs can be detected with noninvasive imaging techniques, providing clinicians with evidence on where vaccines have been delivered, which supports the evaluation of therapeutic efficacy (Dykman et al., 2018). A model of a hyaluronic acid (HA)- and antigen (ovalbumin, OVA)-decorated AuNP-based (HA-OVA-AuNP) vaccine (Cao et al., 2018) was invented for photothermally controlled cytosolic antigen delivery with near-infrared (NIR) irradiation and was discovered to induce antigen-specific $CD8^+$ T-cell responses. HA-OVA-AuNPs promote antigen uptake by DCs through receptor-mediated endocytosis. HA-OVA-AuNPs show the ability to enhance NIR absorption and thermal energy translation. Cytosolic antigen delivery is allowed via the photothermally controlled process of partial heat-mediated endo/lysosome disruption by laser irradiation with reactive oxygen species generation, which helps to increase proteasome activity and downstream MHC I antigen presentation. Therefore, the HA-OVA-AuNP nanovaccine can effectively stimulate a potent anticancer immune response under laser irradiation. In another model of AuNPs mobilized with α-mannose as carriers for a TLR7 ligand to target immune cells (Shinchi et al., 2019). The small molecule synthetic TLR7 ligand 2-methoxyethoxy-8-oxo-9-(4-carboxy benzyl) adenine (1V209) and α-mannose were coimmobilized via linker molecules consisting of thioctic acid on the AuNP surface (1V209-αMan-AuNPs). Compared with the unconjugated 1V209 derivative in mouse bone marrow-derived dendritic cells and in human peripheral blood mononuclear cells, the 1V209-αMan-AuNPs showed higher extracorporeal cytokine production activity. In the internal immunization study, 1V209-αMan-AuNPs induced obviously higher titers of IgG2c antibody specific to ovalbumin as an antigen than unconjugated 1V209, and splenomegaly and weight loss were not apparent. These results suggest that 1V209-αMan-AuNPs could be safe and effective adjuvants for the development of vaccines against cancer.

Kang et al. (Kang et al., 2018) designed a nanodelivery system that delivers TAA to natural killer cells and APCs. The calcium phosphate nucleus of the delivery system was coated with melanoma TAA, αHSP70P protein and adjuvant CpG, and then, the calcium nucleus was coated with a phospholipid bilayer. Effective lymphocyte metastasis and multiepitope T lymphocyte responses were observed when the nanoparticles were injected into mice *in vivo*. This approach can also induce

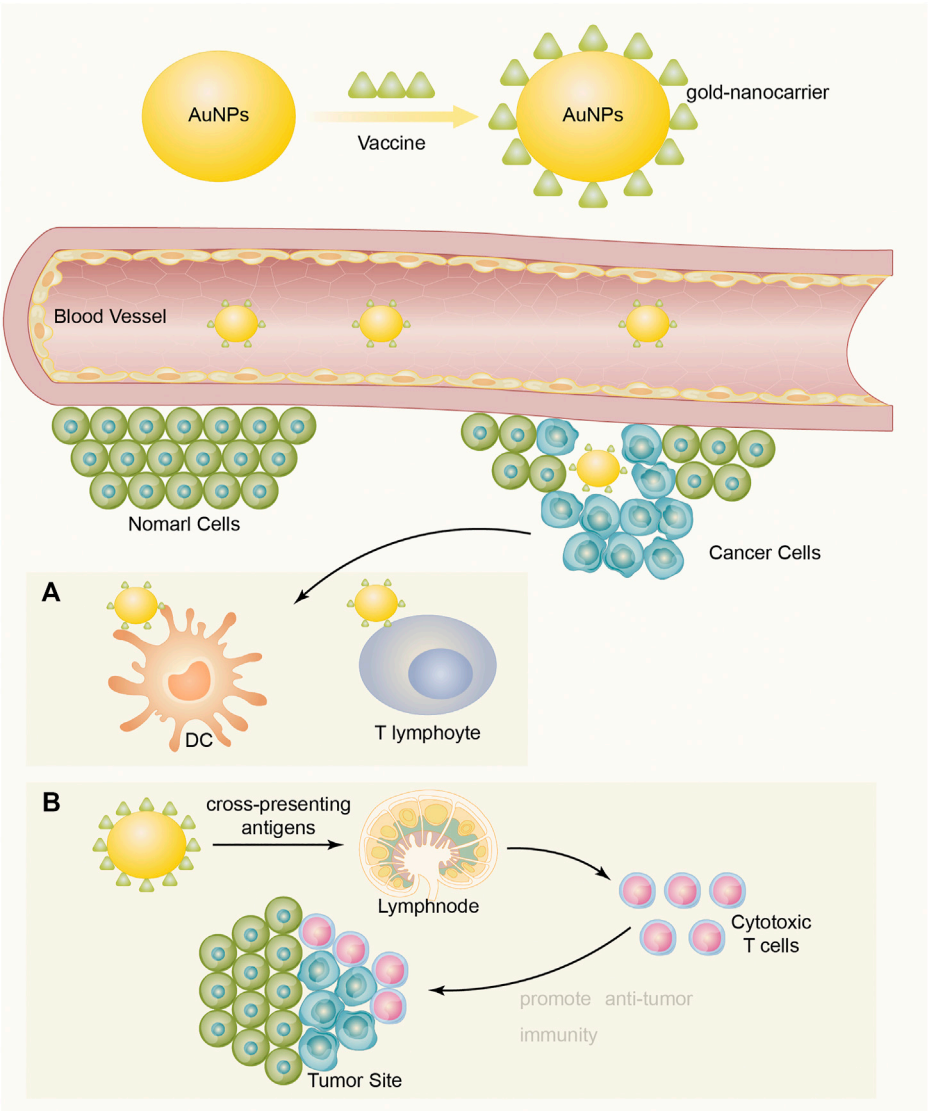

FIGURE 1 | Vaccines connect to and display on the surface of AuNPs to become a formation of vaccine-AuNPs complex, then the complex penetrates blood vessels and delivers vaccine targeting cancer cells to enhance immune response. **(A)** Targeting for delivery to dendritic cells (DCs) or T lymphocytes. **(B)** Cross-present antigens to more effectively stimulate cytotoxic T lymphocytes and to promote antitumor immunity.

the expansion of $CD8^+$ T lymphocytes and NKG2D + NK cell subsets. The nanoparticles also had synergistic effects on the maturation and antigen presentation of DCs derived from bone marrow.

AuNPs, as carriers of tumor vaccines, play an important role in antitumor immunotherapy. Taken together, AuNP-based vaccines are novel and efficient antitumor treatments (Ahn et al., 2014).

Nanocarrier Delivery of Genetic Drugs

The delivery of small interfering RNA (siRNA) mediated by nanocarriers has provided a novel method of intracellular antigen synthesis, which has great application value in antitumor immunotherapy (Yin et al., 2014). Under normal physiological conditions, the inherent negative charge of siRNAs is usually degraded by related enzymes, and siRNA is difficult to be transferred to the target location (Liu et al., 2019a). There are two main reasons why gold nanoparticles (AuNPs) can be used as effective nanomaterials for siRNA delivery applications (Gindy and Prud'homme, 2009; Mahmoodi Chalbatani et al., 2019). First, functional diversity can be easily obtained with the creation of multifunctional monolayers. Second, because of the low toxicity, low size disparity and selective gene silencing and transfection. So far, AuNPs are one of the most extensively used carrier tool for siRNA as an anti-cancer strategy.

In the research of Hou et al. (2016), the authors designed nonviral pDNA/siRNA delivery vectors, that is, generation 5 dendrimer-entrapped gold nanoparticles (Au DENPs)

partially modified with polyethylene glycol monomethyl ether. The entrapped Au DENPs were effectively used to deliver Bcl-2 (B-cell lymphoma 2) siRNA to human cervical cancer cell lines to silence the enhanced green fluorescent protein and luciferase reporter genes. Suman's article reported a model used to treat melanoma comprising layer-by-layer assembled gold nanoparticles (LbL-AuNPs) containing anti-STAT3 siRNA and IM (imatinib mesylate) (Labala et al., 2017). Compared with LbL-AuNPs containing either STAT3 siRNA or IM, the treatment showed greater inhibition of STAT3 protein, reduced cell viability and increased apoptotic events. In summary, combining AuNPs with the RNAi pathway by delivering siRNA and small molecule drugs (IMs) is a way of creating a drug delivery system.

Only if when siRNA was sent to the cytoplasm where gene silencing takes place, it could be of value (Elbashir et al., 2001). Previous studies have shown that functional Au DENPs can transport siPDL1 (programmed siRNA-PD-L1) to cancer cells, effectively down-regulate the expression of PD-L1 protein, and increase the infiltration of $CD8^+$ and $CD4^+$ T cells in tumor tissues and spleen, thereby promoting immunotherapy. Its tumor suppression efficiency is much higher than that of PD-L1 antibody (Xue et al., 2021). So siRNA-AuNPs delivery system may have great application potential in immunotherapy.

AuNPs Delivery of Immune Antibodies

Currently, approved therapies for PD-1/PD-L1 have been effectively used to improve the survival and quality of life of cancer patients with chemotherapy and targeted drug tolerance by using nivolumab, pembrolizumab, cemiplimab, atezolizumab, durvalumab, and avelumab to effectively inhibit the binding of PD-1 to PD-L1 and to prevent the immune escape of cancer cells through the use of antibody drugs, such as nivolumab, pembrolizumab, cemiplimab, atezolizumab, durvalumab, and avelumab (Wang et al., 2019). Despite their numerous advantages, these antibody drugs still have many disadvantages, such as high cost of use, low clinical response rate (approximately 20%), influence of individual differences, large required therapeutic dose, high probability of causing immune side effects, and cases of developing resistance (Luo et al., 2018). To overcome some of these shortcomings, many studies are focusing on immunotherapy strategies that combine immune antibodies with AuNPs.

In fact, the most important antitumor molecules used in clinical practice are PD-1, CTLA-4, Tim-3 and LAG-3, which are the main immune checkpoint molecules associated with tumors (Qin et al., 2019). One extremely promising approach to achieve anticancer immunity is to block the immune checkpoint pathway mechanism of cancer cells and camouflaging the conventional components of the human body (Li et al., 2019). These molecules are expressed in immune cells and can interact with corresponding ligands expressed in cancer cells or immunomodulatory cells in the tumor microenvironment to inhibit the cellular immune response and cause immune escape of cancer cells. Immune checkpoint inhibitors are another kind of mainstream immune anticancer method that has entered the clinic (Darvin et al., 2018). This class of drugs can block the interaction between immune checkpoints and their ligands and restore immune cells to recognize and to kill cancer cells (Han et al., 2020).

PD-1 has received much attention as an immune checkpoint in clinical anticancer therapy (Gong et al., 2018). It is usually expressed in T cells and interacts with the PD-L1 receptor overexpressed on the surface of cancer cells or immunosuppressive cells to inhibit the immune response of T cells to tumor cells and to induce the apoptosis of T cells (Liu et al., 2021; Xia et al., 2019). To overcome some of these shortcomings, Emami et al. (2019) designed doxorubicin (DOX)-conjugated and anti-PD-L1 targeting gold nanoparticles (PD-L1-AuNPs-DOX) for colorectal cancer (CRC). Despite drug resistance, DOX and PD-L1 antibodies are difficult to deliver to tumor sites because of the barrier of the tumor microenvironment (TME) and other factors. Therefore, the authors constructed a model that may improve the drug delivery ability. First, AuNPs exhibit characteristic surface plasma resonance (SRS) absorption in the near-infrared (NIR) region (Banstola et al., 2018), and AuNP-based photothermal therapy (PTT) can be used to ablate tumors by turning NIR light energy into heat and generating of reactive oxygen species (ROS). At the same time, DOX can also be loaded onto AuNP platforms, which enables DOX and heat to be delivered specifically and simultaneously to tumor microenvironments (Chen et al., 2017). Second, some CRC subtypes, especially microsatellite instability-high (MSI-H) CRC (a highly immunogenic cancer), show PD-L1 upregulation on cell surfaces and PD-L1 overexpression, which is known to be distinct for prognosis and survival in CRC patients. Therefore, the authors aimed to construct AuNPs modified with an anti-PD-L1 antibody and drug-covalent conjugation to lipoic acid polyethylene glycol N-hydroxy succinimide (LA-PEGNHS) as a novel drug delivery system for the combined delivery of a drug and heat to CRC cells. In brief, the PD-L1-AuNPs-DOX model (**Figure 2**) successfully facilitated the efficient intracellular uptake of DOX and NIR irradiation obviously and synergistically suppressed the *in vitro* proliferation of CRC cells by increasing apoptosis and cell cycle arrest, and this model in combination with synergistic targeted chemo-photothermal therapy has potential for the treatment of localized CRC. In addition, using AuNPs to deliver PD-1/PD-L1 antibodies or siRNA is another effective way to inhibit PD-1 tumor immune checkpoints. Meir's team (Meir et al., 2017) modified the PD-L1 antibody to adhere to the surface of AuNPs and effectively improved the concentration of antibody drugs at tumor sites by using the efficient targeted drug release ability of AuNPs. Liu et al. (2019b) loaded PD-L1 siRNA into gold nanocarriers to knock down the expression of PD-L1 in tumor cells, which also achieved a good tumor inhibition effect. At present, 5 more research groups have attempted to use AuNPs to deliver PD-1/PD-L1 inhibitors and other anticancer drugs to explore drug combination strategies based on PD-1/PD-L1 targets. The results showed that the combination of PD-L1 antibody mediated by AuNPs and doxorubicin could not only enhance the induction of apoptosis of cancer cells but also inhibit tumor stem cell-mediated angiogenesis by inhibiting autophagy of cancer cells and thus inhibit tumor recurrence (Emami et al., 2019; Ruan et al., 2019).

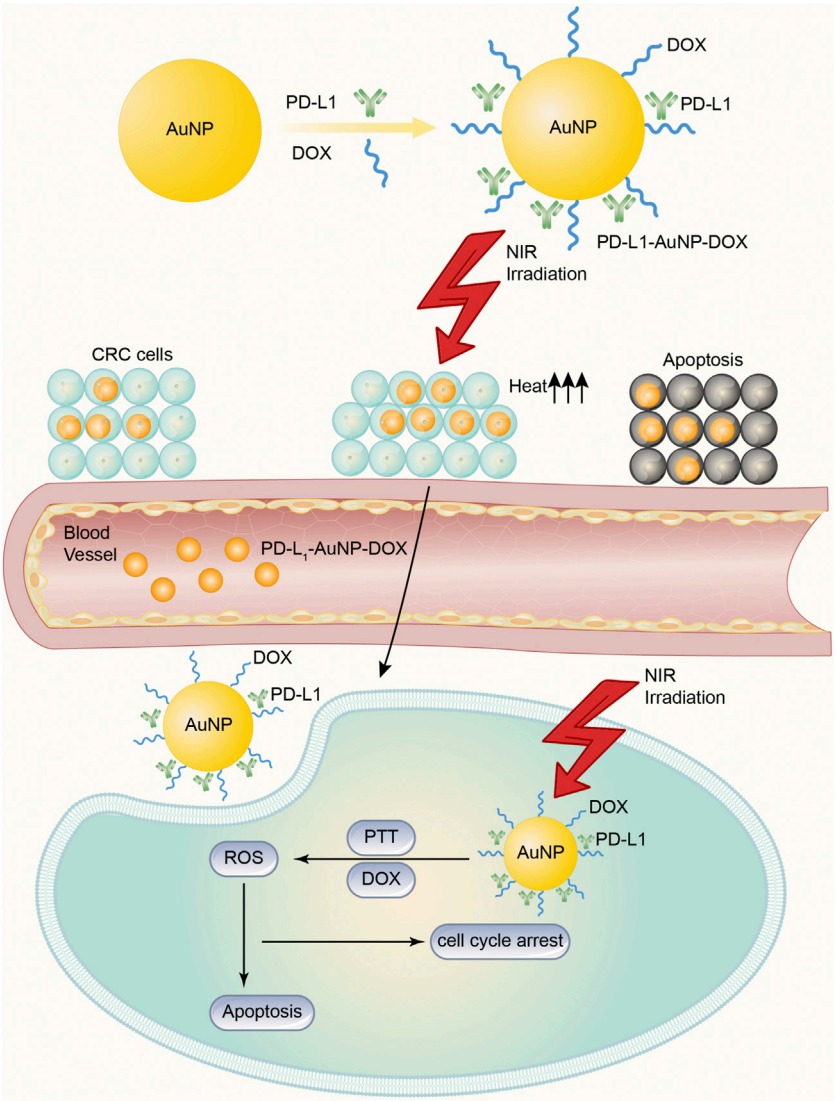

FIGURE 2 | DOX-conjugated and anti-PD-L1 targeting gold nanoparticles (PD-L1-AuNPs-DOX) model for CRC. The PD-L1-AuNPs-DOX model, under the NIR irradiation, may improve the ability of targeted delivery, and gather PD-L1 and DOX together in cancer cells more easily. Then the model effectively generates ROS to increase apoptosis and cell cycle arrest to suppress the *in vitro* proliferation of CRC cells.

APPLICATION STRATEGIES OF AUNPS TO IMPROVE THE TUMOR MICROENVIRONMENT

The Role of AuNPs in the Tumor Microenvironment

Due to the influence of tumor growth, the TME possesses premium physiological characteristics, including hypoxia, slight acidity, and vascular irregularity. In addition, the TME can generate an immunosuppressive microenvironment by releasing cytokine mediators and gathering immunosuppressive cells (Dunn et al., 2004; Estrella et al., 2013). The study by Ibrahim et al. (2018) signified that AuNPs with a size of 5, 20 and 50 nm can up-regulate interleukin (IL)-1β, IL-6, and tumor necrosis factor in the liver, spleen, and kidney of mice at a certain dose. TNF-α and other immune cytokines expression levels. Then the hypoxic state was improved because of the variation of these cytokine level. At the same time, both immune cell infiltration and the efficacy of tumor immunotherapy were enhanced at tumor sites.

Moreover, the special pathological structure of tumor and its inhibitory immune microenvironment jointly limit the efficacy of immunotherapy. Many advances have been made in reshaping the pathological structure of tumor that affects the efficacy of immunotherapy and enhancing the efficacy of immunotherapy. Consequently, remodeling the tumor immunosuppressive microenvironment is of great significance for tumor immunotherapy (Hou et al., 2020). Insufficient tumor vascular perfusion can lead to highly hypoxic TME, which is closely

related to immunosuppressive TME. Regulating the normalization of blood vessels at tumor sites is of great significance for remodeling the immunosuppressive tumor microenvironment and enhancing immune cell infiltration in tumor sites (Mazzieri et al., 2011). AuNPs can improve vascular morphology, increase vascular perfusion, reduce tumor hypoxia, and inhibit the migration of HUVECs and tumor cells (Li et al., 2017). In the research of Li et al. (2017), they connoted that AuNPs can regulate the angiogenin-angiogenin type I receptor pathway, promote the normalization of tumor blood vessels, increase blood perfusion and alleviate hypoxia at the tumor site.

In additional, immune response requires the participation of immune cells, including dendritic cells (DCs) and macrophages, and they mediate innate immune response, which can play the pro- and anti-tumor effect depending on the inflammatory mediators and cytokines in TME (Yang et al., 2021). The growth and development of tumor cells leads to a hypoxic situation, and this state subsequently modifies the physiological function of microenvironment. To this end, anti-tumor drugs can hardly penetrate TME and target tumor cells (Rajendrakumar et al., 2018). With the deepening of the research on AuNPs, how AuNPs work in tumor immunotherapy for TME have been report. Firstly, in comparison with the regular immunotherapy drugs, AuNPs could modulate the immunosuppressive environment in TME through targeting various of abnormal components of TME. For example, the abnormal secretion of vascular endothelial growth factor (VEGF) and transforming growth factor β (TGF-β) inhibit the immune response of DCs, and transfer macrophages to the pro-tumorigenic M2 phenotype. Specifically designed AuNPs combined immunotherapy drugs can target these abnormal components in TME and transform the immunosuppressive TME to an immunosupportive state, thereby improving the efficacy of cancer immunotherapy (Overchuk and Zheng, 2018).

However, what worth noting is that AuNPs alone may be unlikely to directly impact the immune response, because their effects on the immune system remain ill-defined. Dey et al. (2021) created an *in vitro* cell model. They used primary macrophages and DCs of mice as an APC model. Even though AuNPs indistinctively changed the functions of macrophages and DCs, AuNPs had different effects on the response of macrophages and DCs to subsequent stimulation. Firstly, the secretion level of cytokines and chemokine were altered in DCs and macrophages. Moreover, antigen presentation to T cells increased when DCs came into contact with AuNPs resulting in stronger Th1, Th2 and Th17 responses. That is why we emphasize that immunotherapy plus AuNPs do not directly alter the immune response but indirectly influence the function of DCs and macrophages by modulating abnormal components in TME to get twice the result with half the effort. Therefore, AuNPs have been widely designed as drug delivery system.

Anti-Angiogenesis and Vascular Normalization Strategies of AuNPs

Abnormal appearance and impaired function are the most common features of tumor vasculature (Krishna Priya et al., 2016). Hypoxia in the TME induces continued production of proangiogenic molecules, such as vascular endothelial growth factor (VEGF) and transforming growth factor-β (TGF-β) (De Palma et al., 2017; Maity et al., 2000; Hinz, 2015). The imbalance between proangiogenic and antiangiogenic factors leads to rapid but abnormal tumor angiogenesis. Owing to the detachment of the pericytes surrounding the endothelial cells, the blood vessels have increased permeability (Barlow et al., 2013; Huang et al., 2018). Hence, aberrant vasculature contributes greatly to abnormalities in the TME.

If abnormal tumor vasculature is the key issue that hinders the implementation of tumor treatment, then vascular normalization strategies could be a promising solution. Another problem coming to us subsequently is that the therapeutic efficacy of antiangiogenic therapy has been obstructed by acquired resistance of the endothelium toward antiangiogenic drugs such as anti-VEGF therapy (Mattheolabakis and Mikelis, 2019). Zhang et al. (2019a) investigated the use of AuNPs as a therapeutic tool to disrupt multicellular crosstalk in TME cells, with a focus on inhibiting angiogenesis. The authors showed that CM (conditioned media) from ovarian CCs, CAFs, or ECs themselves induced tube formation and migration of ECs *in vitro*. These results prove that AuNPs inhibit angiogenesis by blocking VEGF-VEGFR2 signaling from TME cells to endothelial cells.

Although the antiangiogenic mechanism of AuNPs is still unclear, Pan et al. (2013) revealed that it may be due to the effect of AuNPs on VEGF signaling; AuNPs can reduce VEGF165-induced VEGFR2 and AKT phosphorylation. However, a single vascular normalization strategy cannot improve its penetration in tumor tissues, and there is still a need to develop new formulations based on AuNPs combined with other therapies (Chauhan et al., 2012), such as immunotherapy.

Successful immunotherapy requires not only the infiltration of immune cells, but also an immune-supporting microenvironment to maintain the proliferation and function of T cells. Normalization of blood vessels provides such an environment for immunotherapy (Huang et al., 2013; Jain, 2013). It is also important to note that dysfunctional vessels restrict the infiltration of immune cells as well as the efficient delivery of nanoparticles and therapeutic drugs (Azzi et al., 2013). Meanwhile, some reports have highlighted the effect of mediating tumor vascular normalization of AuNPs. Huang et al. (2020) focused on a model, named targeting polymer and folic acid-modified gold nanoparticles (AuNPP-FA), which can both restrain tumor angiogenesis and promote vascular normalization. It is because of the increased infiltration of $CD3^+CD8^+$ T lymphocytes that the immunotherapeutic response was enhanced by decreasing vascular permeability and improving vascular perfusion. Thereby, the vascular normalization strategy of AuNPs has great potential for tumor immunotherapy.

PHOTOTHERMAL THERAPY AND PHOTODYNAMIC THERAPY OF AUNPS

AuNPs, with their multiple unique functional properties and ease of synthesis, have attracted extensive attention in antitumor tactics. Their inherent features can be altered by changing the

characterization of the nanoparticles, such as shape, size and aspect ratio. They can be applied to a wide range of medical applications, especially photothermal therapy (PTT) and photodynamic therapy (PDT) (Hu et al., 2020). AuNPs exhibit favorable physical properties and tailored surface functionalization, providing a potential platform for developing cancer immunotherapy (Guo et al., 2017).

Application of Photothermal Therapy

The reason why PTT is available under the stimulation of pulsed or continuous-wave Doppler (CW) visible lasers is the surface plasmon resonance (SPR) absorption of AuNPs in the visible region, whereby such treatment may be suitable for shallow tumors (i.e., skin cancer) (El-Sayed et al., 2006; Huang et al., 2007). Despite being a standalone therapy, gold nanoparticle-mediated PTT has recently been developed in combination with other therapies, such as chemotherapy, gene drugs, and immunotherapy, for enhanced antitumor effects (Riley and Day, 2017). Here, we emphatically introduce the combination of PTT and immunotherapy, which is based on the phenomenon that heat causes dying cancer cells to release antigens and heat shock proteins (HSPs) that are captured by antigen-presenting cells such as DCs to mediate an immune response (Almeida et al., 2014; Zhou et al., 2016b). Moreover, the immune environment created by PTT can strengthen immunotherapies to prolong anticancer effects. Liu et al. (2019c) constructed a new nanoplatform GNS@CaCO3/Ce6-NK by loading CaCO3-coated gold nanostars (GNSs) with chlorin e6 molecules (Ce6) into human peripheral blood mononuclear cell (PBMC)-derived NK cells for tumor-targeted therapy. This approach was used because the authors hypothesized that Ce6 would remain stable with characteristic NIR absorption during the endocytic process of NK cells and that the new platform fully utilized the immune function of NK cells and the physical properties of AuNPs to prove the synergistic therapeutic effects of PTT and immunotherapy. Combining PTT with NK cells increased the efficiency and accuracy of the tumor-targeting ability compared to other immunotherapies alone. Thus, this platform reflects a prominent synergistic strategy for enhanced PTT and immunotherapy in the area of anticancer development.

Application of Photodynamic Therapy

PDT is another light therapy developed in recent decades to destroy cancer cells and pathogenic bacteria (Abrahamse and Hamblin, 2016). PDT involves visible light, a photosensitizer (PS), and molecular oxygen (O_2) from tissues. If PDT desires to function successfully, it is completely dependent on the availability of O_2 in tissues. In the process of PDT, the PS absorbed by the tissue is excited by laser light of a specific wavelength. Irradiating the tumor site can activate PS, which selectively accumulates in the tumor tissue, triggering a photochemical reaction to destroy the tumor. The excited PS will transfer energy to the surrounding O_2 to generate reactive oxygen species (ROS) and to increase ROS levels in the target sites. ROS can react with adjacent biological macromolecules to produce significant cytotoxicity, cell damage, and even death or apoptosis (Imanparast et al., 2018; Falahati et al., 2020). Liang et al. (2018) reported a gold nanoparticle system based on core-shell gold nanocage@manganese dioxide (AuNC@MnO2, AM) nanoparticles as tumor microenvironment responsive oxygen producers and NIR-triggered reactive ROS generators for oxygen-boosted immunogenic PDT against metastatic triple-negative breast cancer (mTNBC). In this model, the MnO_2 shell degrades in an acidic TME and generates massive oxygen to boost the PDT effect of AM nanoparticles under laser irradiation and to ameliorate local hypoxia. Furthermore, in the oxygen-boosted PDT effect, immunogenic cell death (ICD) is elicited by damage-associated molecular pattern (DAMP) release, which induces DC maturation and effector cell activation, thereby strongly evoking systematic antitumor immune responses against mTNBC. Hence, this nanosystem offers a promising approach to ablate primary tumors while preventing tumor metastasis through immunogenic endoscopic effects.

Other Application Strategies

In addition to PTT and PDT, radiation therapy (RT) is one of the least invasive and commonly used methods in the treatment of various cancers (Sztandera et al., 2019). RT involves the delivery of high-intensity ionizing radiation (such as γ-rays and X-rays) to tumor tissues while simultaneously protecting the surrounding healthy cells, tissues, and organs, resulting in the death of tumor cells (Retif et al., 2015; Klębowski et al., 2018). Recently, there have been many reports of radiosensitization using AuNPs in RT, which is due to the high atomic number of gold nanoparticles (Jain et al., 2011; McMahon et al., 2011). The most likely mechanism of radiosensitization from AuNPs is that Auger electron production from the surface of the AuNPs can increase the production of ROS, reduce the total dose of radiation, and increase the dose administered locally to the tumor sites, eventually resulting in cell death. These methods also provide potential ways to expose tumor antigens and to enhance the response to cancer immunotherapy (Jeynes et al., 2014; Retif et al., 2015).

CLINICAL APPLICATION OF AUNPS

The use of nanoparticles in therapeutic applications has been improved by coating gold with organic compounds, such as amino acids and amino sugars, which act as carriers to transport nanoparticles to tumor cells (Daniel and Astruc, 2004; Tshikhudo et al., 2004). Colloidal gold-based nanoparticles have been designed to target the delivery of tumor necrosis factor (TNF) and paclitaxel to solid tumors, introducing AuNPs as tumor-targeted drug delivery vectors (Paciotti et al., 2006). AuNPs are recognized as excellent drug and anticancer carriers with many biochemical and therapeutic applications (Singh et al., 2018). In a clinical trial approved by the FDA, whose first phase has been completed, novel PEGylated AuNPs were utilized to deliver TNF into cancer cells, ending with selective TNF storage in tumor cells (Libutti et al., 2010).

However, very few clinical trials are being actively carried out for the approval of AuNPs for cancer diagnostics and therapy

(Tomić et al., 2014; Qiu et al., 2015). As described above, AuNPs show potential for use in cancer diagnostics and therapeutics. However, the absence of coherent information on the actual effect of nanoparticles could have deleterious effects and a negative impact on human health. For example, the toxicity and chain reaction when AuNPs are used *in vivo* are not abundantly clear because few teams have tested them in clinical trials. Thus, there is controversy and disagreement about the potential of AuNPs for clinical use at present (Cheng et al., 2012).

PERSPECTIVE

The advantages of AuNPs make them more effective in the immunotherapy of malignant tumors. For example, the ability to accumulate at tumor sites and the photothermal properties can enable the efficient and targeted delivery of genes, cancer vaccines, immune antibodies and other immune therapeutic-related components. Some studies have shown that in combination with current popular fields of tumor immunotherapy, such as PD-1/PD-1 AuNPs demonstrate excellent efficacy and show great potential clinical application value (Pietro et al., 2016; Liu et al., 2019b). It has been demonstrated that the targeted delivery of immunoregulatory molecules by AuNPs can not only eliminate killed primary tumor tissue but also promote a systemic immune response to treat metastatic lesions and prevent tumor recurrence (Zhang et al., 2019b).

However, AuNPs have various disadvantages in their application (Singh et al., 2018). First, AuNPs cannot be degraded and easily accumulate *in vivo* during long-term treatment, causing uncertain side effects. Second, the application of the photothermal effect of AuNPs is often limited by the penetration depth of near-infrared lasers, and the heating intensity will decrease with increasing laser penetration depth, which limits the directional drug release effect and immunoregulatory activity of AuNPs. More importantly, a series of pharmacokinetic and histocompatibility parameters of AuNPs are changed by surface modification (Weintraub, 2013).

CONCLUSION

Therefore, AuNPs are candidates for enhancing the efficiency and safety of cancer immunotherapy. Every modification of the surface radical groups and ligands of AuNPs requires a reevaluation of their pharmacological and toxicological effects, which increases the amount and cycle of related drug research and development work. Nevertheless, the shortcomings of AuNPs have not deterred researchers from using them to improve the efficacy of tumor immunotherapy. In the future, there will be a series of works to modify the characteristics of AuNPs to overcome these shortcomings.

AUTHOR CONTRIBUTIONS

J-sH wrote the manuscript and designed the figure. S-jL collected the resources and eidted the review. Y-rZ, X-dC, Z-bL and ZZ drawn the figure. S-hQ, Y-gG and HD sorted out references. J-hP and Y-lP supervised the whole work, contributed to writing, and critically revised the paper.

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ROS-Eliminating Carboxymethyl Chitosan Hydrogel to Enhance Burn Wound-Healing Efficacy

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Yang C, Chen Y, Huang H, Fan S, Yang C, Wang L, Li W, Niu W and Liao J (2021) ROS-Eliminating Carboxymethyl Chitosan Hydrogel to Enhance Burn Wound-Healing Efficacy. Front. Pharmacol. 12:679580. doi: 10.3389/fphar.2021.679580

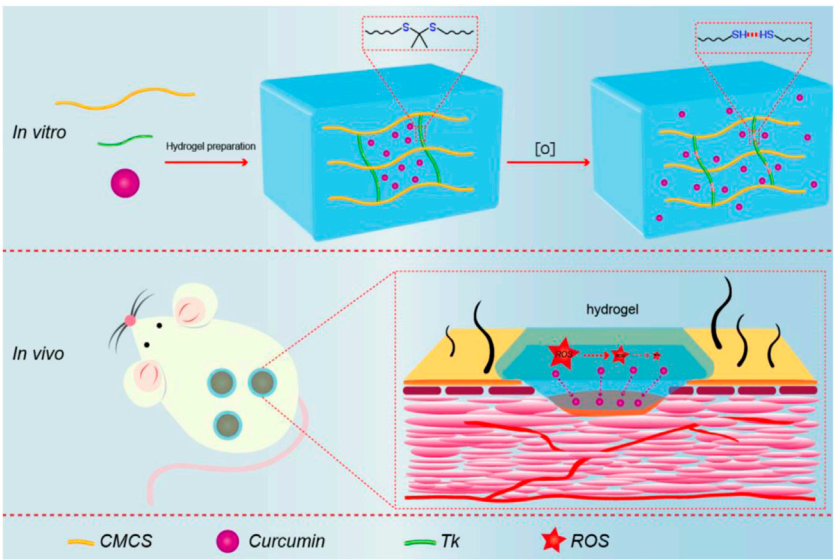
Overexpression of reactive oxygen species (ROS) can lead to chronic inflammation, which limits skin wound healing. Therefore, it is of great significance to develop materials that can locally control the adverse reactions caused by excessive ROS. In this research, an ROS-sensitive hydrogel with strong free radical scavenging ability was prepared by introducing the thione (Tk) group into carboxymethyl chitosan (CMCTS) hydrogel. CMCTS hydrogel was cross-linked by NH_2-Tk-NH_2 agent and loaded curcumin (Cur), which possessed favorable nontoxicity, water absorption, mechanical property, biodegradability, drug release behavior, the M2 phenotype, and inflammatory factor regulating the capacity of macrophages. It is worth noting that Cur@CMCTS-Tk hydrogel can significantly inhibit oxidative damage of human fibroblasts in the H_2O_2-induced microenvironment and protect their viability by reducing the production of intracellular ROS. *In vivo*, ROS-removing hydrogel effectively accelerated the process of wound healing and possessed good regenerative properties, including hair follicle formation, promotion of new blood vessel formation, and highly orderly arrangement of collagen fibers in the full-thickness skin burn defect rat model. Hence, we expect that the Cur@CMCTS-Tk hydrogel could be used for wound treatment and tissue regeneration due to the ability to scavenge excess ROS.

Keywords: ROS-sensitive, carboxymethyl chitosan, hydrogel, macrophages, wound healing

INTRODUCTION

Reactive oxygen species (ROS), which play vital roles in the normal metabolism and pathological process of humans, are signaling molecules, including superoxide radical (O_2-), hydrogen peroxide (H_2O_2), and hydroxyl radical (-OH) (Privat-Maldonado et al., 2019; Yao et al., 2019). Excessive ROS production, however, can induce harmful processes, such as inflammation, necrosis, and cicatrization, to delay the healing of skin wounds and regeneration of damaged tissue (Mittal et al., 2014; Blaser et al., 2016). Therefore, designing a novel biomaterial that can locally control the excess ROS impairing cutaneous wound recovery and accelerate the regeneration process is urgently needed (Thannickal and Fanburg, 2000; Dröge, 2002; Kietzmann, 2010).

Among the many biomaterials, hydrogels were applied to deliver ROS scavengers to targeted sites under controlled therapeutic doses. Considering hydrogels can be used as a sustainable host of ROS



SCHEME 1 | Schematic illustration of synthesis procedure for Cur@CMCTS-Tk and the accelerating release of Cur in the burn wound rat model.

scavengers, here is a growing hotspot that hydrogels as ROS-modulating materials are available for a variety of biomedical applications, for example, wound healing and tissue regeneration (Hu et al., 2020; Martin et al., 2020; Thi et al., 2020; Zhao et al., 2020).

As a special derivative of chitosan, carboxymethyl chitosan (CMCTS) was synthesized by replacing either or both of the amino (NH_2) and hydroxyl (OH) functional groups in the glucosamine units with carboxymethyl ($-CH_2COOH$) substituents (Xu et al., 2021; Zhang et al., 2021). CMCTS possesses high viscosity, low toxicity, and favorable biocompatibility, and all these excellent physical, chemical, and biological properties make this derivative become one of the research focuses in recent years (Fonseca-Santos and Chorilli, 2017; Shariatinia, 2018). Besides, CMCTS also have a unique advantage in forming hydrogel because the presence of carboxyl groups allows CMCTS to be constructed into materials *via* chemical cross-linking methods. It is well-known that curcumin (Cur) extracted from the rhizome of turmeric is one kind of natural polyphenols and has anti-inflammatory and antioxidant properties. In recent studies, curcumin was reported to not only scavenge excess ROS but also reduce cellular expression of pro-inflammatory cytokines (IL-6 and TNF-α) (Kasiewicz and Whitehead, 2016; Barchitta et al., 2019; Liczbiński et al., 2020; Vallée and Lecarpentier, 2020).

Therefore, CMCTS-based hydrogel cross-linked by an ROS-sensitive linker and loaded with curcumin (Cur) was prepared to promote burn wound healing. In the microenvironment of the burn wound, such a hydrogel can clear superfluous ROS due to the presence of ROS-sensitive cross-linkers. In the meantime, with the degradation of the hydrogel, the loaded Cur was released from the interior of the hydrogel to further sweep away ROS and inhibit inflammation. After the preparation, we assessed the morphology, FTIR, Cur delivery property, water absorption, water vapor transmission, mechanical property, cytotoxicity, and macrophages phenotype *in vitro*. Subsequently, the hydrogel in full-thickness skin burn defect rats was applied to investigate the recovery efficiency, inflammatory factor expression, neovascularization, and collagen fiber alignment in the wound areas. As displayed in **Scheme 1**, we expect that the Cur@CMCTS-Tk hydrogel could be used for wound treatment and tissue regeneration due to the ability to scavenge excess ROS.

MATERIALS AND METHODS

Materials

Carboxymethyl chitosan ((CMCTS, viscosity 1,000 mPs), substituted ratio >90%) was purchased from Dalian GlycoBio Co., Ltd (China), 2,2′-(propane-2,2-diyldisulfanediyl) diethanamine (NH_2-Tk-NH_2, Tk) was purchased from Cassim (Xi'an) Biotechnology Co., Ltd. (China), and enhanced green fluorescent protein (EGFP) plasmid was purchased from Hanbio (Shanghai) Co., Ltd. (China). The following materials were all obtained from Aladdin: H_2O_2 (30 wt% in H_2O), Cur, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), N-hydroxysuccinimide (NHS), and lipopolysaccharide (LPS). All drugs and reagents were of analytical grades so that no additional refinement was required.

Synthesis of Cur@CMCTS-Tk Hydrogels

EDC/NHS–mediated reaction was used to synthesize CMCTS-Tk polymers especially, and amide coupling reaction happened between the carboxylic groups (CMCTS) and the amino groups (NH_2-Tk-NH_2). In brief, first, 5 g of CMCTS was dissolved in 150 ml of distilled water at 40°C. Simultaneously, 2 g NH_2-Tk-NH_2 was dissolved in 125 ml buffer solution and 2 g EDC (10 mmol) and 1 g NHS (13.9 mmol) was also added to

activate the carboxylic groups of CMCTS. Subsequently, the pH value of the solution stayed at 6.0 after stirring for 1 h. The purification of CMCTS-Tk was divided into two steps. The first step is to remove excess EDC/NHS, the product is placed into a dialysis bag with a molecular cutoff point of 3,500 Da for 3 days to remove the excess EDC and NHS. The second step is to remove the excess or unreacted Tk, the product is placed into a vessel filled with anhydrous acetone under a shaker (200 rpm) for 24 h. The product is washed with anhydrous acetone thrice and with deionized water once. Then 100 mg Cur was added to 10 ml ethyl alcohol solution and then CMCTS-Tk hydrogel (5 g) was placed in a shaker (100 rpm/s) overnight at 40°C to fully loading 48 h. After loading Cur, the excess unloading Cur is taken to measure the concentration for calculating the loading quality into CMCTS-Tk hydrogel *via* the UV-Vis method. Finally, the Cur@CMCTS-Tk hydrogel was obtained following the process of filtration and lyophilization. In addition, a control group of hydrogel (named Cur@CMCTS) was prepared in which Tk-c [NH_2-$(CH_2)_6$-NH_2] was used to replace NH_2-Tk-NH_2, and the other steps remained unchanged.

Morphology and FTIR of Hydrogels

Morphology: The final hydrogels were cut and coated with gold by sputtering, and a field emission scanning electron microscope (SEM) (Philips LEO1530 VPSEM) was used to observe the cross section morphology of the hydrogel.

FTIR analysis: The cross-linked reaction between NH_2-Tk-NH_2 and CMCTS molecules was identified through an FTIR spectrophotometer (Bruker Optics Inc.). For the measurement, KBr was introduced to the sample to form transparent pallets. The test was conducted at room temperature with wavenumber ranges from 4,000 to 400 cm^{-1} and a resolution of 4 cm^{-1}.

^1H NMR analysis: The purity of CMCTS-Tk hydrogel, CMCTS, and Tk were determined by the proton nuclear magnetic resonance (^1H NMR) spectra.

In Vitro Delivery of Cur by Hydrogels

To examine the release behavior of Cur under H_2O_2 condition, 2.0 g Cur@CMCTS and Cur@CMCTS-Tk hydrogel were placed into 10 ml of cell lysate (RIPA lysis buffer) medium at 37°C. After 5 h, the releasing medium was added into 50 mM H_2O_2. At the appointed time, we took 8 ml of the mixed solution to investigate the release ratio *via* the ultraviolet spectrophotometer and replaced it with the same volume of fresh buffer solution instead.

Swelling Ratio of Hydrogels

In order to measure the swelling rate of CMCTS hydrogels, 200 mg Cur@CMCTs and Cur@CMCTS-Tk hydrogels were prepared with Tk-c and Tk cross-linking agents separately and then immersed in deionized water for 22 h after freeze-drying, respectively. After removing the excess water, the hydrogels were weighed (wet weight). Then the hydrogel sample was frozen and lyophilized to weigh again (dry weight), and the formula for determining the swelling ratio is given in **Eq. 1**(Augustine et al., 2021; Balakrishnan et al., 2005).

$$\text{Swelling Ratio (SR)} = \frac{W_{wet}}{W_{dry}}. \quad (1)$$

Equation 1. Swelling ratio determination for hydrogels.

Water Vapor Transmission Rate and Mechanical Characterization of Cur@CMCTS-Tk Hydrogel

Based on the American Society for Testing Material (ASTM) standard E96-00 (Queen et al., 1987), the moisture permeability of Cur@CMCTS-Tk hydrogel was determined by water vapor transmittance (WVTR). Briefly, hydrogels were placed over the mouth of a 40-mm-diameter cylindrical glass bottle containing deionized water, and the rim of the bottle is tightened to prevent water vapor from escaping from the edge. The bottle was placed in an environment of 37°C and 35% humidity for 24 h, while the relation curve between time and weightlessness was recorded and drew. WVTR was calculated by the following equation based on the slope of the curve.

$$WVTR = \frac{slope \times 24}{A} g/m^2/day. \quad (2)$$

Eq. 2. WVTR of hydrogels, A indicates the test area of hydrogels (m^2).

Mechanical strength: Before the analysis of mechanical strength using a rheometer, the hydrogels were treated with different times of H_2O_2. Subsequently, storage modulus (G′) was measured through frequency (range from 0.1 to 10 Hz), strain sweeping (at a maximum strain of 10%), and oscillatory (with a frequency of 1.0 and 100 Hz) mode.

Cytotoxicity of Hydrogels

The biocompatibility of hydrogels was studied using the fibroblast (L929) cells. First of all, the L929 cells were cultured with the DMEM media containing 10% fetal bovine serum (FBS), penicillin (100 UmL^{-1}), and streptomycin (100 UmL^{-1}) under appropriate humidified incubating conditions (at 37 C in 5% CO_2). Then the cells were transfected by EGFP plasmid to emit green fluorescence for a laser scanning confocal microscope (LSCM, 510Meta Duo Scan, Zeiss, Germany) observation. After 7 days of culture, the cells were separated *via* trypsinization and centrifugation processes. For the biocompatibility analysis, the CMCTS-Tk and Cur@CMCTS-Tk hydrogels were sterilized by Co-60 for 10 kGy. Finally, the L929 cells were seeded in 5×10^4 cells per well of a 24-well plate, and the LPS was also added to the culture medium to simulate the ROS environment *in vitro*.

LSCM fluorescence imaging: It was used to visualize the L929 cells after culturing with different hydrogels. The cells were captured using a laser scanning confocal microscope (LSCM, 510Meta Duo Scan, Zeiss, Germany) with the EGFP excitation wavelength 486 nm and emission wavelength 509 nm at the 7 days.

Cells migration: The effect of hydrogel on the migration property of L929 cells was assessed by *in vitro* wound healing migration experiment. Hydrogels were placed at the bottom of

the 24-well plate and then L929 cells were inoculated on it. After 24 h, cell scratches were formed with the tip of the sterile pipette. Cultured for 24 h later, the cells were fixed with 4% paraformaldehyde at room temperature for 15 min and DAPI staining and microscope photography were performed.

MTT assessment: The cell cytotoxicity was tested by the MTT method, and the cell survival percentage was defined as OD_{exp}/OD_{con}. OD_{exp} represented the optical density in the experimental group, while OD_{con} was for the control group.

Effects of Hydrogels on RAW 264.7 Cells Polarization, Inflammatory Response, and Cytokine Expression

To analyze the effects of Cur@CMCTS-Tk hydrogel on macrophages phenotype switch and inflammatory response, a flow cytometer (FCM) and a Western blot analysis were applied. To stimulate the ROS microenvironment, LPS (100 ng/ml) was chosen and co-cultured with Cur@CMCTS-Tk hydrogel. At last, 1.0×10^5 RAW 264.7 cells were cultured with CMCTS-Tk or Cur@CMCTS-Tk hydrogel and LPS condition.

Western blot analysis: Fibroblasts were rinsed in phosphate buffer saline (PBS) and mixed with a radioimmunoprecipitation assay (RIPA) buffer containing 1% (v/v) phenylmethylsulfonyl fluoride (PMSF). The protein was electrophoretically resolved (120 V) on a 12% SDS-polyacrylamide gel and transferred (350 V) to PVDF membrane for 90 min and incubated with 5% skim milk. Afterward, the PVDF membrane was incubated overnight with primary antibodies at 4°C and washed with TBST thrice, for 10 min each time. Next, the membrane was blotted with peroxidase-conjugated secondary antibodies and washed the same number of times as in the previous step with TBST. Visualization of proteins was performed by the chemiluminescent signal following the instructions of the manufacturer. Primary antibodies for TNF-α (ab255275) and IL-10 (ab189392) monoclonal antibodies were used.

FCM analysis: After incubation for 48 h, 10% mouse serum was used to block the RAW 264.7 cells for 30 min. The cells were then incubated in the mixed solution combining rabbit CD86 (ab242142) and CD206 (ab223961) monoclonal antibody (dissolved in PBS) with 0.05% proclin300 and 1% BSA for at 4°C 30 min. After washing with PBS thrice, the RAW 264.7 cells were placed in PBS and analyzed by flow cytometry (Beckman Coulter, California, United States). Besides, the inflammation-associated protein of TNF-α and IL-10 was analyzed by using a flow cytometer which is incubated with rabbit TNF-α (ab255275) and IL-10 (ab215975) monoclonal antibody.

In Vivo Wound Repair

Based on previous studies (Lin et al., 2020), the effect of hydrogels on wound healing *in vivo* was evaluated in a full-thickness burn rat model for up to 21 days. Animal experiments were performed according to the approval of the Animal Ethics Committee of Jinan University, in accordance with relevant laws and institutional guidelines. Specifically, 36 male Sprague Dawley (SD) rats (2–3 months of age) weighing 250 g were intraperitoneally injected with ketamine and thiazide at 40 and 5 mg/kg, respectively. After shaving the operative dorsal skin region of rats and disinfecting with 75% ethanol, a scalding machine was applied to burn for 10 s at 95 ± 1 C. A full-thickness circular wound was created about 2 cm in diameter by using forceps and scissors to remove the damaged tissue. PBS, CMCTS, Cur@CMCTS, CMCTS-Tk, and Cur@CMCTS-Tk hydrogels were placed on the wound fixed with an elastic bandage to promote healing. All rats were kept alone in cages and fed with enough food and water till they were sacrificed. At regular intervals, the wound appearance was photographed *via* camera and the wound trace was plotted by Adobe Illustrator (AI) software. According to the wound area at different times (days 0, 7, 14, and 21), the percentage of wound contraction was calculated using **Eq. 3**.

$$Wound\ contraction = \frac{Area_{d0} - Area_{dn}}{Area_{d0}} \times 100\%. \quad (3)$$

Eq. 3. The percentage of wound contraction, where d0 is on day 0, and dn is on days 7, 14, and 21, respectively.

Histological and Immunohistochemical Staining

The rats were sacrificed on day 21 after skin burns, and the wound with the surrounding skin was excised for histological detection. Skin tissues were fixed in formaldehyde for 24 h, dehydrated in an ethanol solution, and embedded in paraffin waxes. Histological sections were cut as 4.5 μm and stained with hematoxylin and eosin (H&E) and Masson staining for histological analysis.

The expression levels of TNF-α and CD31 were detected by immunohistochemistry. The slides were incubated with the primary antibody at 4°C overnight and with a secondary antibody at room temperature for 90 min. Photomicrographs were observed under a light microscope (DS-Fi3; Nikon, Japan).

Statistical Analysis

Data were evaluated using GraphPad Prism 6 software followed by the Student's unpaired *t*-test. We define $p < 0.05$ as statistically significant.

RESULTS AND DISCUSSION

Morphologies, Compositions, and Delivery Property of Hydrogels

Cur@CMCTS-Tk and Cur@CMCTS hydrogels were synthesized *via* CMCTS monomers in an aqueous phase system by using Tk and Tk-c as the cross-linker, EDC/NHS as the activating agent. The Cur@CMCTS hydrogel cross-linked and degradation procedures were shown in **Figure 1**.

In **Supplementary Figure S1**, the ^{1}H NMR spectrum of CMCTS, Tk, and CMCTS-Tk was shown. For CMCTS ^{1}H NMR, the peaks shows broader. The resonance of acetyl (-C(O)CH3) protons can be found at the chemical shift 2.0 ppm. The methylene proton in O- and N-substituted carboxymethyl chitosan appeared at the shift 3.91 and

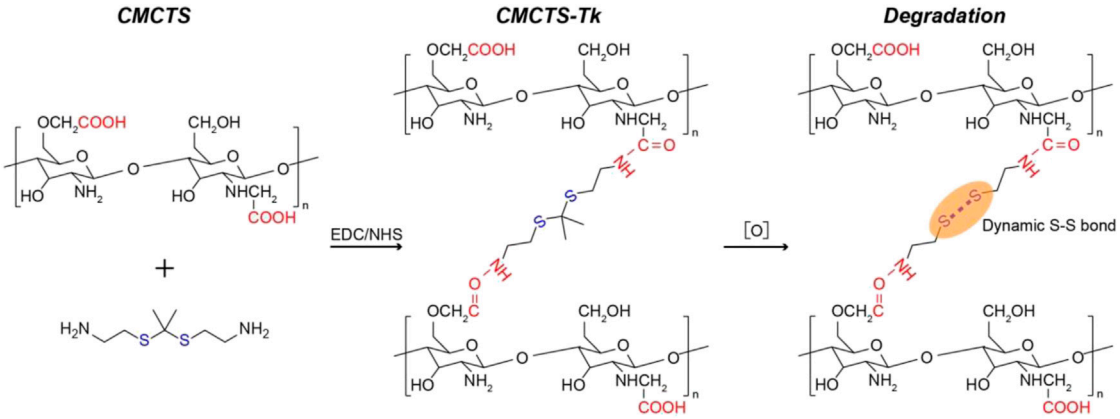


FIGURE 1 | Schematic representation of Cur@CMCTS hydrogel preparation and degradation procedures.

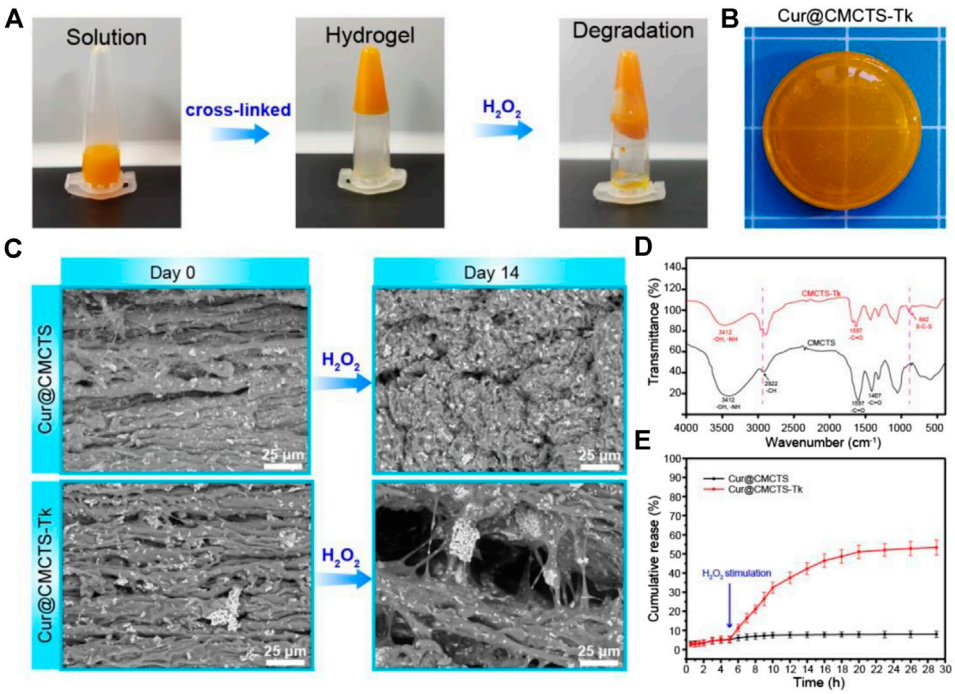


FIGURE 2 | (A) Sol–gel transition behavior of Cur@CMCTS-Tk hydrogel. **(B)** Image of Cur@CMCTS-Tk hydrogel for burn wound repair. **(C)** SEM image of Cur@CMCTS and Cur@CMCTS-Tk hydrogels treated with H_2O_2. **(D)** FTIR analysis of CMCTS and CMCTS-Tk hydrogels. **(E)** Cur delivery curve from Cur@CMCTS and Cur@CMCTS-Tk hydrogels treated with H_2O_2 at 5 h.

3.31 ppm, respectively. The shift in the range of 3.5–4.0 ppm is corresponding to the proton from carbon atom C3–C6 from glucopyranose unit. For the Tk ^{1}H NMR, the peaks appear at 2.81, 2.64, 1.51, and 1.22 ppm, which are kept in accordance with the previous study (Li et al., 2020). For the ^{1}H NMR of CMCTS-Tk, the curve kept similarly with the CMCTS. In addition, the Tk characteristic peak appeared, indicating the successfully cross-linked between CMCTS and Tk.

In **Figure 2A**, the results of the tube inversion test indicated Cur@CMCTS-Tk hydrogel showed good gelation within the appropriate reaction time (Kim et al., 2016). When Cur@CMCTS-Tk hydrogel was treated with H_2O_2 for 7 days, the hydrogel became degradation indicating the ROS sensibility ability. The appearance of Cur@CMCTS-Tk hydrogel (Cur/Cur@CMCTS-Tk is 1.76 wt%) developed for rats full-thickness burn repair is shown in **Figure 2B**.

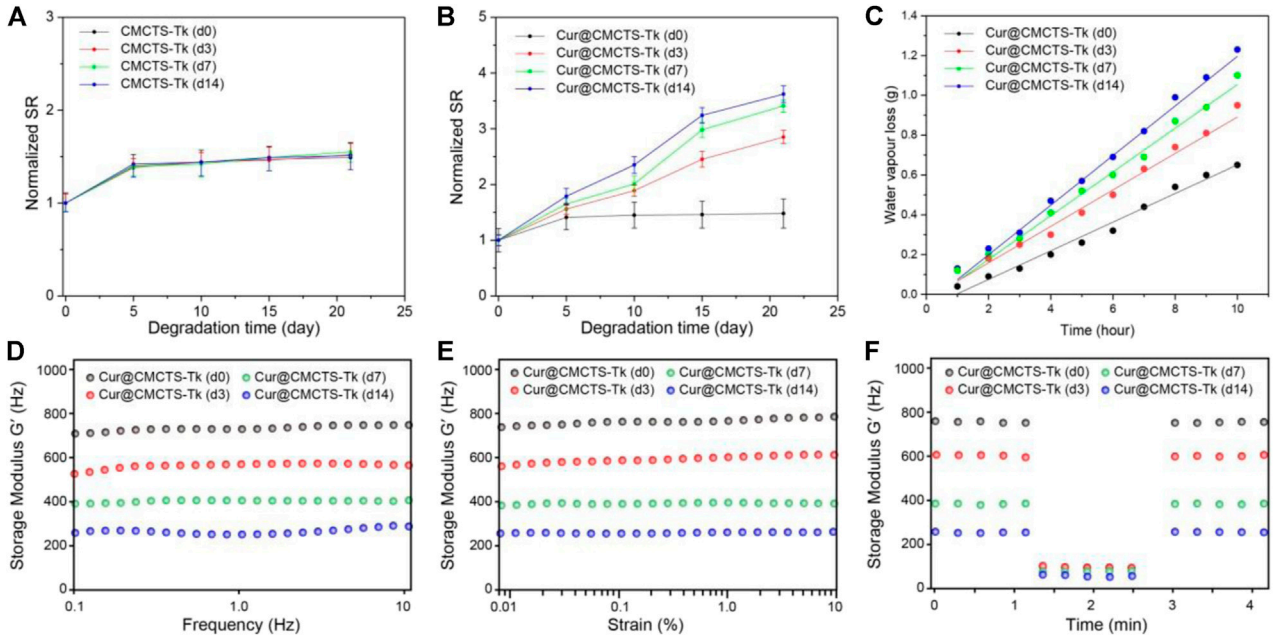


FIGURE 3 | Normalized swelling ratio of CMCTS-Tk **(A)** and Cur@CMCTS-Tk **(B)** hydrogels treated with H_2O_2 for 0, 3, 7, and 14 days. **(C)** Water vapor loss of Cur@CMCTS-Tk hydrogel treated with H_2O_2 for 0, 3, 7, and 14 days. **(D)** Frequency-dependent (from 0.1 to 10 Hz), **(E)** strain sweeping (at a maximum strain 10%), and **(F)** oscillatory mode with a frequency (1.0 and 100 Hz) of storage modulus (G′).

The cross-section morphology of Cur@CMCTS and Cur@CMCTS-Tk hydrogels treated with H_2O_2 were observed by SEM (**Figure 2C**). Both Cur@CMCTS and Cur@CMCTS-Tk hydrogel possessed a relatively tight structure with a limited micro-pore that favored the micromolecule gas permeation but did not favor Cur delivery. Notably, obvious Cur residues were seen on the hydrogel surface, indicating the complete dispersion inside hydrogel. After being treated with H_2O_2, the pore diameter became larger for the cross-linked structure degradation.

As depicted in **Figure 2D**, the FTIR spectra of CMCTS-Tk and CMCTS were clearly displayed. CMCTS showed strong peaks at 1,597 cm^{-1} and 1,407 cm^{-1}, which corresponded to the carboxy group and carboxymethyl group, respectively (Kalaithong et al., 2021). Besides, a wideband at 3,412 cm^{-1} meant stretching vibrations of O–H and N–H bonds, while 2,922 cm^{-1} for C–H bonds. After being cross-linked by NH_2-Tk-NH_2, CMCTS-Tk presented the enhanced characteristic absorption bands of methylene stretching peak at 2,890 cm^{-1} and a new stretching peak of S-C-S at 845 cm^{-1}, indicating that NH_2-Tk-NH_2 was successfully grafted onto the CMCTS.

The H_2O_2-sensitivity delivery curve of Cur from Cur@CMCTS and Cur@CMCTS-Tk hydrogel is exhibited in **Figure 2E**. For Cur delivery in Cur@CMCTS-Tk hydrogel, a fast release of 53.4 ± 3.9% was observed after 5 h because the thioketal chain was broken by H_2O_2 attack, while almost no delivery was observed in Cur@CMCTS hydrogel due to the close integration of Cur and CMCTS chain (Shim and Xia, 2013; Pu et al., 2014). This Cur release feature is favorable for applying in ROS redundant wound. For the ROS-sensitivity hydrogel, if the burn wound possessed abundant ROS, the ROS-sensitivity hydrogel molecular chain will soon break and release curcumin to absorb the excess ROS. Once the ROS is controlled in a low level, the hydrogel molecular chain will stop the breakage and the curcumin will keep a stable release rate. The results demonstrated that Cur@CMCTS-Tk hydrogel could deliver continuously Cur under H_2O_2 condition. It have been demonstrated that when the curcumin concentration is more than 2.5 μg/ml, it will reduce the inflammatory factor production in LPS-induced macrophages (Ternullo et al., 2019). In this study, those continuously released curcumin concentrations are enough for cellular uptake. It can be supposed that curcumin cellular uptake is time dependent and occurs through a concentration gradient mechanism *via* membrane partitioning (Shefa et al., 2020).

The Water Absorption and Water Vapor Transmission Rate of Hydrogels

The results of the water absorption test indicated that the water equilibrium swelling rate of Cur@CMCTS and Cur@CMCTS-Tk hydrogels in PBS solution was ~48% (**Figures 3A,B**). Such a high fluid absorption capacity was essential for absorbing wound exudate and edema fluid. However, after being treated with H_2O_2, Cur@CMCTS-Tk hydrogel was significantly more swollen than the PBS-treated hydrogel, showing up as the bigger aperture.

The ideal wound dressing also could keep the wound moist by controlling water loss at the optimal rate. The evaporation rate of water from wound evaporation ranged from 2000 to 2,500 g/m^2/day provides sufficient water to get rid of the risk of wound

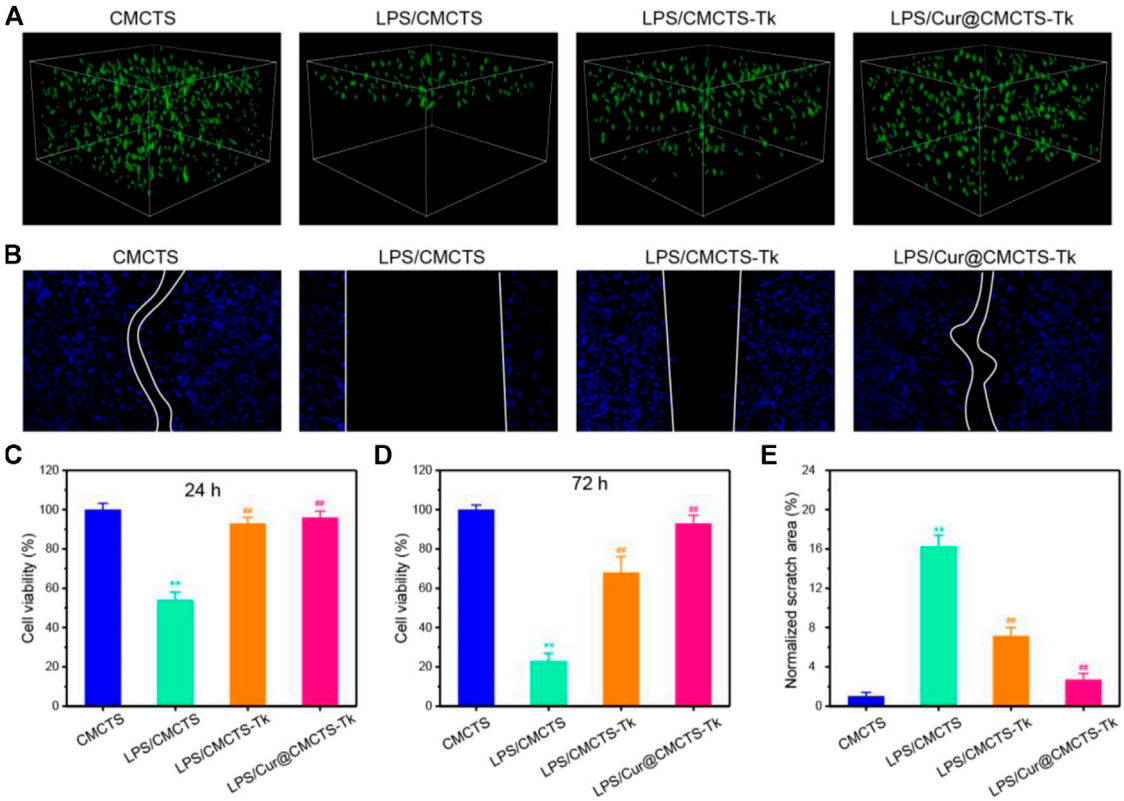


FIGURE 4 | (A) The GFP-plasmid transfected cells were cultured with CMCTS (Control), LPS/CMCTS (LPS), LPS/CMCTS-Tk, and LPS/Cur@CMCTS-Tk hydrogel for 72 h. **(B)** Images for cell wound scratch assay of L929 cells at 72 h. **(C)** Cytotoxicity of Cur@CMCTS-Tk hydrogel to fibroblast cells by MTT assay at 24 and 72 h. **(D)** Quantitative analysis of the scratch area. The data were represented as mean ± SD (n = 6), $^{**}p < 0.01$ vs. control; $^{\#\#}p < 0.01$ vs. LPS/CMCTS (LPS) group.

dehydration. Based on the slope of the chart (**Figure 3C**), the WVTR of Cur@CMCTS-Tk hydrogel treated with H_2O_2 for 0, 3, 7, and 14 days were ~1,376, ~1749, ~2098, and ~2,381 g/m^2/day, respectively. The WVTR of Cur@CMCTS-Tk treated with H_2O_2 possessed an appropriate WVTR treated with H_2O_2 for 3 days, which is beneficial to maintain appropriate liquid balance on the surface of the wound.

Rheology Characterization of Cur@CMCTS-Tk Hydrogels

To evaluate the degradation property of Cur@CMCTS-Tk hydrogels *in vitro*, the hydrogel was incubated with different concentrations of H_2O_2 and characterized by rheological property. As shown in **Figures 3D–F**, the rheological analysis suggested that the storage modulus (G′) of Cur@CMCTS-Tk hydrogel was irrelevant to frequency, which confirmed its hydrogel properties. In addition, the G′ of Cur@CMCTS-Tk hydrogel was time-dependent when treated with H_2O_2 and G′ on day 0 was ~2.69 times than on day 14 in the frequency mode. The result demonstrated that the fluid property of Cur@CMCTS-Tk hydrogel became better to fit the wound healing after being treated with H_2O_2.

On the other hand, the G′ frequency at high (100 Hz) and low (1.0 Hz) shear frequency were measured to assess the self-healing ability of Cur@CMCTS-Tk hydrogel. The sharp drops of G′ of Cur@CMCTS-Tk hydrogel at high frequency verified its shearing refinement performance, while the fast recovery of G′ at low frequency after high frequency indicated that G′ has good self-healing property due to the formation of dynamic "S–S" bond. The results showed that Cur@CMCTS-Tk hydrogel treated with H_2O_2 could significantly accelerate its degradation *in vitro* and improve rheological properties.

Cytotoxicity of Hydrogels

It should be biocompatible if a dressing material aims at repairing the wound. To explore the cytotoxicity of Cur@CMCTS-Tk hydrogel, the GFP plasmid–transfected fibroblast cells were implanted into Cur@CMCTS-Tk hydrogel surface (**Figure 4A**). For the CMCTS-Tk hydrogel group, a large number of fibroblast cells are proliferated into the hydrogel interior after culturing for 72 h. While for the LPS/CMCTS hydrogel group, the cells were only dispersed on the hydrogel superficial layer. For LPS/CMCTS-Tk and LPS/Cur@CMCTS-Tk group, the cells could continue to adhere to the hydrogel's interior. The results may be due to the CMCTS-Tk hydrogel could eliminate ROS aroused by LPS and further promotes cell proliferation.

Here, the cell migration property was evaluated using the L929 cells (**Figure 4B**). The CMCTS-Tk and Cur@CMCTS-Tk

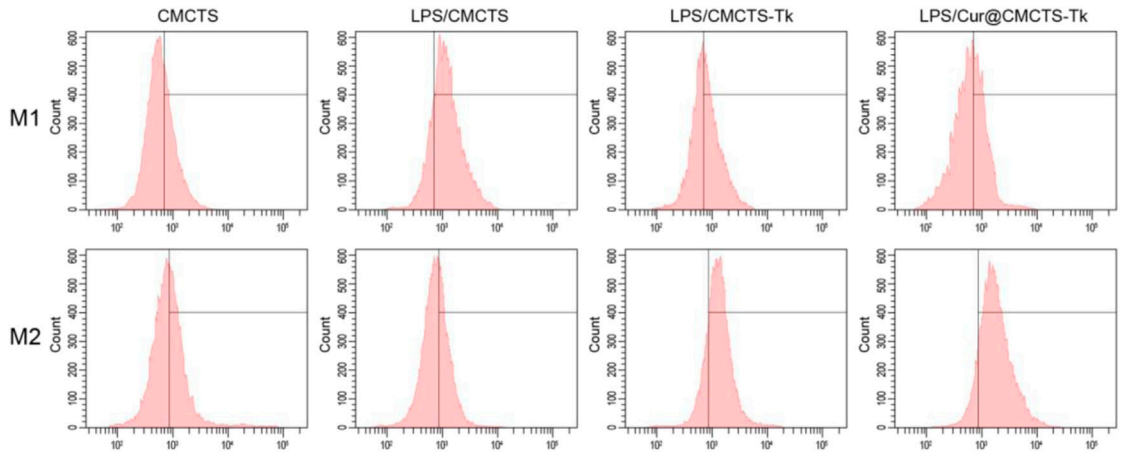


FIGURE 5 | FCM results of RAW cells cultured with normal CMCTS hydrogel (control) and LPS/CMCTS (LPS), LPS/CMCTS-Tk, and LPS/Cur@CMCTS-Tk hydrogel after 24 h. RAW cells were stained with M1 marker CD86 and M2 marker CD206. The ratios of M1 and M2 phenotypes of macrophages were also presented.

hydrogels both could promote L929 cells migration when compared with the LPS groups (**Figure 4E**) because the CMCTS-Tk hydrogel could absorb redundant ROS brought by LPS. Besides, the released Cur also could eliminate cell inflammation, improve cell activity, and then promote cell proliferation and migration. The MTT assay was used to evaluate the toxicity of Cur@CMCTS-Tk hydrogel to fibroblast cells at 24 and 72 h (**Figures 4C,D**). Based on the international standard [ISO 10993-5:2009(E)], the cytotoxicity was divided into 0, 1, 2, 3, 4, and 5 grades, which correspond to the cell survival rate as 100%, 75 ~ 99%, 50 ~ 74%, 25 ~ 49%, 1 ~ 24%, and 0 grade, respectively. Among these six grades, grade 0 and grade 1 are considered as non-cytotoxic. When fibroblast cells are cultured with LPS/CMCTS hydrogel, the cytotoxicity is 54.1 ± 4.3 (24 h) and 22.7 ± 3.8 (72 h) corresponding to the 2nd grade and 3rd grade, which is harmful to cells. When cells were cultured with LPS/CMCTS-Tk or LPS/Cur@CMCTS-Tk hydrogel, the cytotoxicity was significantly decreased compared to that in the LPS/CMCTS group both on days 3 and 7. The cell survival percentage of these two hydrogels could both reduce the cytotoxicity brought by H_2O_2.

Macrophage Phenotype Converted by Hydrogels

Macrophages are immune cells with a variety of functions and can be divided into M1 type and M2 type according to their activation state and function (Shen et al., 2020). M1 macrophages possess enhanced anti-inflammatory ability, secrete pro-inflammatory cytokines (such as TNF-α, IL-1, IL-6, and IL-23), and arouse ROS, while M2 macrophages can inhibit pro-inflammatory cytokines and secrete extracellular matrix components that may be necessary for the late stage of tissue repair (Chen et al., 2014). Granulation tissue formation, myofibroblast differentiation, matrix deposition, and angiogenesis also rely on the M2 phenotype (Murray et al., 2014).

To analyze the macrophage phenotypic switch influenced by Cur@CMCTS-Tk hydrogel, the M1 and M2 phenotypic markers of macrophages were detected. Macrophages expressing M1 marker CD86 and M2 marker CD206 were detected by FCM as shown in **Figure 5**. It can be seen that the RAW cells M2 phenotype was inhibited by LPS. While cultured with LPS/CMCTS-Tk and LPS/Cur@CMCTS-Tk hydrogel, the macrophages M2 were increased to 73.3 ± 4.8% and 83.4 ± 3.1%, respectively. On the other hand, RAW cells M1 phenotype was evoked by IPS when compared with the control group. While cultured with LPS/CMCTS-Tk and LPS/Cur@CMCTS-Tk hydrogel, the macrophages M1 were decreased to 42.1 ± 3.7% and 37.9 ± 4.5%, respectively. The results suggested that CMCTS-Tk and Cur@CMCTS-Tk hydrogels could reduce the inflammatory phenotype evoked by LPS. In addition, the detailed M1 and M2 phenotype statistical data were recorded in **Table 1**.

Effects of Hydrogels on Inflammation-Related Cytokine Expression in RAW Cells

To investigate the inflammation-related cytokine expression in RAW cells after cultured with Cur@CMCTS-Tk hydrogel, RAW cells were cultured on Cur@CMCTS-Tk hydrogel with LPS and then analyzed by Western blot, FCM, and immunofluorescence method. **Figure 6A** shows the Western blot results that Cur@CMCTS-Tk hydrogel could activate the macrophages to boost the anti-inflammatory factors IL-10 expressions (**Figure 6C**) and inhibit the pro-inflammatory factors TNF-α expression (**Figure 6B**) under LPS environment. In order to evaluate cytokine levels quantitatively, the FCM was applied to measure the expression of TNF-α and IL-10 in RAW cells (**Figures 6D,E**). The FCM result was consistent with the Western blot result, guaranteeing that CMCTS-Tk and Cur@

TABLE 1 | The RAW cells M1 and M2 phenotype statistical data (mean ± SD, n = 3).

	CMCTS (%)	LPS/CMCTS (%)	LPS/CMCTS-Tk (%)	LPS/Cur@CMCTS-Tk (%)
M1	35.8 ± 3.4	73.1 ± 4.5	42.1 ± 3.7	37.9 ± 4.5
M2	43.1 ± 2.7	37.8 ± 3.6	73.3 ± 4.8	83.4 ± 3.1

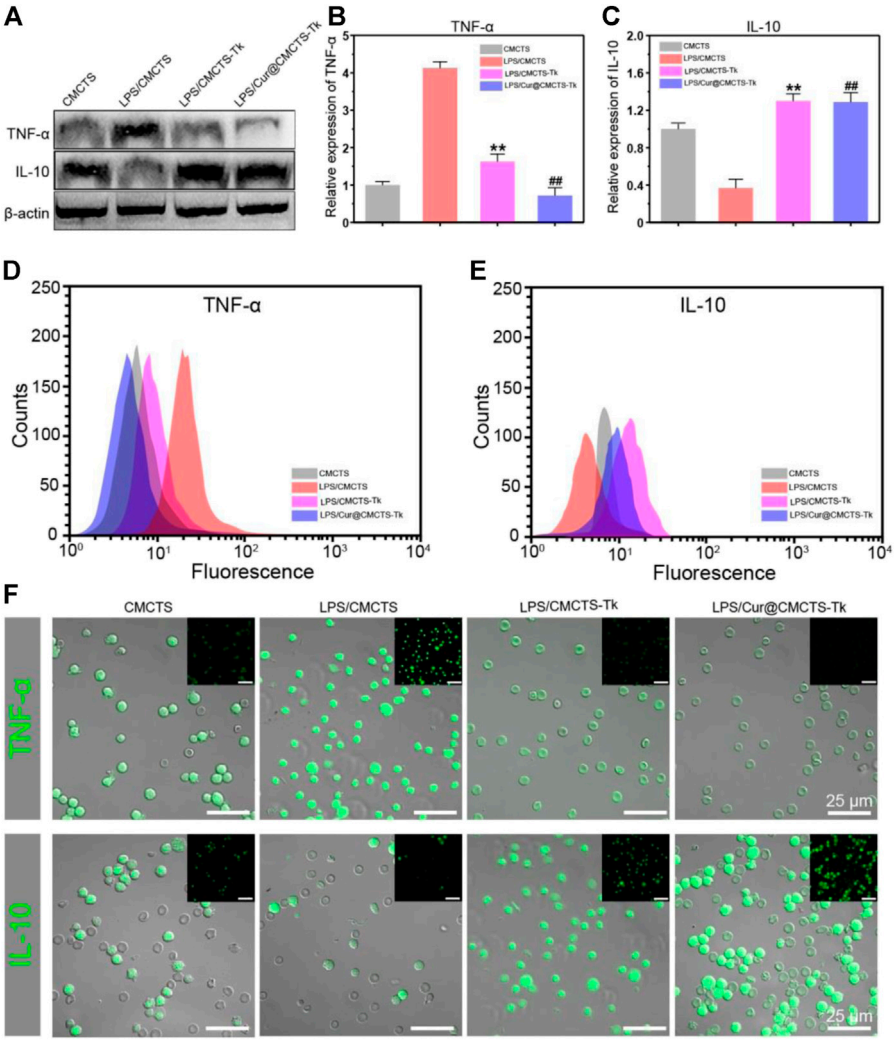


FIGURE 6 | The protein expressions in RAW cells cultured with normal CMCTS hydrogel (control) and LPS/CMCTS (LPS), LPS/CMCTS-Tk, and LPS/Cur@CMCTS-Tk hydrogel after 24 h were analyzed using Western blot **(A)** and the TNF-α **(B)**, IL-10 **(C)** relative expression analysis. The FCM analysis **(D–E)** and immunofluorescence staining **(F)** of TNF-α and IL-10 in RAW cells. The data were represented as mean ± SD (n = 3). $^{**}p < 0.01$ vs. control; $^{\#\#}p < 0.01$ vs. LPS group. The scale bar is 25 μm.

CMCTS-Tk hydrogel could suppress the secretion of inflammatory cytokines TNF-α and promote anti-inflammatory cytokines IL-10 expression. Finally, to more visually reflect the expression of TNF-α and IL-10, their antibodies were used to measure RAW cells using immunofluorescence staining. It can be seen from **Figure 6F** that Cur@CMCTS-Tk hydrogel reduced the expression of TNF-α but improved IL-10 in macrophages. All the above results could co-prove that CMCTS-Tk and Cur@CMCTS-Tk hydrogel inhibited the expression of the inflammatory cytokines and enhanced anti-inflammatory cytokines expression under the LPS environment.

In Vivo Wound Repair

CMCTs, Cur@CMCTS, and CMCTS-Tk hydrogels were used as the control groups to evaluate the wound healing effect of Cur@CMCTS-Tk hydrogel *in vivo*. **Figure 7A** illustrates the progress of wound closure after hydrogels treatment and the wound trace

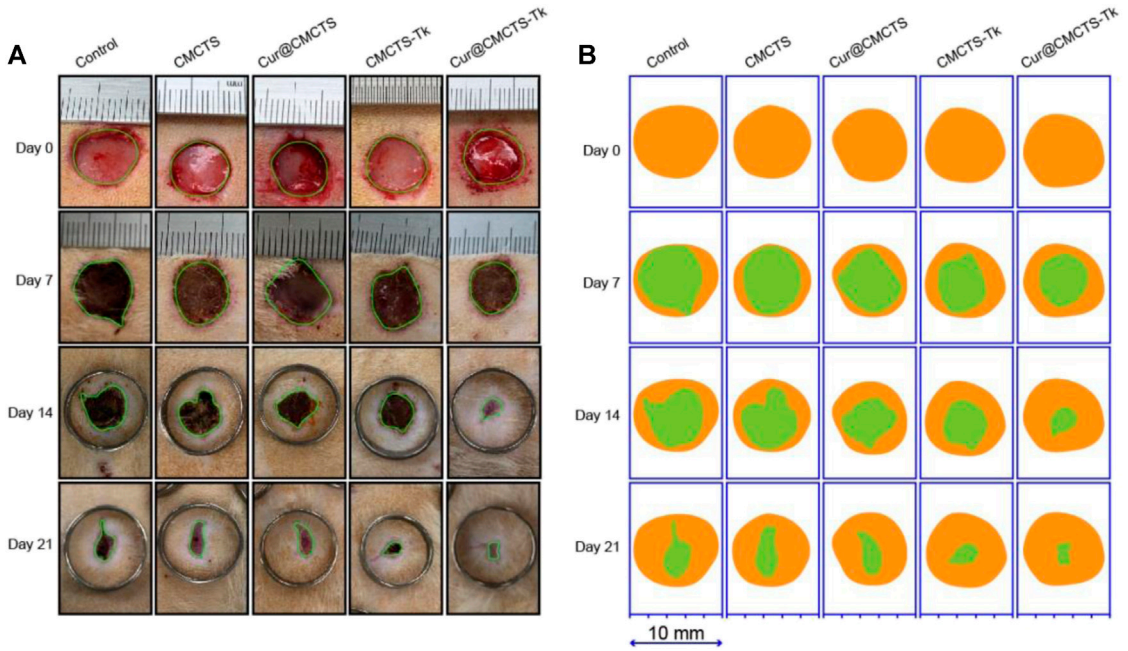


FIGURE 7 | (A) Digital photos of wounds on the dorsum of rats after treatment by Cur@CMCTS-Tk hydrogel for up to 21 days using no treatment, CMCTS, Cur@CMCTS, and CMCTS-Tk hydrogel as the controls. In the early seven days, the wound was rewetted at an interval of 8 h by two to three drops of PBS (37°C), allowing Cur delivery; **(B)** wound trace was drawn by Adobe Illustrator software. Yellow: 0 d, green: real-time day. The data were represented as mean ± SD ($n = 3$) (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

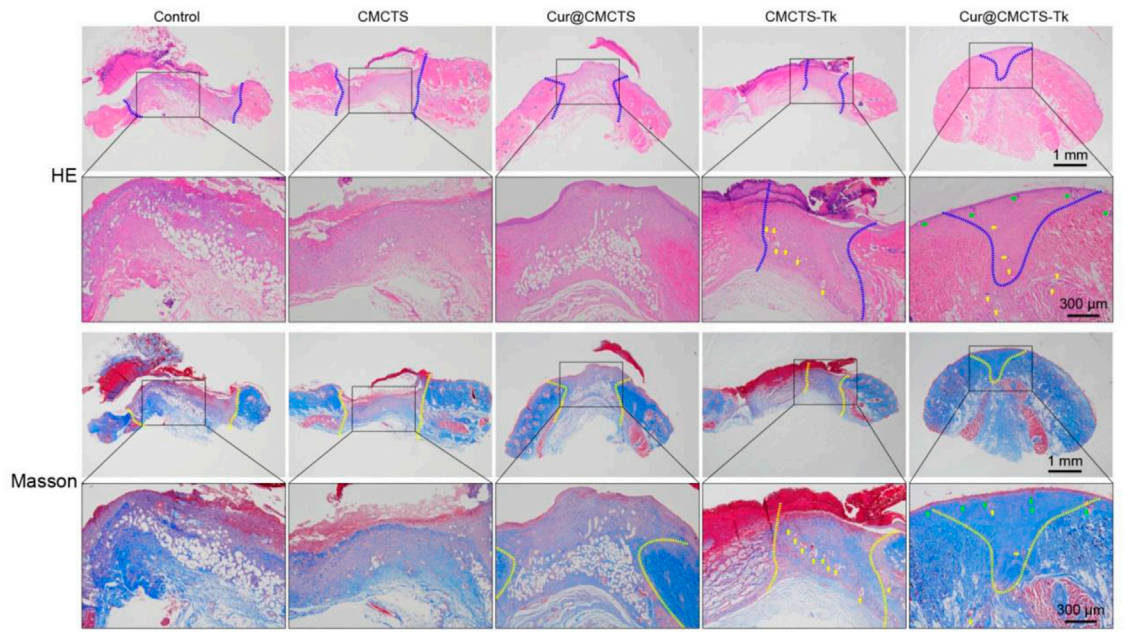


FIGURE 8 | *In vivo* wound healing effect of Cur@CMCTS-Tk hydrogels. Representative images of sections stained with H&E **(top)** and Masson's trichrome **(bottom)** from normal skin and wounded skin treated with/without hydrogels at day 21 post-wounding (the area within the blue or yellow dashed line is not healed and the yellow arrows represent micrangium). Diminished image scale bars are 1 mm, enlarged image scale bars are 300 μm.

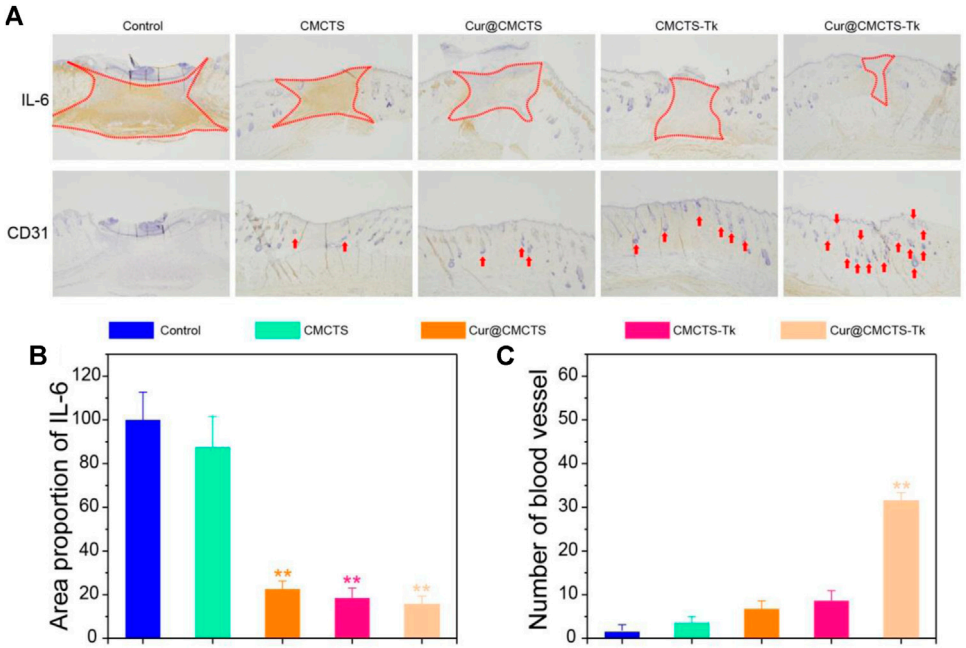


FIGURE 9 | Inflammatory factor and vascularization in the burn wounds treated with Cur@CMCTS-Tk hydrogel. **(A)** IL-6 and CD31 immunohistochemistry staining in the wounds treated with Cur@CMCTS-Tk hydrogel in full-thickness burns rat model at day 21. Expression of IL-6 **(B)** and quantification of the number of blood vessels **(C)** in the wounded area using ImageJ software (n = 3).

drawn by AI software was presented in **Figure 7B**. During the early 7 days, rewetting all hydrogels with two to three drops of PBS every 8 h to keep the wound moist. On the 7th day, the wound surface of each part was significantly reduced when some suppuration appeared, indicating the wound became inflamed. On day 14, all wounds of the control groups contracted into irregular circles with a contraction range of 56–60% and red granulation tissue was formed. At the same time, the scab of the Cur@CMCTS-Tk hydrogel group basically disappeared, and the wound recovery efficiency was about 83%, which was significantly better than the control groups. Here, this accelerated repair efficiency could be explained in two steps. For the first step, Cur@CMCTS-Tk hydrogel was attacked by excrescent ROS, the redundant ROS was absorbed by Cur@CMCTS-Tk hydrogel when the thioketal group is broken. Second, as the Cur@CMCTS-Tk hydrogel degraded, its fluidity enhanced, and then Cur released from the Cur@CMCTS-Tk hydrogel interior into the wound defect area. These results indicated that Cur@CMCTS-Tk hydrogels exhibited the most significant healing effect in all groups due to the gradual transmission of the elimination of Cur and ROS during wound healing.

H&E and Masson's Trichrome Staining

On day 21, we performed histological analysis of the wound using H&E staining and Masson's trichrome staining (**Figure 8**). We were surprised to find that the Cur@CMCTS-Tk hydrogel improved reepithelialization and wound remodeling more effectively than the control groups. First of all, the images suggested that the epidermis was similar in thickness to the skin tissue of healthy rats and much thicker than the other groups. These results implied that the burn wound covered by Cur@CMCTS-Tk would obtain limited scar formation due to the ROS elimination ability. In the second place, more blood vessels and hair follicles appeared in the Cur@CMCTS-Tk hydrogel group. Besides, the results of Masson's trichrome staining showed the Cur@CMCTS-Tk hydrogel group existed higher collagen deposition (blue staining) and more regular fiber arrangement. In the meantime, more microvessels were found in the Cur@CMCTS-Tk group, which was beneficial to wound healing. And this phenomenon can be interpreted as the biological effect of Cur@CMCTS-Tk hydrogel, such as the removal of ROS, continuous release of Cur, inhibition of ROS aggregation, improvement of cell viability, and promotion of angiogenesis. In conclusion, Cur@CMCTS-Tk hydrogel with controllable ROS scavenging activity can accelerate the speed of wound healing and significantly improve the quality of skin tissue regeneration.

IL-6 and CD31 Expression in the Wound Regeneration Area

To verify the inflammation expression in the wound area *in vivo*, we selected IL-6 as the represented cytokine that was closely related to inflammation, so an immunohistochemical method was used to assess the total IL-6 level in the wound area. As **Figure 9A** shows, the IL-6 expression was higher in the control group, and inflammation was more serious in the CMCTS group on day 21 ($^{**}p < 0.01$), whereas, the IL-6

expression in CMCTS-Tk and Cur@CMCTS-Tk group was lower in the wound area (**Figure 9B**), which was due to the efficient ROS scavenging ability of the CMCTS-Tk hydrogel and Cur delivery ability to against inflammatory response. The contents of platelet endothelial cell adhesion molecule-1 (CD31) (DeLisser et al., 1997) that can promote angiogenesis (**Figure 9C**) in the Cur@CMCTS-Tk group were significantly highest compared to the other groups indicating the fastest angiogenesis and the wound healing rate appeared on day 21 ($^{**}p < 0.01$). Overall, the intrinsic ROS scavenging ability and Cur delivery capacity of Cur@CMCTS-Tk hydrogel can reduce inflammatory responses and increase angiogenesis to promote wound healing.

CONCLUSION

In this study, we developed a Cur@CMCTS-Tk composite hydrogel dressing that progressively delivers Cur to eliminate redundant ROS during inflammation and new tissue formation in the process of wound healing. The Cur@CMCTS-Tk hydrogel, as a continuous phase, could react with ROS and quickly eliminate ROS. With the occurrence of the reaction, the thioketone group broke and the hydrogel degraded, leading to the accelerated release of Cur. Cur@CMCTS-Tk hydrogel presented good water vapor transmittance, mechanical properties, and biocompatibility after H_2O_2 treatment and treating with H_2O_2 at 37°C could accelerate the delivery of Cur. *In vivo*, Cur@CMCTS-Tk hydrogel could improve the efficiency of wound contraction, reduce the response of inflammation, and promote angiogenesis in the full-thickness burn rat model. Thus, Cur@CMCTS-Tk hydrogel could be a dressing for burn wound treatment.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, and further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Ethics Committee of Jinan University.

AUTHOR CONTRIBUTIONS

JL and WN conceived and directed this research. CY and YC performed the experiments. HH, SF, CY, and LW analyzed the data. WL processed the figures. JL and WN wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.679580/full#supplementary-material

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Functional Nanocarriers for Delivering Itraconazole Against Fungal Intracellular Infections

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Infectious diseases caused by intracellular microorganisms represent a significant challenge in medical care due to interactions among drugs during coinfections and the development of resistance in microorganisms, limiting existing therapies. This work reports on itraconazole (ITZ) encapsulated into functional polymeric nanoparticles for their targeted and controlled release into macrophages to fight intracellular infections. NPs are based on poly (lactic acid-co-glycolic acid) (PLGA) polymers of different compositions, molecular weights, and lactic acid–to–glycolic acid ratios. They were self-assembled using the high-energy nanoemulsion method and characterized by transmission electron microscopy, Fourier transform infrared spectroscopy (FT-IR), and differential scanning calorimetry. It was studied how the polymer-to-drug ratio, changes in the aqueous phase pH, and type and concentration of surfactant affected nanocarriers' formation, drug-loading capacity, and encapsulation efficiency. Results showed that drug-loading capacity and encapsulation efficiency reached 6.7 and 80%, respectively, by lowering the pH to 5.0 and using a mixture of surfactants. Optimized formulation showed an initial immediate ITZ release, followed by a prolonged release phase that fitted better with a Fickian diffusion kinetic model and high stability at 4 and 37°C. NPs functionalized by using the adsorption and carbodiimide methods had different efficiencies, the carbodiimide approach being more efficient, stable, and reproducible. Furthermore, linking F4/80 and mannose to the NPs was demonstrated to increase J774A.1 macrophages' uptake. Overall, *in vitro* assays showed the nanosystem's efficacy to eliminate the *Histoplasma capsulatum* fungus and pave the way to design highly efficient nanocarriers for drug delivery against intracellular infections.

Keywords: intracellular infection, poly (lactic acid-co-glycolic acid), itraconazole, nanocarriers, macrophages, bioreceptor

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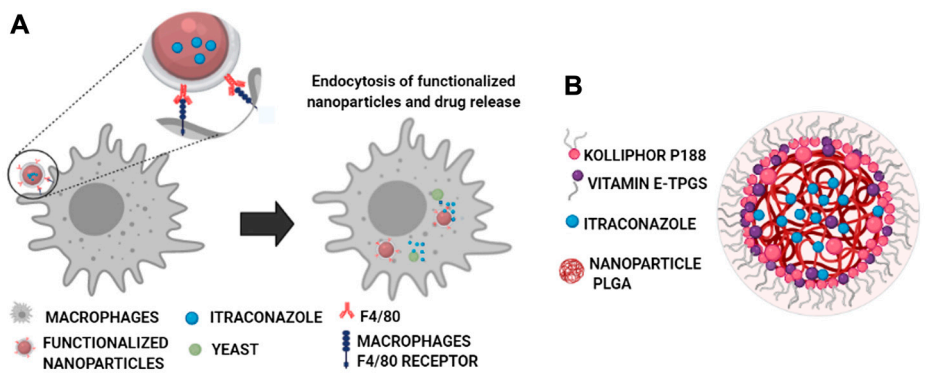
INTRODUCTION

Limited effectiveness of conventional therapies to combat intracellular infectious agents is commonly related to phagocytic evasion mechanisms, low-specificity treatments, manipulation of intracellular machinery by the pathogen, and the appearance of coinfections and drug resistance (Abushaheen et al., 2020). In this context, nanoencapsulation of therapeutic principles in NPs has been introduced as a powerful alternative to improve therapeutic efficacy and provide higher specificity, reducing doses and adverse effects (Begines et al., 2020; Eleraky et al., 2020; Sánchez et al., 2020). The most

commonly explored nanoencapsulation systems are based on nanoemulsions, lipid NPs, natural and synthetic polymeric nanocarriers, nanocapsules, nanogels, liposomes, niosomes, etc. (Endsley and Ho, 2012; Li et al., 2017; Bentz and Savin, 2018; George et al., 2019; Colorado et al., 2020; Sánchez et al., 2020). Among them, PLGA-based polymeric nanosystems enjoy high biocompatibility and biodegradability and high reproducibility and scalability. NPs fulfill the functions of reservation and protection of the active principle. They can be designed to cross biological barriers and functionalized with targeting ligands (Mena-Giraldo et al., 2020; Sánchez et al., 2020) at the outermost NP surface for enhanced cellular uptake, releasing the therapeutic load in a site-specific manner. Functional nanocarriers have been demonstrated to have the potential for the management of intracellular infections caused by viruses, certain bacteria (e.g., *Mycobacterium tuberculosis*), some protozoa (e.g., *Toxoplasma gondii* and *Leishmania* spp.), and some fungi (e.g., *Histoplasma capsulatum*) (Eleraky et al., 2020; Sánchez et al., 2020).

The size, shape, physicochemical properties, and surface chemistry of NPs are tuned on demand for a specific purpose (Duan and Li, 2013; Sánchez et al., 2020). One of the most significant challenges in developing functional nanocarriers is their interaction with physiological proteins, which may form a biomolecular corona (or protein corona), changing their superficial properties, affecting their circulation time in the body, and decreasing their specificity and effectiveness. The protein corona impacts antibody-coated NPs' targeting and may depend on the functionalization method owing to their partial or total NP masking (Mahmoudi et al., 2016; Tonigold et al., 2018). Antibodies may bind to the NPs reversibly through ionic, electrostatic, hydrophobic, or Van der Waals interactions (Tonigold et al., 2018) or irreversibly through covalent bonds. Interactions depend not only on NPs' affinity, composition, and pending functional groups but on antibody isoelectric point, surface chemistry, and pH conditions. Although physical adsorption may show low reproducibility and low stability at different pH conditions, good results have been reported regarding antibodies' orientation (Oliveira et al., 2019). Covalent coupling depends on the NPs' surface and ligand reactive groups. Linking ligands to NPs by covalent bonding, such as carbodiimide chemistry, offers high stability despite possible aggregation, polymerization, and random antibody orientation, thus affecting the accessibility of the antigen-binding sites (Oliveira et al., 2019).

This work aims to develop a new therapeutic strategy based on nanoencapsulation of a therapeutic agent into NPs with high specificity toward macrophages to fight intracellular infections (Scheme 1A), with antifungal itraconazole (ITZ) as a hydrophobic drug model (Scheme 1B). This drug is commonly prescribed to patients with serious fungal infections such as histoplasmosis, an infection produced by the etiological agent *H. capsulatum*. This fungus, together with *Pneumocystis* spp, is currently associated with the development of coinfections in HIV-positive patients, whose presence in some geographical regions is even higher than that of bacteria such as *M. tuberculosis* (Agudelo et al., 2012; Caceres et al., 2018; Carreto-Binaghi et al., 2019). By encapsulating ITZ in NPs, we expect to reduce the limitations related to its high lipophilicity and low absorption ability (Ling et al., 2016; Alhowyan et al., 2019; Biswaro et al., 2019). ITZ nanoformulations include nanocrystals (Wan et al., 2018), NPs (Alhowyan et al., 2019; Jana et al., 2019), and solid lipid nanoparticles (Kim et al., 2010), among others (Hong et al., 2006; Chen et al., 2008; Sharma et al., 2016; Karashima et al., 2017). However, few studies have addressed directing functionalized NPs toward macrophages. The current study develops a biocompatible formulation of ITZ encapsulated into PLGA NPs with optimal colloidal properties regarding size, moderate polydispersity, and surface charge and optimal DLC and EE for adequate ITZ release (Scheme 1B). NPs were further functionalized with the F4/80 antibody and mannose by physical adsorption and chemical



SCHEME 1 | Functionalized NPs' specific interaction of the F4/80 antibody (in red) with macrophages' F4/80 receptor (in blue) through non-covalent interactions (hydrogen bonding, Van der Waals, and hydrophilic interactions) with the N-terminal region of the receptor, composed of six EGF-like domains (Lin et al., 2010) **(A)**, and core–shell–like type schematic representation of the components of the self-assembled nanocarriers with the optimized ITZ-PLGA-TPGS-pH5 formulation without the targeting ligand **(B)**.

coupling for targeted cargo delivery into macrophages (Scheme 1A), demonstrating efficacy in eliminating the *H. capsulatum* fungus. To the best of our knowledge, this is the first report showing that F4/80-functionalized NPs can help improve macrophage-targeted therapy and with similar efficiency to that of mannose-coupled NPs. Therefore, functional nanocarriers could be a platform for drug encapsulation as a promising therapeutic alternative to fight infectious diseases.

MATERIALS AND METHODS

Chemicals

Poly (lactic acid-co-glycolic acid) (PLGA); LA:GA 75:25 (RG 752H) with an inherent viscosity of 0.14–0.22 dl/g (4–15 kDa) and PLGA; LA:GA 50:50 (RG 503H) with an inherent viscosity of 0.32–0.44 dl/g (24–38 kDa) were generously donated by Evonik (Essen, Germany). Sigma-Aldrich provided Nile red (CAS 7385-67-3), itraconazole (ITZ, CAS 84625-61-6), poloxamer 188 (CAS 9003-11-6, Kolliphor®), D-α-tocopherol polyethylene glycol 1,000 succinate (vitamin E-TPGS, CAS 9002-96-4), voriconazole (CAS 137234-62-9), and phosphate buffer saline (PBS D8573). D-mannose (CAS 3458-28-4), Kit MTT TOX-1, HMM broth (nutrient media F12 HAM, N6760), Janus Green (CAS 2869-83-2), tween 80 (CAS 9005-65-6), sodium periodate (CAS 7790-28-5), Hoechst (CAS 23491-45-4), and ethylenediamine (EDA, CAS 107-15-3) were purchased from Sigma-Aldrich (St Louis, MO, United States). Dulbecco's modified Eagle medium (DMEM, Ref. 10569010) and penicillin–streptomycin (Ref. 15140122) were purchased from Gibco (Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, United States). Ethyl acetate (CAS 141-78-6), ethanol (CAS 64-17-5), acetonitrile (CAS 75-05-8), and dimethyl sulfoxide (DMSO, CAS 67-68-5) were bought from Merck. Sucrose (CAS 57-50-1) and citric acid (CAS 77-92-9) were purchased from VWR Chemicals. Sodium chloride (CAS 7647-14-5), potassium chloride (CAS 7447-40-7), and potassium phosphate monobasic (CAS 7778-77-0) were provided by J. T. Baker (J.T. Baker, Thermo Fisher Scientific, Inc., Waltham, MA, United States). Sodium citrate dihydrate (CAS 6132-04-3) and sodium phosphate dibasic (CAS 7558-79-4) were acquired from Panreac. F4/80 antibody (ab100790), IgG isotype (ab171870), and Alexa flour 488 (ab150077) were obtained from Abcam (Abcam, Cambridge, United Kingdom). Brain–heart infusion (BHI) was obtained from Difco Laboratories (Difco Laboratories, Ref. DF0418, Thermo Fisher Scientific, Inc., Waltham, MA, United States). Fetal bovine serum (FBS) was acquired from Trynyty TEK (01010102, Cartagena, CO). 0.1 M citrate buffer (pH 4.5–5.5), 50 mM MES buffer (pH 6.0–6.5), 50 mM HEPES buffer (pH 7.0), and 0.1 M PBS buffer (pH 6.5) were prepared by dissolving the reagents as received in Milli-Q water and filtrating using a 0.2-μm filter.

Microorganisms and Cell Lines

Histoplasma capsulatum strain CIB 1980 was isolated from a human clinical case and deposited in the collection of the Experimental and Medical Mycology Group, CIB, Medellín-Colombia. Yeasts were grown in BHI broth and supplemented with 0.1% L-cysteine and 1% glucose at 37°C with aeration on a mechanical shaker (150 rpm, Innova® 44, Thermo Fisher Scientific, Inc., Waltham, MA, United States) and routinely collected during the exponential growth phase (at 48 h). Fungal growth was collected and passed 20 times through a tuberculin syringe with a 1/2-inch needle to eliminate clumped fungal cells. Viability and number of yeasts were determined by staining with Janus Green. The fungal unit was considered to be equivalent to a single cell or mother cell with 2–7 daughter cells. Fungal cells were counted in a hemocytometer and resuspended in HMM culture medium to obtain the desired number. Murine macrophages J774A.1 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, United States). Cells were cultured in suspension in DMEM medium supplemented with 10% FBS and 1% penicillin–streptomycin at 37°C with 5% CO_2. Cells were passaged every 2–3 days. Viability was determined by visual microscopic inspection of the nuclei stained with trypan blue.

Nanoemulsion Formulations

NPs were prepared using the high-energy emulsification method using a variety of PLGA polymer compositions, that is, different glycolic acid–to–lactic acid ratios (50:50 or 75:25) and molecular weights (24–38 and 7–14 kDa, respectively), namely, Resomer® RG 503H and RG 752H. Briefly, 75 mg of PLGA was dissolved in 5 ml ethyl acetate, and the organic phase was poured into 10 ml 1% Kolliphor® P188 solution under vortex mixing at 3,200 rpm for 20 s. Immediately after mixing, the microemulsion was subjected to three ultrasonication pulses using a 1/8-inch probe at 20% amplitude (Ultrasonicator Q500, Qsonica, Newton, CT, United States) to make the nanoemulsion. Afterward, the nanoemulsion was put in a rotary evaporator under 300 mbar at 150 rpm for 10 min at 40°C (Rotavapor® R-300, Buchi, Germany). The resulting NPs were purified by dialysis with 20% ethanol. Finally, the colloidal dispersion in ultrapure water was lyophilized. To obtain NPs with the proper size, surface charge, drug-loading capacity, and release kinetic, preliminary studies were carried out with various surfactants either in the aqueous or in the organic phases. **Table 1** summarizes the colloidal properties of the formulations studied.

Encapsulation of Model Hydrophobic Compounds Into NPs

Three different polymer-to–active ingredient ratios (1:25, 1:11, and 1:10) were evaluated to encapsulate ITZ in PLGA NPs, which allowed the determination of how the ITZ concentration influenced its encapsulation process. The emulsification method was used as described above. 3.0–7.5 mg ITZ was dissolved in 5 ml of ethyl acetate. A certain quantity of freeze-dried NPs was weighed, dissolved in acetonitrile, and analyzed using a validated HPLC chromatography method in the reverse phase using a C18 column (Eclipse XDB, 150 × 4.6 mm, 5 μm), a UV-VWD detector, voriconazole as internal standard, a 40:60 ratio of water to acetonitrile mobile phase, isocratic mode at 1 ml/min, and a wavelength of 261 nm (Cáceres et al., 2016) to find out

TABLE 1 | Colloidal properties of PLGA NPs with encapsulated ITZ.

Polymer (PLGA)	ITZ (mg)	Size (nm)	PDI	ζ-potential (mV)	DLC (%)	EE (%)
50:50	3	189.9 ± 7.5	0.21 ± 0.01	–50.7 ± 3.3	2.8 ± 0.1	72.1 ± 0.2
	6.6	188.5 ± 3.0	0.23 ± 0.04	–39.9 ± 2.0	4.0 ± 0.2	47.6 ± 3.2
	7.5	272.5 ± 43.3	0.41 ± 0.12	–46.4 ± 2.4	8.2 ± 0.2	89.4 ± 2.8
75:25	6.6	150.4 ± 6.4	0.16 ± 0.02	–40.9 ± 2.9	6.0 ± 0.2	75.2 ± 2.9
50:50-pH5	6.6	217.7 ± 5.5	0.24 ± 0.04	–46.9 ± 1.3	5.6 ± 0.1	68.0 ± 0.3
50:50-TPGS-pH5	6.6	165.0 ± 9.7	0.23 ± 0.05	–38.9 ± 2.1	6.4 ± 0.7	78.3 ± 8.6
75:25-TPGS	6.6	157.7 ± 7.7	0.16 ± 0.01	–38.8 ± 2.1	6.1 ± 1.1	75.0 ± 13.0
75:25-TPGS-pH5	6.6	147.3 ± 7.7	0.19 ± 0.01	–38.0 ± 0.3	6.7 ± 1.3	80.1 ± 11.8

the amount of ITZ loaded into the NPs. As described above, the emulsification method was used to encapsulate Nile red as a model molecule (at a concentration of 120 μM). Quantification of encapsulated Nile red was evaluated by UV–visible spectrophotometry at 520 nm.

Determination of the Encapsulation Efficiency and Drug-Loading Capacity

Determination of DLC and EE in NR-PLGA-NPs and ITZ-PLGA-NPs, respectively, was estimated from the following:

$$\mathrm{DLC} = \frac{\text{Encapsulated mass (NR or ITZ) into nanoparticles (mg)} \times 100\%}{\text{Total mass of nanoparticles (mg)}},$$

$$\mathrm{EE} = \frac{\text{Encapsulated mass (NR or ITZ) into nanoparticles (mg)} \times 100\%}{\text{Mass (NR or ITZ) initially added (mg)}}.$$

Physicochemical Characterization of Nanoparticles

NP size and size distribution (PDI) were obtained by dynamic light scattering (DLS) and ζ-potential (mV) was obtained by electrophoretic light scattering (ELS) in a Zetasizer-pro (Malvern Instruments, United Kingdom) at 25°C after adequate aqueous dilution in triplicate. Transmission electron microscopy (TEM) determined the morphology of the NPs. A drop of the nanoparticle dispersion was added to a copper grid with a carbon membrane and allowed to dry at room temperature to obtain the TEM images (Tecnai F20 Super Twin, FEI). Digital micrograph software was used for image treatment. Thermal analysis was performed by differential scanning calorimetry (DSC) using a TA Instruments Q20 model. The analysis was carried out with the lyophilized nanoparticles loading itraconazole and its precursors, causing thermal deletion before analyzing each sample. The method consisted of weighing 4- to 8-mg samples, sealing them in a hermetic aluminum capsule, and analyzing at temperatures ranging from 20 to 350°C at 10°C/min in a nitrogen atmosphere to obtain the respective thermograms. NPs with the encapsulated drug, functionalized nanoparticles, physical mixture of the precursors, and precursors alone were characterized by FT-IR spectroscopy using the transmittance accessory in Thermo iS50 equipment. Materials were prepared as potassium bromide (KBr) pellets, and the measurements were carried out using 32 scans with a resolution of 4 cm^{-1}. Spectra were collected from 4,400 to 350 cm^{-1} wavenumbers. Before the analysis, the baseline was measured under the same conditions using a pure KBr tablet as a reference.

Drug Release and Stability

For evaluating the ITZ release profile, 3 ml dilution of the original solution of fresh NPs was prepared, diluted in 25 ml of sterile purified water, and centrifuged at 10000 rpm and 4°C for 30 min. After this time, the supernatant was discarded, and the pellet was resuspended in 25 ml of release medium simulating physiological conditions (PBS solution at pH 7.2) containing 1% v/v of tween 80. 1.0 ml of this solution was added to each 1.5-ml tube for a total of 11 analysis times (0, 0.5, 1, 2, 3, 4, 6, 7, 24, 48, and 72 h, respectively). Subsequently, the tubes were incubated in a thermal mixer at 800 rpm and 37°C, and each vial was removed at the selected times. Then, the vials were centrifuged at 10,000 rpm and 25°C for 1 h. The supernatant and the pellet were used for the direct and indirect determination of ITZ by HPLC chromatography.

The stability of NPs from two types of polymers was evaluated after lyophilization and cryopreservation with 5% sucrose. 4 and 25°C were studied as storage temperatures for up to one month. The dispersion of optimized NPs stored at 4°C without lyophilization for one month was also evaluated. Additionally, the stability of dispersion at 37°C was analyzed under the same kinetic release conditions (PBS pH 7.2, tween 1% v/v at 37°C). For this purpose, 20 mg of the NPs was added to 1.5-ml tubes, and the samples were extracted at the beginning and at least five times between the first and fourth evaluation weeks. The lyophilized and dispersed NPs were resuspended in 1 ml of sterile purified water, and size, surface charge, and PDI were evaluated as mentioned above.

Antifungal Susceptibility Testing

The antifungal activity of ITZ loaded into PLGA-NPs was compared against ITZ–aqueous suspensions. The fungal growth inhibition was tested using the microdilution method for yeast (M27-A3) following the National Committee for Clinical Laboratory Standards (NCCLS), with empty PLGA-NPs and each component of the NPs (PLGA, TPGS, and Kolliphor P188) as a control. Yeast suspensions were prepared

in HMM medium adjusted to a concentration of 3×10^5 CFU. Aliquots of 0.1 ml of suspension yeast were added per well. The dilutions of each treatment were prepared in HMM medium with 1% DMSO. Final drug concentrations were 16–0.007 μg/ml for each treatment, using the medium with 1% of solvent without the drug as the control. The plate was incubated at 37°C with aeration on a mechanical shaker (150 rpm, Innova® 44, Thermo Fisher Scientific, Inc., Waltham, MA, United States) for 7 days. When the incubation was finished, the fungal growth was checked by visible turbidity, and fungus viability was determined through the 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) method. All experiments were performed in triplicate.

Immobilization of Antibodies on Poly (Lactic Acid-Co-Glycolic Acid) NPs and Mannose-Coated NPs

Functionalization of NPs with antibodies by physical adsorption and covalent coupling were compared in terms of efficiency. For the physical immobilization of the antibodies on the surface of the PLGA75:25-TPGS-pH5 NPs with encapsulated Nile red, 25 μl of the particle dispersion (1.5 mg/ml in 0.1 M citrate buffer with a pH value between 4.5 and 5.5, 50 mM MES buffer with a pH value between 6.0 and 6.5, 0.1 M PBS with a pH value of 6.5, or 50 mM HEPES buffer with a pH value of 7.0) was diluted with 4 ml of MES buffer. The NP dispersion was poured dropwise into 0.36 mg/ml anti-F4/80 antibody, depending on the 1:5, 1:10, and 1:20 ratios, dissolved in 50 or 300 μl of the buffer according to the pH to be evaluated. This solution was incubated at room temperature for 4 or 12 h or at 4°C for 24 h under constant shaking. A button of functionalized NPs was obtained by ultracentrifugation at 10,000 rpm at 4°C for 30 min, followed by two washing steps with 600 μl of water by 10,000-rpm ultracentrifugation at 4°C for 30 min. The final button was diluted to a final volume of 17 μl with sterile 1X PBS.

The covalent attachment of antibodies to the surface of PLGA NPs was by the EDC/NHS coupling reaction, using a two-step process involving the activation of a carboxyl group and subsequent conjugation with primary amines. PLGA75:25-TPGS-pH5 NPs with encapsulated Nile red were diluted to 1.5 mg/ml in 50 mM MES buffer (pH 6.5). 23 mg (100 mM) of NHS and 153 mg (400 mM) of EDC-HCl were dissolved in 1 ml 50 mM MES buffer (pH 6.5), added to 1 ml of this dispersion, and shaken at 800 rpm at room temperature for 30 min. The reaction mixture was then washed three times with MES buffer by centrifugation at 10,000 rpm for 30 min to remove the unreacted material and diluted to a final volume of 2 ml with 10 mM PBS (pH 6.5). 5.7 μl of the activated NP dispersion (6.5 mg/ml) was diluted in 50 μl PBS (pH 6.5). 10.4 μl of F4/80 (0.36 mg/ml) or 3.75 μl of mouse IgG (1 mg/ml) were dissolved in 50 μl of PBS. The NP dispersion was added dropwise to the antibody solution, and the combined reaction mixture was shaken at 4°C for 2 h. The functionalized NPs were then washed twice with 10 mM PBS (pH 7.2) by centrifugation at 3,000 rpm for 30 min. The final volume of the dispersion was adjusted to 17 μl with 10 mM PBS (pH 7.2).

Mannose-covered NPs were prepared based on previous works (Ghotbi et al., 2011), with modifications. Briefly, 3 mg of each PLGA NP was resuspended in 2 ml of MES (pH 6.5) and mixed with 500 μl 0.1 M NHS and 500 μl 0.4 M DCC. After 30 min, 0.2 μl of EDA was added and incubated for 2 h. Simultaneously, the D-mannose ring was opened by treating 3 mg of it with 10 mM $NaIO_4$ solution and incubated under constant agitation for 30 min at room temperature. Then, the mannose was added to the solution with PLGA NPs and incubated for 12 h at room temperature (RT) under agitation at 800 rpm. Afterward, the NPs were washed with 10 mM PBS through centrifugation at 10,000 rpm at 4°C for 30 min thrice to remove the excess EDA and unreacted mannose.

Detection of Antibodies on NPs

4, 10, 20, and 30 μg/ml goat antiRabbit IgG Alexa Fluor 488 (AF 488) were added to the solution of functionalized NPs. 1X sterile PBS was added until the final volume of 20 μl was reached. This solution was incubated at room temperature for 30 min under constant shaking. Once the incubation was finished, it was brought to a final volume of 200 μl to measure them by flow cytometry or fluorescence spectrophotometry, respectively. A calibration curve was made with different concentrations of AF488 between 25 and 0.195 μg/ml, the fluorescence intensity values were plotted against concentration (**Supplementary Figure S4A,S4B**), and the values of the unknown antibody concentrations were calculated from the straight-line equation.

Study of Protein Corona Formation

The functionalized NPs were incubated with 10–100% fetal bovine serum at 37°C for 1 h to allow protein adsorption in a final volume of 20 μl, and then, 3.4 μl was taken to perform size and surface charge measurements as previously stated. The functionalized NPs gestated with the protein corona were incubated with 20 μg/ml of secondary AF 488 antibody at room temperature for 30 min, brought to a volume of 100 μl with sterile PBS, and measured in terms of fluorescence intensity by spectrophotometry to evaluate the accessibility of the primary F4/80 antibody after the protein corona was formed (Tonigold et al., 2018).

In vitro Assay of Specificity

5×10^4 J774.1A cells were adhered onto a 96-well plate for 16 h in DMEM with 10% BFS. Then, 1.5 μg/ml of functionalized NPs using the method that presented a higher degree of functionalization or uncoated NPs were added and incubated at 37°C for 3 h with 5% CO_2. Therefore, monolayers were washed three times with PBS that was previously tempered to eliminate no endocytosed NPs. The cell cultures were characterized by fluorescence microscopy. Determination of Nile red inside the cells (area of Nile red) was measured indirectly to find out the differences in ligands' specificity. To corroborate the NPs' uptake, we used Hoechst dye to stain cells' nuclei and colocalize the NPs. The area of Nile red (%) was determined using ImageJ software version 1.48 (National Institutes of Health). Ten images were captured using a 40X objective and analyzed randomly from different regions. Individual cells and Nile red areas were framed

with freehand selection to measure the inner region. These areas were taken as the total area, and the area of Nile red was calculated using the Microsoft Excel 2013 package using the data (Mejía et al., 2015).

Experimental Design and Statistical Analysis

A Plackett–Burman type screening experimental design was used randomly with three central points in duplicate, and the statistical package Statgraphics® Centurion XVII was used to evaluate the nanoemulsion method's critical parameters to get a total of 15 tests. All statistical analyses used GraphPad Prism software (version 8.0); normal distribution was determined using ANOVA and verified using the Kolmogorov–Smirnov normality test. According to the Gaussian distribution of data, differences between groups were analyzed using Student's t-test or the Mann–Whitney test. A p-value ≤ 0.05 was considered to be statistically significant.

RESULTS

Preparation and Characterization of NPs

A rational Plackett–Burman experimental design screened the most important factors influencing nanocarriers' self-assembly, using the nanoemulsion method with Nile red as a hydrophobic molecule model. The design consisted of fifteen experiments that evaluated the influence on the NPs' size, PDI, and surface charge as response variables. The factors evaluated were concentrations of PLGA (5–15 mg/ml), Nile red (20–40 μM), and surfactant (3–5%), sonication amplitude (50–80%), time (30–40 s), and organic phase–to–water phase ratio (v/v; 1:2, 1:1.5, and 1:1). The process was studied with PLGA of two different compositions (50:50 or 75:25 glycolic acid–to–lactic acid ratio) and molecular weights (24–38 and 7–14 kDa). Optimal experimental conditions were established to assemble PLGA nanocarriers with a size of 200 nm, PDI <0.3, and ζ-potential ≤−30 mV, which are considered adequate for our purpose. From the experimental design, the optimal conditions were 15 mg/ml PLGA, 30 μM Nile red, 3% surfactant, 20% sonication amplitude, 30 s sonication time, and 1:2 organic-to-water phase ratio, in which the diameter of NPs from PLGA 50:50 (148.2 ± 23.3 nm) was larger than that of PLGA 75:25 NPs (119.9 ± 18.1) (**Supplementary Figure S1A**). This behavior is explained by the increase in the inherent viscosity of the PLGA 50:50 system (0.32–0.44 dl/g), with a higher molecular weight than that of PLGA 75:25 (0.14–0.22 dl/g). Nanocarriers from PLGA 50:50 had a particle size distribution with a slightly lower polydispersity (0.12 ± 0.02) than nanocarriers from PLGA 75:25 (0.16 ± 0.04). There were no statistically significant differences between the ζ-potential values, those being negative in both systems (−34.5 ± 9.0 and −32.9 ± 6.8 mV) for NPs from PLGA 50:50 and 75:25, respectively.

At the optimized conditions, named from now on as "base formulation," the influence of the PLGA-to-ITZ ratio on the physicochemical properties, DLC, and EE was studied. Nanocarriers were prepared from PLGA 50:50 by changing the ITZ weight (3, 6.6, and 7.5 mg) but keeping constant the PLGA weight (15 mg/ml) to obtain different ITZ-to-PLGA ratios (1:25, 1:11, and 1:10). HPLC achieved quantification of ITZ using the isocratic method to determine DLC and EE. DLC (8.2 ± 0.2%) and EE (89.4 ± 2.8%) of the 1:10 ITZ-to-PLGA system increased significantly with respect to the 1:25 ITZ-to-PLGA system, which has a DLC of 2.8 ± 0.1% and an EE of 72.1 ± 0.2%. However, the particle size increased to 272.5 ± 43.3 nm with a PDI of 0.4 ± 0.1. Such formulation was tested with NPs of PLGA 75:25, showing proper size (150.4 ± 6.4 nm) and suitable properties (−40.9 ± 2.9 mV, PDI 0.16 ± 0.02, DLC 6.0 ± 0.2%, and EE 75.2 ± 2.9%) (**Figure 1A**; **Table 1**). The size, distribution, degree of aggregation, and morphological homogeneity of the NPs were studied by TEM analysis of air-dried unstained samples showing low and high magnification images (**Figure 1B**; **Supplementary Figure S2A**). The particle dense core image of the as-assembled NPs presented spherical and quasi-spherical shape morphology with a smooth surface, absence of vesicular structures, and low degree of aggregation or coalescence. NPs presented an average particle size of 132.0 ± 36.6 nm (PLGA 75:25) and 145.0 ± 28.2 nm (PLGA 50:50) (**Figure 1B**; **Supplementary Figure S2A**), with n = 30, respectively.

FT-IR and DSC characterized the nanoencapsulation process to determine ITZ–PLGA interactions. **Figures 1C,E** show the FT-IR spectra of the individual precursors (a–c), the physical mixture (d), and NPs without (e) and with (f) encapsulated ITZ in both types of polymers. The physical mixture shows signals mostly from the contribution of peaks at 1,767 cm^{-1} (C=O stretching) from PLGA polymers, a little peak at 1,697 cm^{-1} (C=O stretching) from the amide group of ITZ, and at 2,844 cm^{-1} (C–H stretching) and 1,106 cm^{-1} (C–O stretching) from the stabilizer. The physical mixture pattern changed in the NPs with encapsulated ITZ (f) with respect to empty NPs (e), showing a peak at 1767 cm^{-1} (C=O stretching) of higher intensity. The ITZ characteristic peaks at 1,697 cm^{-1} (C=O stretching from the amide group), 1,520 cm^{-1} (C=N stretching), and 1,232 cm^{-1} (C–N stretching) could not be observed in NPs with encapsulated ITZ, thus indicating PLGA–ITZ interactions. **Figures 1D,F** show the DSC analysis of the individual precursors (a–c), the physical mixture (d), and NPs without (e) and with (f) encapsulated ITZ in both types of polymers. For example, the thermograms 1 F show the glass transition temperature and the single endothermic melting peak at 49.1 and 55.9°C from the PLGA 75:25 polymer (a) and the surfactant Kolliphor P188 (c), respectively. A single-phase transition at 53.8°C was observed for the physical mixture of the components. In comparison, free ITZ (b) shows a single endothermic melting peak at 169.9°C, typical of a drug in the crystalline form, whereas empty NPs (e) display a single endothermic melting peak at 53.1°C. Remarkably, the melting peak from ITZ completely disappeared in the NP-encapsulated ITZ thermogram, thus indicating that PLGA and ITZ might be interacting through the triazole group (or the amine group) of ITZ and the carboxylic group of the PLGA hydrophobic tail (Yi et al., 2007). A similar analysis is derived from the thermogram in **Figure 1D** for PLGA 50:50.

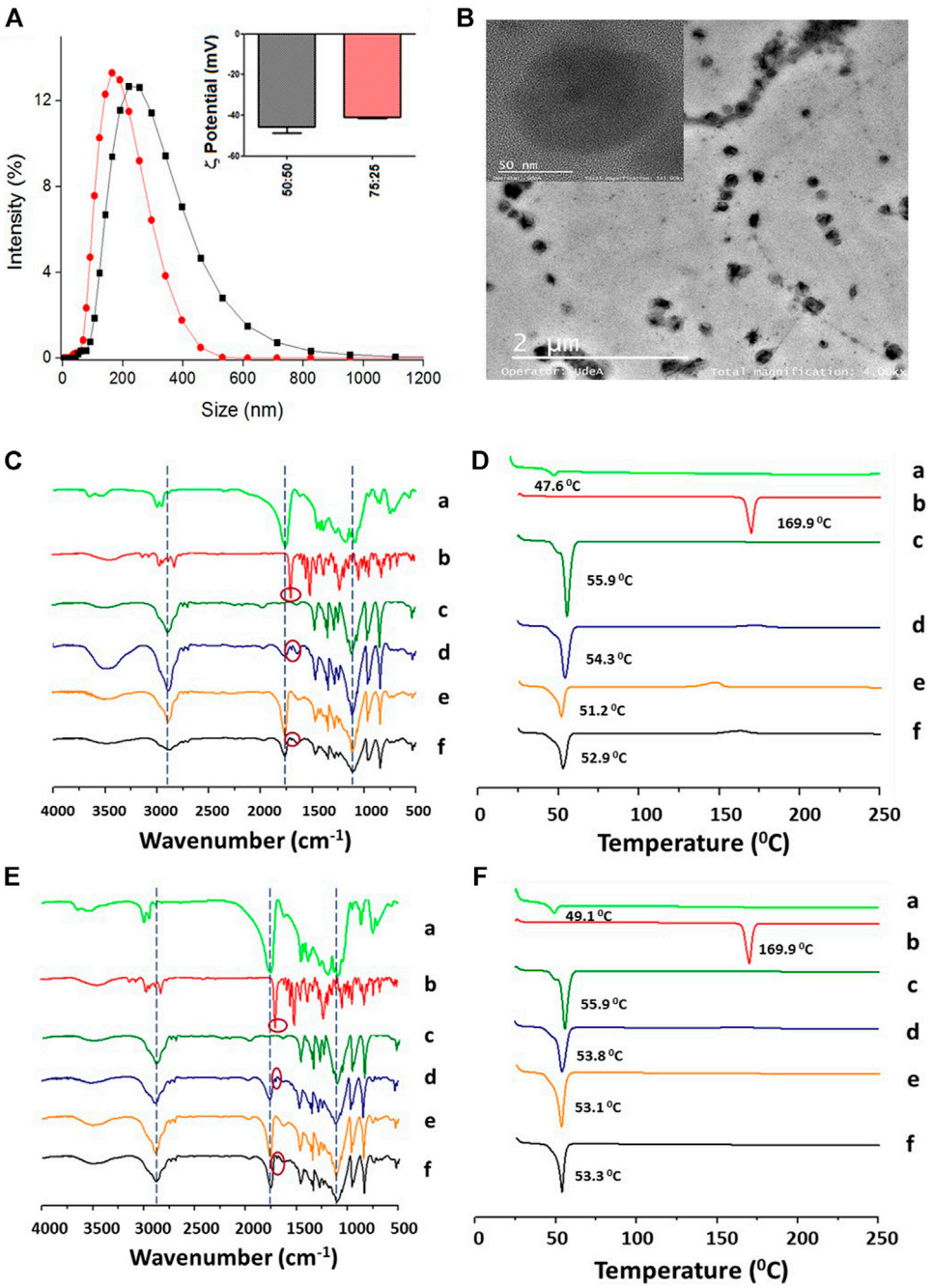


FIGURE 1 | Physicochemical characterization of the PLGA NPs (base formulation) with encapsulated ITZ. **(A)** Hydrodynamic size and ζ-potential of PLGA 50:50 (black line and bars) and PLGA 75:25 (red line and bars). **(B)** Morphologic characterization of PLGA 50:50 NPs by TEM. **(C, D)** Characterization of PLGA 50:50 and **(E, F)** PLGA75:25 NPs, respectively, by FT-IR **(left)** and DSC **(right)**. PLGA (a), ITZ (b), Kolliphor P188 (c), physical mixture of 1:11:37.9 ITZ-to-PLGA-to–Kolliphor P188 weight ratio (d), and NPs from the mixture without (e) and with (f) encapsulated ITZ.

The next set of experiments introduced some base formulation modifications to improve its physicochemical and structural properties, evaluated by the DLC, EE, and drug-release profile. Modifications consisted of lowering the pH of the aqueous solution to five and including a multipurpose amphiphilic excipient such as vitamin E-TPGS in a 1:10 ratio by weight with respect to PLGA, one at a time. When ITZ was encapsulated in PLGA 50:50 NPs at a pH value of 5, the DLC (5.60 ± 0.03%) and EE (68.0 ± 0.3%) increased with regard to NPs at neutral pH (**Table 1**), with other characteristics within the expected values (217.7 ± 5.5 nm, 0.24 ± 0.04, and −46.9 ± 1.3 mV) for size, PDI, and ζ-potential, respectively. Then, a combination of surfactants

(Kolliphor P188 and vitamin E-TPGS) at different concentrations was assessed. The resultant NPs showed an enhanced DLC (6.1 ± 1.1%) and EE (75.0 ± 13.0%) compared to the base formulation (**Table 1**), with other characteristics at optimal values (157.7 ± 7.7 nm, 0.16 ± 0.01, and −38.8 ± 2.1 mV) for size, PDI, and ζ-potential, respectively. When the mix of surfactants at the pH value of 5 was assessed altogether, both polymers (PLGA 50:50 and PLGA 75:25) showed improved DLC and EE (**Table 1**), moderate polydispersity, and an adequate size and surface charge. Similarly, an optimized formulation with PLGA 75:25 and the mix of surfactants (Kolliphor P188 and vitamin E-TPGS) at the pH value of 5 showed an increase in the DLC and EE values with regard to the base formulation, going from 6.0 to 6.7% of DLC and from 75.2 to 80.1% of EE, maintaining similar characteristics of size, distribution, and surface charge.

To investigate the interaction of ITZ with the components present in the NPs with optimized formulation, the chemical composition and physical changes of the materials were studied by FT-IR and DSC, respectively. **Figures 2C,E** show the FT-IR spectra of the individual precursors (a–e), the physical mixture (f), and NPs without (g) and with (h) encapsulated ITZ in both types of polymers. The physical mixture shows mostly the peaks at 1,767 cm^{-1} (C=O stretching) from the PLGA polymer, a little peak at 1,697 cm^{-1} (C=O stretching) from the amide group of ITZ and a small band of carbon–carbon double bonds at 1,511 cm^{-1}, and at 2,844 cm^{-1} (C–H stretching) and 1,106 cm^{-1} (C–O stretching) from the stabilizer. The physical mixture pattern changed in the NPs with respect to empty NPs (e) and encapsulated ITZ (f), showing a peak at 1,760 cm^{-1} (C=O stretching) of higher intensity. The ITZ characteristic peaks at 1,697 cm^{-1}(C=O stretching from the amide group), 1,520 cm^{-1} (C=N stretching), and 1,232 cm^{-1} (C–N stretching) could not be observed in NPs with encapsulated ITZ. **Figures 2D,F** show peaks of Tg and endothermal fusion peaks for PLGA polymers, ITZ, and Kolliphor P188 (a–c), as previously mentioned regarding the DSC analysis for the optimized formulation. Endothermal fusion peaks at 36.87°C for vitamin E-TPGS (d) and for the physical mixture of sodium citrate and citric acid (2:1) at 160 and 195.38°C (e) were observed. Besides, using the PLGA 75:25 polymer (**Figure 2F**), a unique phase transition was observed for the physical mixture (f), blank NPs (g), and encapsulated ITZ NPs (h) at 52.4, 50.0, and 50.1°C, respectively. A similar analysis is derived from the thermogram in **Figure 2D** for PLGA 50:50 optimized formulation (Yi et al., 2007).

In vitro Release Kinetic Models

The ITZ release kinetics from the nanocarriers with the optimized formulation was evaluated in a release medium containing 1% v/v tween 80 to ensure infinite "sink" dilution conditions and emulate physiological conditions (PBS with a pH value of 7.2 at 37°C). The kinetics was determined by obtaining the fraction of ITZ released at a certain time (M_t/M_0) for 72 h by HPLC. **Figure 3A** shows a kinetic profile of similar ITZ release, with a maximum ITZ release of 46 and 43% from the PLGA50:50-TPGS-pH5 and PLGA75:25-TPGS-pH5 nanocarriers, respectively, in agreement with reports in the literature (Ling et al., 2016; Varga et al., 2019). Data were adjusted using mathematical models searching for the best fitting by analyzing the correlation coefficient and other parameters related to the studied models (**Supplementary Table S1**) to inquire about the ITZ-release mechanism's optimized formulation. It was found with regard to both types of particles that the release kinetics did not fit the zero-order, Higuchi, or Korsmeyer–Peppas models, as judged by their low correlation coefficients. The adjustment of the release profiles employing the Lindner and Lippold model to evaluate the "burst" effect presented relatively low values of correlation coefficients; therefore, the studied profiles did not fit this model either. However, when the systems were evaluated using the Ritger–Peppas and Peppas–Sahlin models, the correlation coefficients were closer to unity, showing a better fitting.

Nanocarrier Stability

The stability of the PLGA-TPGS-pH5 optimized nanocarriers was evaluated in aqueous suspension at 4°C and under the release kinetics conditions, that is, PBS (pH 7.2), with 1% v/v tween at 37°C. **Figure 3B** shows that during the month of evaluation, aqueous dispersions of the formulations stored at 4°C presented high physical stability in terms of their particle size, with a variation of less than 5.5% of the relative standard deviation (RSD) (upper plot), moderate polydispersity (middle plot), and a medium-to-high negative potential (bottom plot). Similarly, **Figure 3C** shows the suspension under release kinetics conditions for both types of NPs. It demonstrated high colloidal stability during the 14 days of evaluation with particle size variations less than 10% of the RSD (upper plot), moderate polydispersity (middle plot), and medium-to-high negative potential (bottom plot). Due to PLGA75:25-TPGS-pH5 NPs presenting optimal characteristics (size, PDI, and ζ-potential) and higher DLC, EE, stability, and reproducibility, they continued to the *in vitro* studies.

Antifungal Activity

The minimum inhibitory concentration (MIC) of both free and encapsulated ITZ in the optimized nanocarriers was determined with a Colombian strain of *H. capsulatum* (CIB 1980). For this purpose, different concentrations ranging from 16 to 0.015 μg/ml of free and encapsulated ITZ in PLGA75:25 TPGS-pH5 NPs and empty NPs (as a control) were evaluated. The MIC was found to be 0.031 and 0.061 μg/ml for free and encapsulated ITZ using the macroscopic turbidity method (data not shown) and MTT assays (**Supplementary Figure S3**). The empty NPs at the high concentration (16 μg/ml) showed a slight inhibition of the fungal growth. The control with each component of NPs did not inhibit the fungal growth (data not shown). On the other hand, the mean inhibitory concentration (IC50) was estimated to be 0.031 μg/ml with both treatments (**Figure 4A**).

Functionalization of Nanocarriers

The effect of pH, temperature, incubation time, and antibody-to-NP ratio of PLGA75:25 TPGS-pH5 NPs on the functionalization efficiency was evaluated by flow cytometry. It was observed that at pH 4.5 and 7.0, the NPs increased in size and PDI, suggesting agglomeration processes. At such extreme pH, peaks were

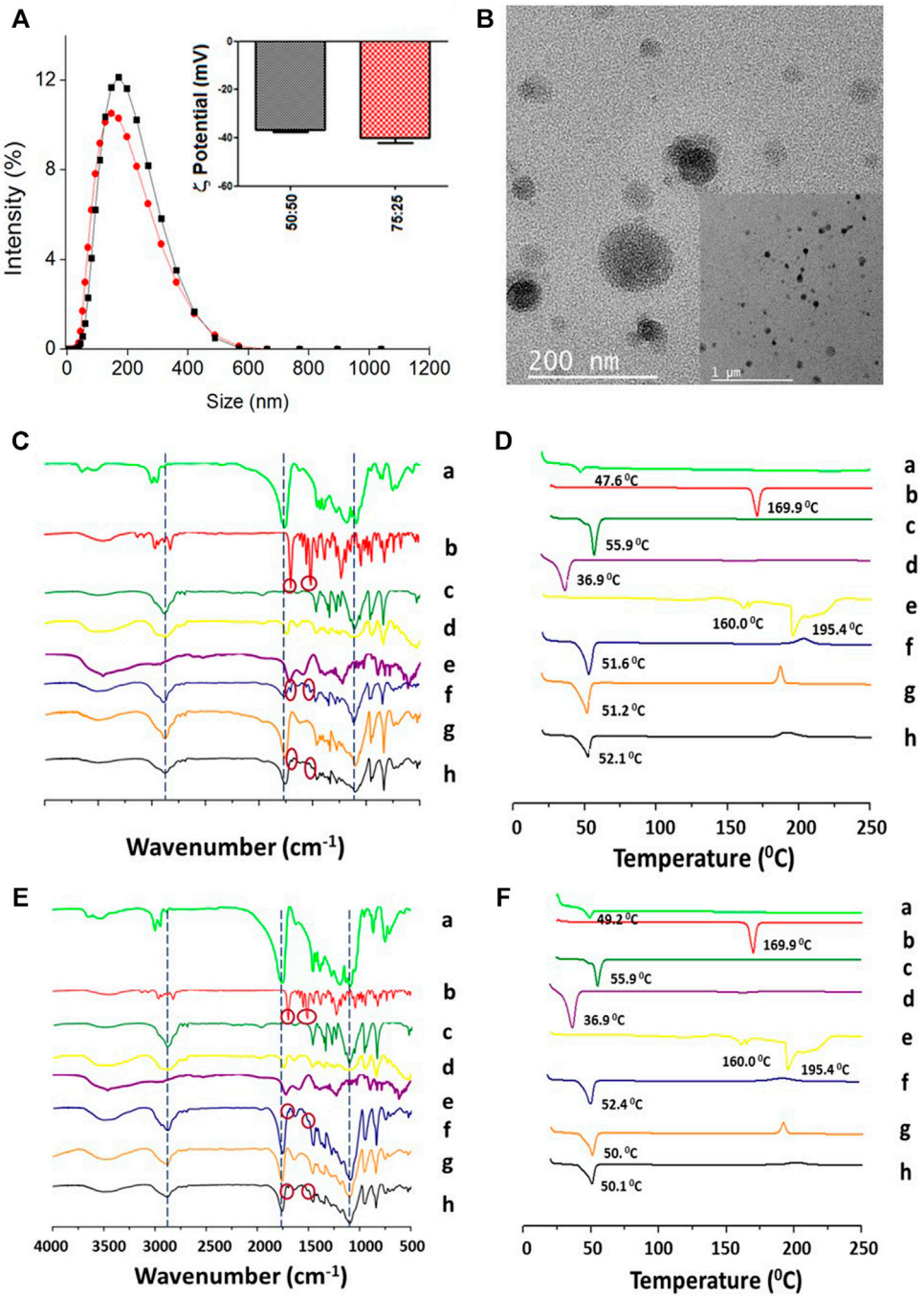


FIGURE 2 | Physicochemical characterization of the PLGA-TPGS-pH5 (optimized formulation) NPs with encapsulated ITZ. **(A)** Hydrodynamic size and ζ-potential of PLGA 50:50 (black line and bars) and PLGA 75:25 (red line and bars). **(B)** Morphologic characterization of PLGA 50:50 NPs by TEM. **(C, D)** Characterization of PLGA 50:50 and **(E, F)** PLGA75:25 NPs, respectively, by FT-IR **(left)** and DSC **(right)**. PLGA (a), ITZ (b), Kolliphor P188 (c), vitamin E-TPGS (d), sodium citrate–citric acid mixture (2:1) (e), physical mixture of 1:11:14.2:1.1 ITZ-to-PLGA-to–Kolliphor P188–to–vitamin E-TPGS weight ratio (f), and NPs from the mixture without (g) and with (h) encapsulated ITZ.

generated in both negative and positive voltages, which could indicate the agglomeration and/or precipitation of non-absorbed and possibly denatured antibodies and/or the destabilization of NPs (data not shown). A significant decrease in ζ-potential was evident at 12 h, which could indicate a decrease in the stability of the NPs (data not shown). Overall, the optimal conditions for the adsorption method were 1:10 antibody-to-NP ratio (**Figure 4B**), a pH value of 6.5 (**Figure 4C**), and 24 h of incubation at 4°C (**Figure 4F**), increasing the size but maintaining a moderate polydispersity of 0.3 with a remained negative surface charge

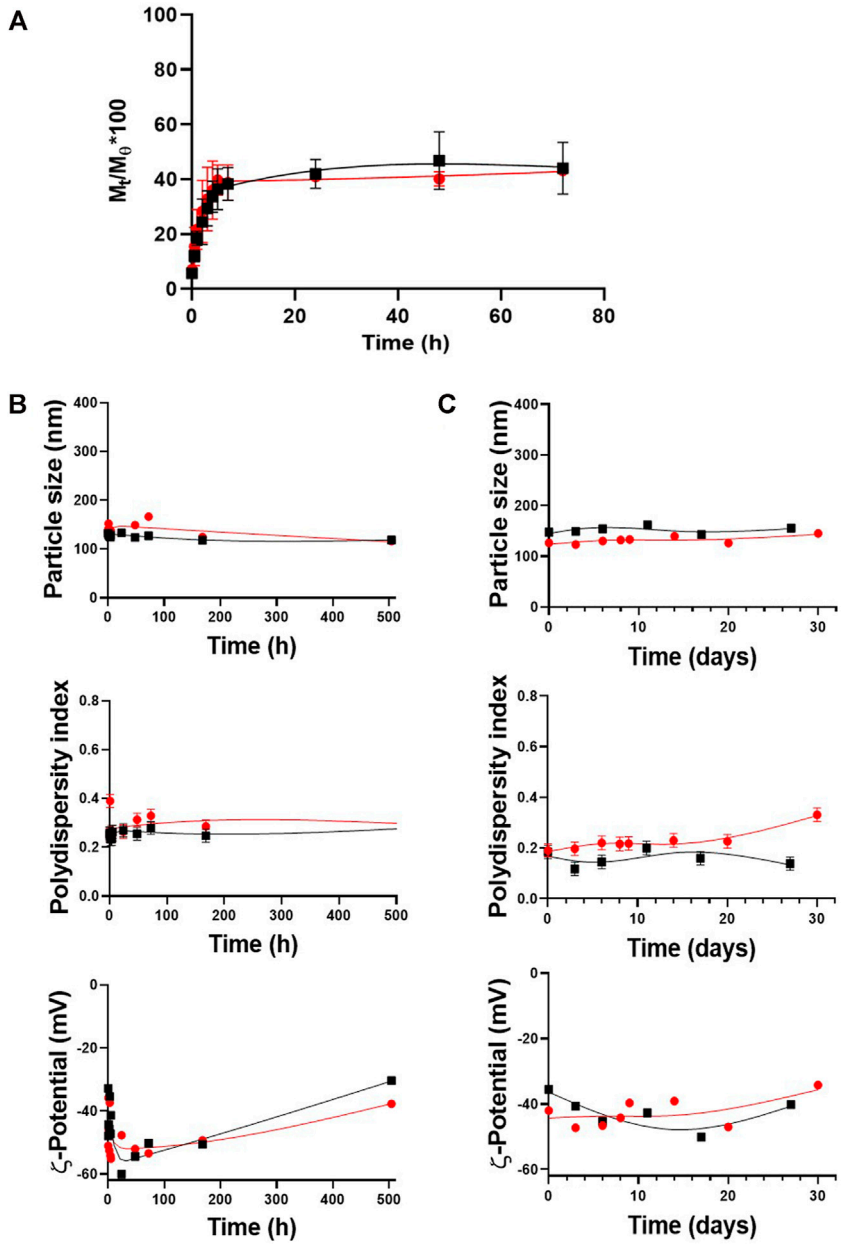


FIGURE 3 | Release kinetics and stability of the optimized formulation upon time. **(A)** Release profiles for PLGA50:50-TPGS-pH5 and PLGA75:25-TPGS-pH5. Stability of PLGA50:50-TPGS-pH5 (black squares) and PLGA75:25-TPGS-pH5 (red circles) in terms of particle size, PDI, and ζ-potential **(B)** stored for one month at 4°C in an aqueous dispersion and **(C)** under the same kinetic release conditions (PBS with a pH value of 7.2, tween 1% v/v at 37°C). The error bars indicate the standard deviation of three successive measurements.

of −46 mV, indicating colloidal stability. A maximum extent of 1.48% of marked NPs was the highest value compared with samples functionalized at other conditions (**Figure 4D**). Furthermore, the optimal secondary antibody concentration was determined to be 20 μg/ml (**Figure 4D**).

For covalent coupling, available carboxyl groups were first semi-quantified through esterification with EDC/NHS, followed by FTIR analysis. **Supplementary Figure S5A** shows the FTIR spectrum of the esterified NPs (NP_PLGA_75: 25_Ester), unesterified NPs (NP_PLGA_75: 25), and EDC, NHS, and urea (which can be reaction residues) (Wang et al., 2011). The esterified NPs did not show the characteristic peaks of NHS, EDC, or urea, which indicates that the reaction was efficient and that the NPs' washing process was adequate. Furthermore, esterified NPs showed a decrease in the area under the curve of the peak at λ = 3,492 cm^{-1}, corresponding to the OH group, and an increase in the peak at λ = 1,758 cm^{-1}, corresponding to the RC = O group. This fact is explained by the presence of the C= O attached to the

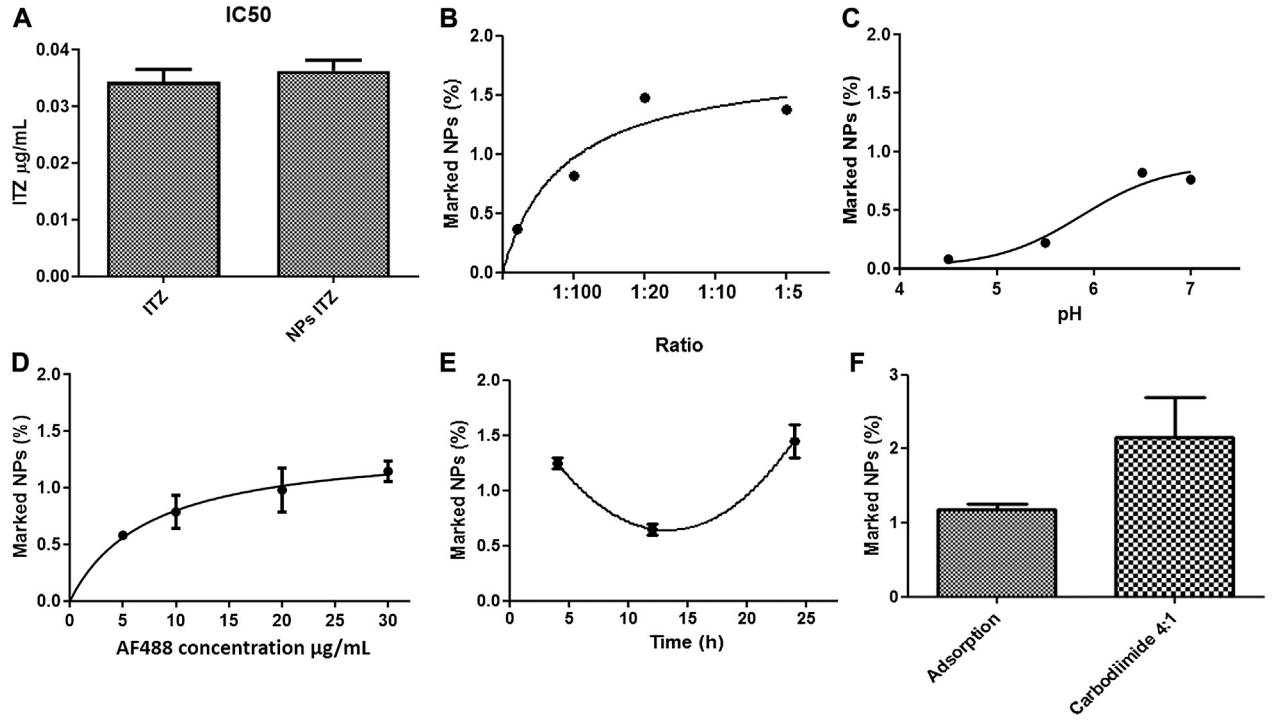


FIGURE 4 | (A) IC50 of *Histoplasma capsulatum* treated with free itraconazole and itraconazole encapsulated into PLGA-TPGS-pH5 NPs. Effect of **(B)** pH, **(C)** antibody-to-NP ratio, **(D)** concentration of the secondary antibody, **(E)** incubation time in the adsorption of anti-F4/80 on NPs, and **(F)** evaluation of the functionalization method characterized by spectrophotometry (Ex: 490 nm–Em: 520 nm).

cyclic chain. By measuring the areas under the curve of the esterified NPs (final areas) with respect to the control (initial areas) (**Supplementary Figure S5C**), the efficiency of the reaction could be estimated and related to the extent of activated carboxyl groups on the NP surface. About 40% of the carboxyl groups in NPs were available for activation and covalent antibody anchoring. The antibody's coupling was assessed by the carbodiimide chemistry, obtaining 2.7% of functionalized NPs under the optimized conditions using the adsorption method based on protocols reported in the literature (**Figure 4F**).

Formation of a Protein Corona

As an initial analysis of the protein corona formation at the outermost functionalized NP surface, depending on the functionalization method, they were incubated with 10–100% of FBS at 37°C for 1 h to simulate circulation conditions in the body. The nanoparticles modified using the adsorption method presented a larger size than NPs modified using the carbodiimide chemistry. With a concentration of 10% of FBS, the NPs of both methods tended to ±260 nm in size (**Supplementary Figure S6A**). The ζ-potential showed a significant decrease when modified using the adsorption method (only with 10% FBS), going from −58 to −37 mV, compared with that from covalent coupling that did not show a drastic change in the surface charge, going from −37.2 to −33.85 mV (**Supplementary Figure S6B**). Finally, when analyzing whether there was masking of the anti-F4/80 by the protein corona, there was no significant change in the anti-F4/80 functionalization efficiency under adsorption, varying from 0.92 to 0.98%. In contrast, the covalent coupling presented a significant decrease from 1.88 to 0.98% of anti-F4/80 at the NP surface (**Supplementary Figure S6C**).

Specificity for Macrophages

Nile red was used as a model of the hydrophobic compound to evaluate the functionalized NPs' specificity due to its high hydrophobicity (solubility in water <1 µg/ml), relatively high molecular weight (318.37 g/mol), simple detection (UV–visible spectroscopy and fluorescence microscopy), and high photostability. Fluorescence microscopy was used to evaluate the specificity of functionalized NPs by J774A.1 macrophages. While NPs functionalized with the F4/80 antibody or D-mannose by covalent coupling were evaluated and compared, expected to present high specificity by macrophages, bare NPs and those functionalized with the IgG isotype were used as controls. The merged images show that F4/80 and D-mannose increased NP endocytosis (NPs—Nile red and blue cell nucleus) (**Figures 5C,D**), but bare NPs and NPs coated with the IgG isotype were less endocytosed by macrophages (NPs—Nile red and merged; **Figure 5B**). The uptake of NPs occurred in macrophages after 3 h of incubation, as shown in **Figure 5**. Nile red fluorescence was estimated as described in the Materials and Methods section to confirm the uptake differences among the differently functionalized NPs. In this fashion, the macrophage's internalization extent was greater

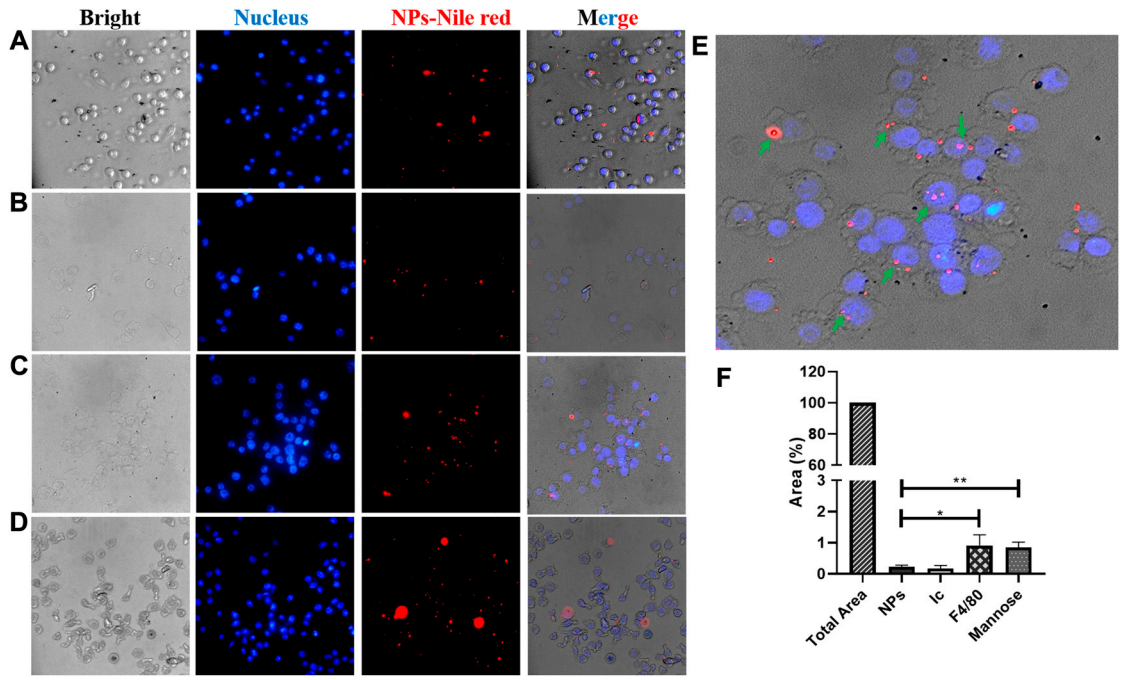


FIGURE 5 | Fluorescence microscopy images. Endocytosis evaluation of functionalized NPs with Nile red encapsulated into the J774A.1 macrophage. NPs **(A)** without and **(B–D)** with different ligands, **(B)** control isotype, **(C)** F4/80 antibody, and **(D)** mannose, upon 3 h of incubation. Images are the bright field **(left)**, DAPI filter **(left-center)**, TRITC filter **(right-center)**, and merged **(right)**, respectively. The scale is 100 μm. **(E)** Enlarged image of the F4/80 merged image; green arrows indicate macrophages with endocytosed NPs. **(F)** Endocytosed NPs estimated by measuring Nile red by fluorescence intensity as described in the Materials and Methods section.

for those treated with F4/80 antibody–covered NPs (0.91%) than for those treated with D-mannose–functionalized NPs (0.84%), with bare (0.23%) and IgG isotype–coated (0.17%) NPs being used as controls, respectively (**Figure 5F**).

DISCUSSION

NPs for site-specific delivery of antifungal drugs to fight intracellular infections were developed to improve therapeutic efficacy. In this context, scientific literature shows that ITZ has been encapsulated into polymeric micelles by nanoemulsion but has relatively low DLC. Besides, most of them are not specific to macrophages, a principal target cell in infectious diseases and the main problem in developing antibiotic resistance. Functionalized NPs are a valuable option to regulate biodistribution through a target cell or tissue and can direct drugs across biological barriers (Scheme 1A) to a specific target through particle size modulation and surface modification with ligands to increase cell penetration and achieve specificity of action. By using this strategy, NPs can be directed to the infection foci, where the main burden of pathogens is found to release its cargo. In addition, higher drug doses at the infected site can be administered (Scheme 1A), diminishing existing microorganism resistance or eliminating microorganisms, thus causing fewer adverse effects on patients.

In this study, NPs' properties were tuned to encapsulate an optimal ITZ concentration in a highly biocompatible system. With the base formulation, when studying the influence of the PLGA-to-ITZ ratio on the physicochemical properties of NPs prepared by nanoemulsion, the system containing 7.5 mg of ITZ produced a larger particle size, which increased as relative viscosity increased, in agreement with reports in the literature (Wooster et al., 2008; Gupta et al., 2016). Furthermore, a 1:5 ITZ-to-PLGA ratio produced NPs with a higher polydispersity (PDI = 0.41) than a 1:10 ratio (PDI = 0.19), as reported elsewhere (Bian et al., 2013). This effect may be related to the inadequate adsorption of surfactant at a lower ITZ-to-PLGA ratio and a higher amount of ITZ at the surface level that did not effectively coat the emulsified droplets, causing their destabilization (Bian et al., 2013; Lakkireddy and Bazile, 2016; Wilkosz et al., 2018) and further agglomeration. Furthermore, a 1:11 ITZ-to-PLGA ratio produced NPs with higher DLC values (4.0 ± 0.3%) but lower EE values (47.6 ± 3.2%). The higher molecular weight of PLGA 50:50 possibly increased its hydrophilic character, avoiding an adequate partitioning of ITZ in the NPs by hydrophobic and electrostatic PLGA–ITZ interactions so that ITZ might be migrating toward the continuous phase during the nanoemulsion process, producing lower EE. However, such PLGA 50:50 NPs' base formulation presented optimal size (188.5 ± 3.0 nm), PDI (0.23 ± 0.04), and ζ-potential (−39.9 ± 2 mV), compared to other ratios evaluated (1:10 and 1:25) (**Figure 1**; **Table 1**). The

TABLE 2 | Comparison of average particle size by DLS and TEM.

Polymer (PLGA)	Size (nm), DLS	Size (nm), TEM
50:50	188.5 ± 3.0	145.0 ± 28.2
75:25	150.4 ± 6.4	132.0 ± 36.6
50:50-TPGS-pH5	165.0 ± 9.7	140.3 ± 26.7
75:25-TPGS-pH5	147.3 ± 7.7	131.0 ± 30.8

smaller average particle size of PLGA 75:25 NPs compared to that of PLGA 50:50 may be related to the lower molecular weight of PLGA 75:25, which decreases the system's viscosity. In contrast, the increased DLC may be explained by the higher hydrophobic interaction between the aliphatic carbons of the ITZ and the methyl groups of PLGA 75:25, which resulted in higher stabilization of the active principle within NPs. In other words, higher PLGA 75: 25–ITZ hydrophobic interactions produced higher packaging and, therefore, smaller nanostructures.

The next set of experiments introduced some base formulation modifications to improve its physicochemical and structural properties, evaluated by the DLC, EE, and drug-release profile. Modifications consisted of lowering the pH of the aqueous solution to five and including a multipurpose amphiphilic excipient such as vitamin E-TPGS in a 1:10 ratio by weight with respect to PLGA, one at a time. Such a slightly acidic medium increased ITZ ionization as its amide carbonyl group (R–N–C=O– $H\delta^+$) was protonated, thereby increasing its solubility and dispersibility (Prentice and Glasmacher, 2005; Kapoor et al., 2015; Alhowyan et al., 2019). Therefore, the higher amount of stabilizing electrostatic interactions among the formulation components decreased ITZ diffusion toward the continuous phase during the nanoemulsion process to avoid considerably affecting PLGA composition but impacting the enhanced DLC and EE. Then, a combination of surfactants (Kolliphor P188 and vitamin E-TPGS) at different concentrations was assessed. Unlike the base formulation, Kolliphor P188 was added in a lower concentration (9.37 mg/ml) and dissolved into the aqueous phase, while vitamin E-TPGS was used in a 1:10 ratio of PLGA 75:25 to vitamin E-TPGS and dissolved into the organic phase. This formulation increased DLC and EE, and such enhancement may be explained by increased hydrophobic interactions of the aliphatic tail (18 carbons) from vitamin E-TPGS with the aliphatic carbons of ITZ in addition to electrostatic interactions of the carbonyl ester group from vitamin E-TPGS with the triazole groups of ITZ. PLGA 75:25 NPs' DLC (6.7 ± 1.3%) was slightly higher than PLGA 50:50 NPs' DLC (6.4 ± 0.7%), and this PLGA 75:25–based formulation went to the next set of experiments. On the other hand, it is known that high Kolliphor P188 concentrations might be involved in the production of free radicals and reactive oxygen species during the sonication process when NPs are assembling, thus generating biological responses such as affectation of mitochondrial respiration and ATP synthesis, among other adverse effects, when tested *in vivo* (Batrakova and Kabanov, 2008; Wang et al., 2012). Therefore, it is important to highlight that through this improved formulation, the content of Kolliphor P188 was reduced more than 2.5-fold with respect to the base formulation, thereby expecting higher biocompatibility.

FT-IR and DSC results for both formulations (base and optimized) showed the polymer–drug interactions in the ITZ-encapsulated NPs. In this context, these interactions occur possibly due to a complex formed between the triazole (or amine) group of ITZ and the carboxylic group from the PLGA hydrophobic tail, as pointed out by Yi and collaborators (Yi et al., 2007). Additionally, the polymer's characteristic band at 1,745 cm^{-1} (C=O stretching) in the NPs with ITZ was highly intense. This band is indicative of carboxylic end groups available for further functionalization with ligands. Remarkably, the melting peak from ITZ completely disappeared in the NP-encapsulated ITZ thermogram, indicating the absence of the crystalline state of the encapsulated drug. The interaction among the formulation precursors allows a certain freedom of molecular movement to organize themselves in a crystalline way, reflected by small exothermic peaks of low intensity between 150 and 210°C for the physical mixture and the empty and loaded NPs in the two types of nanocarriers.

TEM images determined structural and morphological characteristics of the NPs from the two PLGA polymers with optimized formulation (**Figures 2B**; **Supplementary Figure S2**), showing spherical core–shell–like structures. NPs consist of the PLGA hydrophobic core and the hydrophilic shell from the hydrophilic tails of the Kolliphor P188 and vitamin E-TPGS surfactant mixture. Schemes 1A,B illustrate a diagram representing the composition of core–shell–like NPs and the corresponding receptor–ligand interactions in the cellular surface to accomplish internalization processes (Lin et al., 2010). Steric repulsion made by hydrophilic polymer chains can improve nanocarriers' circulation time in the blood by overpassing the phagocytic mononuclear system (Mu and Feng, 2003; Mu and Feng, 2003). The particle size of PLGA 75:25 NPs, estimated by TEM, was smaller than that of PLGA 50: 50 NPs for the optimized formulations and comparable with those obtained from DLS (**Table 2**). Moreover, the particle size by DLS was overestimated, as expected.

ITZ release kinetics from the nanocarriers with the optimized formulation was studied. Although it was expected that the more hydrophilic PLGA50:50-TPGS-pH5 NP system would degrade faster than the PLGA75:25-TPGS-pH5 ones, the fact of similar behaviors being present in both types of nanocarriers may be explained by the higher molecular weight of the PLGA 50:50 polymer, which has a higher amount of hydrophobic interactions with ITZ, comparable to the hydrophobic interactions of PLA in the PLGA 75:25 of lower molecular weight. In other words, the higher molecular weight of PLGA 50:50 impacts the release kinetics more than the hydrophilic character of this polymer as compared to PLGA 75:25 of lower molecular weight (Fredenberg et al., 2011). Furthermore, the multipurpose behavior of vitamin E-TPGS as a matrix component allows the establishment of electrostatic and hydrophobic interactions within the nanoparticle that equates the two types of systems with different chemical natures. In both types of particles, it was found that the release kinetics does not fit the zero-order, Higuchi, or Korsmeyer–Peppas models if it is judged by the low correlation

coefficients obtained. The fact that it does not follow a zero-order model allows us to ensure that the ITZ release rate is not constant over time and that the polymer chains' relaxation does not control the release process. As it does not follow a kinetic model like Higuchi or Korsmeyer–Peppas, it implies that the swelling/contraction phenomena must be taken into account. Release kinetics fit better with the Ritger–Peppas and Peppas–Sahlin models. It is speculated that there is a coupled mechanism or a superposition of apparently independent mechanisms such as Fickian diffusion and the polymeric matrix's swelling/relaxation. It is important to highlight that the Fickian mechanism's contribution (K1) is greater than the contribution of the polymer chains' relaxation mechanism (K2), as indicated by the higher value of K1 compared to K2. Furthermore, in both types of NPs, the *n* value was very close but higher than 0.5, indicating a quasinormal Fickian diffusion (Bruschi, 2015).

The PLGA-TPGS-pH5 formulations stored at 4°C presented high physical stability. By decreasing the temperature, the kinetic and diffusive energy and the collision frequency among the NPs decreased accordingly, and therefore, particle aggregation was less. Similarly, **Figure 4C** shows the suspensions under release kinetics conditions for both types of NPs. It demonstrated high colloidal stability during the 14 days of evaluation with particle size variations less than 10% of the RSD (upper plot), moderate polydispersity (middle plot), and medium-to-high negative potential (bottom plot). These results support the hypothesis that the ITZ-release kinetic profile has a low contribution caused by hydrolytic erosion/degradation of the PLGA matrix.

By evaluating MICs with the different free- and encapsulated-ITZ treatments in PLGA-TPGS-pH5 NPs, it was possible to show that encapsulated ITZ preserved its antifungal activity against the fungus requiring a higher concentration of 0.061 μg/ml with respect to 0.031 μg/ml of free ITZ. However, when the IC50 of both treatments was calculated, it was evident that the same amount of free or encapsulated ITZ was needed to obtain 50% control of the fungus (**Figure 4A**). These results are related to the type of kinetic release that the NPs presented (**Figure 3A**), where after 72 h, only 43% of the encapsulated ITZ was released. Therefore, to inhibit 100% of the fungal growth, a higher concentration of encapsulated ITZ would be necessary than the free one, but the amount of the ITZ released from the NPs was high enough to inhibit 50% of the fungal growth. The concentration of ITZ in the assay with NPs may be achieving the maximum saturation point, hindering the diffusion of more ITZ from the hydrophobic NPs' core to the hydrophilic phase. Furthermore, the empty NPs at the higher concentration (16 μg/ml) presented an inhibitory action against the fungal growth, which was not related to the NP components considering the controls' results, where they did not show any inhibition. Therefore, the inhibition might be related to the fact that NPs at high concentrations can bind easier to proteins or other fungus molecules, affecting their growth.

Methods for functionalizing NPs have a strong influence on the NPs' performance with regard to their specificity for target cells. They can influence the amount and orientation of ligands coupled onto the NPs, improving the uptake of NPs for the macrophages. When comparing the methods, we observed that the chemical coupling presented a higher degree of functionalization than the physical adsorption one, being more reproducible and maintaining the stability of the NPs. Furthermore, results from **Supplementary Figure S6** show that the NPs functionalized by covalent and adsorption coupling in the presence of 10% FBS had an insignificant decrease in the antibody's coating extent, indicating low masking of the ligands for both methodologies. On the contrary, the presence of 100% FBS, functionalized by covalent coupling, caused a significant decrease in the antibody's coating extent, indicating more effective masking with regard to those modified by adsorption. However, this effect may be related to the higher amount of the ligand in the NPs with covalent coupling than in the NPs coated by adsorption, suggesting the formation of the more extensive protein corona on the NPs as reported in the literature (Tonigold et al., 2018).

Regarding evaluating the specificity of functionalized NPs, results showed that NPs functionalized with the F4/80 antibody and mannose using the covalent method increased their endocytosis into macrophages significantly. Therefore, we demonstrated that NP functionalization might increase the number of endocytosed NPs, depending on the type of (bio) molecule coating. In this sense, it is well known that different macrophage populations exist, characterized by their heterogeneity, plasticity, and expression of diverse receptors. For example, lung macrophages in mice (alveolar macrophages) have high expression of mannose and the siglec-F receptor and low expression of the F4/80 receptor as compared to peritoneal macrophages that present an intermediate expression of F4/80, low expression of mannose, and no expression of the siglec-F receptor (Gordon and Plüddemann, 2017). Additionally, some reports have demonstrated that mannose-functionalized NPs controlled leishmaniasis infection by increasing the distribution of the functional NPs in the selected organs such as the liver and spleen and decreasing the amount in the peripheral blood as compared to NPs without mannose (Asthana et al., 2015; Barros et al., 2015). This is the first report showing that functionalization of NPs with F4/80 antibodies can help to improve macrophage-targeted therapy and with similar efficiency to that of mannose-coupled NPs. Therefore, F4/80-functionalized NPs open up the possibility of use in therapies directed toward other subpopulations of macrophages that do not present a high expression of mannose, that is, peritoneal macrophages (Hussell and Bell, 2014). Overall, functionalized NPs are a versatile technological platform that might be extended to a broad spectrum of applications for the treatment of intracellular infectious diseases.

CONCLUSION

We successfully encapsulated ITZ into core–shell–like NPs based on two types of PLGA, obtaining stable and moderately polydisperse nanocarriers with adequate size and optimal DLC (6.6%) and EE (80%) by lowering the pH and by modulating the type and concentration of a mixture of surfactants. Whereas FT-IR and DSC analysis demonstrated the ITZ–PLGA interactions, FT-IR showed the presence of carboxylic end groups available to react with ligands. The release profile of PLGA 75:25 and PLGA 50:50 NPs fitted well with the Fickian diffusion model. The NPs showed stability in water at 4°C and under release kinetics

conditions. Encapsulated ITZ efficiently eliminated *H. capsulatum*, with a similar IC50 to that of free ITZ. The covalent coupling to functionalize the NPs was more efficient than the adsorption method, but the protein corona masking was similar in both methods. *In vitro* assays showed that the NPs functionalized with F4/80 and mannose increased the uptake of NPs by J774 macrophages. Therefore, the F4/80-coupled NPs can be an alternative for tagging other subpopulations of macrophages. Due to the multiple mechanisms presented by pathogens that cause intracellular infections, the use of functionalized NPs would allow a much more specific treatment of these infections, reducing undesired effects in patients. In this context, current research on macrophage target therapies is directed toward finding different types of ligands for targeted drug release into specific macrophage subpopulations.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**; further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

SM designed and performed most of the experiments. AS contributed to the experimental design and performed the nanocarrier experiments. VV made the functionalization experiments. SM wrote the first draft of the manuscript with contributions from AS and VV. JO got the funding, supervised the work, and edited the manuscript. All authors participated in discussions and have read and agreed to publish this version of the manuscript.

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SUPPLEMENTARY MATERIAL

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REVIEW
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Nanomaterials Manipulate Macrophages for Rheumatoid Arthritis Treatment

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Rheumatoid arthritis (RA) is a chronic, progressive, and systemic inflammatory autoimmune disease, characterized by synovial inflammation, synovial lining hyperplasia and inflammatory cell infiltration, autoantibody production, and cartilage/bone destruction. Macrophages are crucial effector cells in the pathological process of RA, which can interact with T, B, and fibroblast-like synovial cells to produce large amounts of cytokines, chemokines, digestive enzymes, prostaglandins, and reactive oxygen species to accelerate bone destruction. Therefore, the use of nanomaterials to target macrophages has far-reaching therapeutic implications for RA. A number of limitations exist in the current clinical therapy for patients with RA, including severe side effects and poor selectivity, as well as the need for frequent administration of therapeutic agents and high doses of medication. These challenges have encouraged the development of targeting drug delivery systems and their application in the treatment of RA. Recently, obvious therapeutic effects on RA were observed following the use of various types of nanomaterials to manipulate macrophages through intravenous injection (active or passive targeting), oral administration, percutaneous absorption, intraperitoneal injection, and intra-articular injection, which offers several advantages, such as high-precision targeting of the macrophages and synovial tissue of the joint. In this review, the mechanisms involved in the manipulation of macrophages by nanomaterials are analyzed, and the prospect of clinical application is also discussed. The objective of this article was to provide a reference for the ongoing research concerning the treatment of RA based on the targeting of macrophages.

Keywords: nanomaterials, macrophages, rheumatoid arthritis, inflammation, treatment

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INTRODUCTION

Rheumatoid arthritis (RA) is the most common inflammatory autoimmune disease and is characterized by immune cell infiltration (e.g., macrophages) and chronic inflammation in the synovium tissue. The global prevalence of RA in patients aged 5–100 years was approximately 0.24% (95% confidence interval: 0.23–0.25%). An approximately three-fold higher incidence of RA was observed in females vs. males, that is, 0.35% (0.34–0.37%) vs. 0.13% (0.12–0.13%), respectively (Cross et al., 2014). The exact etiology of RA is currently unknown. However, it has been demonstrated that a number of effector cells (e.g., macrophages, T lymphocytes, and dendritic cells), inflammatory

cytokines, such as tumor necrosis factor α (TNF-α), interleukin 1 (IL-1), and interleukin 6 (IL-6), and their interactions contribute to the pathological process of RA (**Figure 1**) (Smolen et al., 2007; Aletaha and Smolen, 2018).

Promonocytes exist in the bloodstream and can differentiate into monocytes. Subsequently, these cells extravasate into tissues, where they further differentiate into a specific type of "resident" tissue macrophage (Maruotti et al., 2007). The number of macrophages is often increased in the synovium lining of patients with RA (Athanasou, 1995). Macrophages are important immune cells causing a sustainable chronic inflammatory response in the synovial tissue of patients with RA. Activated macrophages act through the release of pro-inflammatory cytokines (e.g., TNF-α, IL-1β, and IL-6) and inflammatory mediators (e.g., prostaglandins and chemokines), which maintain the chronic inflammation and result in pain, an increase in inflammatory cell infiltration, the formation of synovial pannus, and joint destruction (Ma and Pope, 2005). More importantly, the recruitment of macrophages to the site of inflammatory response results in persistent and amplified inflammation (Gao et al., 2021). Therefore, the targeting of macrophages is an important approach to treating inflammation and RA.

Although substantial evidence and experience with regard to the treatment of RA have been accumulated over the past few decades, the effective management of this disease remains a challenge. Currently, the major objectives of drug therapy are to alleviate the symptoms of RA and reduce the disease activity (Smolen et al., 2010). Several drugs are recommended by the European League Against Rheumatism for the management of RA. Based on the guidelines, treatment with synthetic and biological disease-modifying antirheumatic drugs (DMARDs), including conventional synthetic DMARDs [methotrexate (MTX), leflunomide, and sulfasalazine] and biological DMARDs (adalimumab, certolizumab pegol, and etanercept), should be promptly initiated following the diagnosis of RA. This highlights that DMARDs play an irreplaceable role in the pharmacological strategies for the treatment of RA. Meanwhile, glucocorticoids (GCs) can also be used as bridging therapy until conventional synthetic DMARDs produce observable effects. In China, traditional Chinese medicine (TCM) (e.g., sinomenine preparations, total glycosides of paeony capsules, and

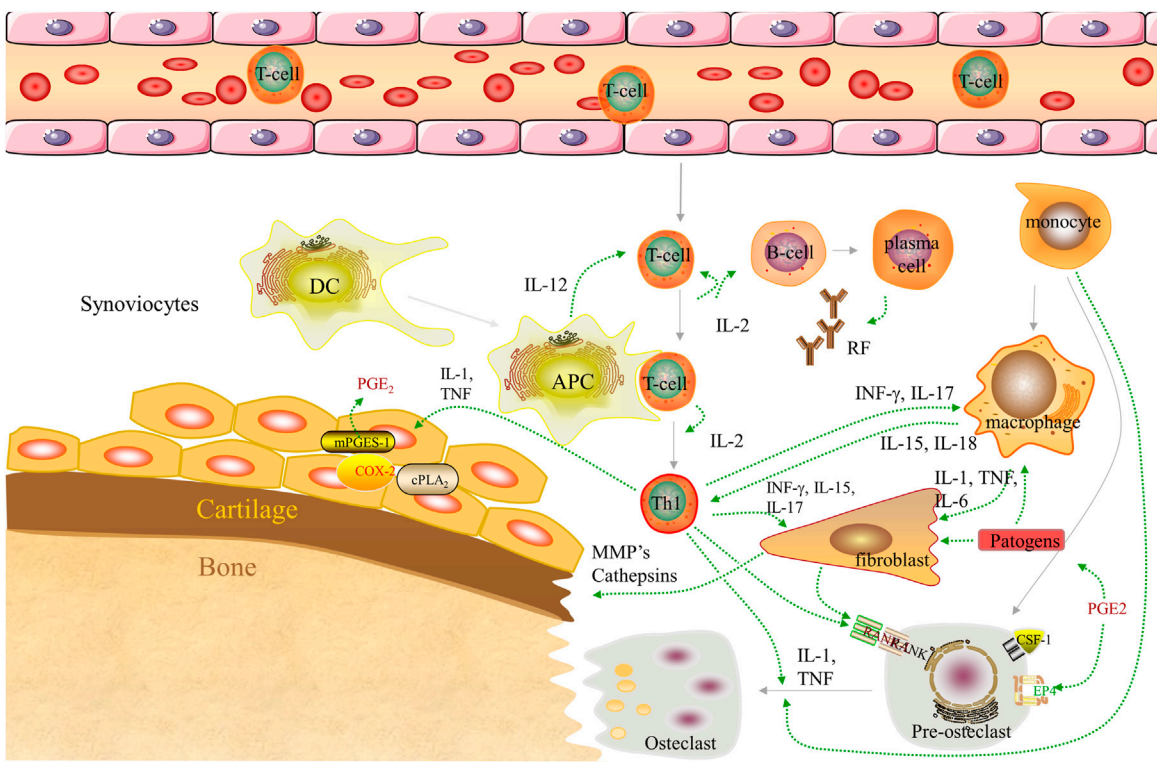

FIGURE 1 | Pathogenesis of RA. Dendritic cells complexed with autoantigen to activate naive T cells. Differentiated T cells stimulate B cells to produce autoantibodies [rheumatoid factor (RF)]. B cells present autoantigens to T cells, leading to their activation. Synovial macrophages are activated by T cells to release pro-inflammatory cytokines (TNF-α, IL-1, and IL-6), driving the activation of synovial fibroblasts and inducing osteoclast production. In addition, the monocyte subpopulations of the arthritic synovium can differentiate into macrophages and osteoclasts. Collectively, these components play an important role in the destruction of bones, cartilage, and the synovium. APC, antigen-presenting cell; COX-2, cyclooxygenase-2; cPLA2, cyosolic phospholipase A2; CSF-1, colony-stimulating factor-1; DC, dendritic cell; EP4, prostaglandin E receptor subtype 4; IFN-ɣ, interferon-gamma; IL-, interleukin; MMP, matrix metalloproteinase; mPGES-1, microsomal prostaglandin E synthase-1; PGE2, prostaglandin E2; PGF2, prostaglandin F2; RA, rheumatoid arthritis; RANK, receptor activator of nuclear factor-kB; Th1, T helper 1; TNF-α, tumor necrosis factor α.

TABLE 1 | Strategies using nanomaterials to manipulate macrophages for Rheumatoid arthritis treatment.

Route of administration	Drugs/agents	Carrier systems		Consequent			Reference
		Organic material	Inorganic material	Inflammatory cytokines		Polarization and apoptosis	
				Reduction	Increase		
Intravenous injection	Methotrexate	FA-PPLNPs[a]	—	TNF-α, IL-6	—	—	Zhao et al. (2017)
		&HSA[a]	—	TNF-α, IL-1β, IL-6	—	Polarization	Liu et al. (2019)
		Sta-R8-FA-PLPNs[a]	—	TNF-α, IL-1β, IL-6	—	—	Zhao et al. (2018)
		DS-5β-cholanic acid[a]	—	TNF-α, IL-1β, IL-6	—	—	Heo et al. (2017)
		DS-micelle[a]	—	TNF-α, IL-1β, IL-6	—	—	Yang et al. (2017)
		FGCN[a]	—	TNF-α, IL-1β, IL-6, IL-17	IL-10	Apoptosis	Kumar et al. (2020)
		—	Fe3 + @HA MOFs[a]	TNF-α, IL-1β, IL-6	—	—	Guo et al. (2021)
		—	Au-DEN-NPs[a]	TNF-α, IL-1β, IL-6	—	—	Pandey et al. (2019)
	Methotrexate and minocycline	PLGA	—	TNF-α, IL-1, IL-6	—	—	Janakiraman et al. (2019)
	Prednisolone	HA-SLNs[a]	—	TNF-α, IL-1β, IL-6	—	—	Zhou et al. (2018)
	Prednisolone and curcumin (Chinese medicine monomer, derived from *Curcuma longa* L.)	&HAS[a]	—	TNF-α, IL-1β, IL-6	IL-10	—	Yan et al. (2019)
	Celastrol (Chinese medicine monomer, derived from *Tripterygium wilfordii* Hook. f.)	&HSA-HS15[a]	—	TNF-α, IL-1β	—	—	Gong et al. (2019)
		PEG-b-PPS	—	TNF-α, IL-1β, IL-6	TGF-β1, M-CSF	—	An et al. (2020)
	Triptolide (Chinese medicine monomer, derived from *Tripterygium wilfordii* Hook. f.)	GDR[a]	—	TNF-α, IL-1β, IL-6, IFN-γ, IL-17A	—	—	Li et al. (2020)
		&PAT	—	TNF-α, IL-1β, IL-6	—	—	Zhang et al. (2018)
	Benzoylaconitine (Chinese medicine monomer, derived from *Aconitum carmichaelii* Debx.)	mPEG-PLGA	—	TNF-α, IL-1β	—	—	Gai et al. (2020)
	Dexamethasone	HA-PNPs[a]	—	TNF-α, IL-1β	—	—	Yu et al. (2019)
	Dexamethasone palmitate	DEPE-PEG2000	—	TNF-α, MCP-1	—	—	Lorscheider et al. (2019)
	Tacrolimus	MNP[a]	—	TNF-α, IL-1β, IL-6	—	—	Li et al. (2019)
	Superoxide dismutase	F-CNM[a]	—	IL-6	—	—	Srivastava et al. (2018)
	Ag+	LA-PEG-FA[a]	—	TNF-α, IL-1β, IL-6	—	Polarization	Yang et al. (2021)
	Fumagillin prodrug	Rv-β3-FFC[a]	—	TNF-α, IL-1β, IL-6, MCP-1	—	—	Zhou et al. (2014)
	Mcl-1 siRNA	FA-PPNPs[a]	—	TNF-α, IL-1β, IL-6	—	—	Sun et al. (2019)
	IL-1β siRNA	FS14-NPs	—	TNF-α, IL-1β, IL-6	—	—	Song et al. (2019)
	NF-κB p65 siRNA	FA-PEG-liposome[a]	—	TNF-α, IL-1β	—	Polarization	Duan and Li, (2018)
	Notch1 siRNA	tGC	—	TNF-α, IFN-γ, MCP-1, IL-6, IL-12, IL-17	—	—	Kim et al. (2015)
	BTK siRNA	CLAN	—	TNF-α, IL-1β, IFN-γ	—	—	Zhao et al. (2019)
	—	—	Au-NP		—	Apoptosis	James et al. (2015)
Percutaneous absorption	Methotrexate	PLC-PEG-PLC	—	TNF-α, IL-1β, IL-6	—	—	Qindeel et al. (2020a)
		NLCs	—	TNF-α, IL-1β, IL-6	—	Apoptosis	Garg et al. (2016)
	Quercetin (Chinese medicine monomer, derived from *Quercus iberica*)	NE	—	TNF-α, IL-1β, IL-6	—	—	Gokhale et al. (2019)
Intra-articular injection	Methotrexate	—	MFC-MSNs	TNF-α, IL-1β	—	Polarization	Kim et al. (2019)
	Methotrexate and teriflunomide	—	HAP-NPs	TNF-α, IL-1β, IL-6	—	—	Pandey et al. (2021)
	Dexamethasone	—	ND-ODA	TNF-α, IL-1β	—	Polarization	Pentecost et al. (2017)
	Resveratrol (Chinese medicine monomer, derived from *Vitis* spp.)	—	QRu-PLGA-DS	TNF-α, IL-1β, IL-6	IL-4, IL-10, TGF-β	Polarization	Chen et al. (2019a)

(Continued on following page)

TABLE 1 | (*Continued*) Strategies using nanomaterials to manipulate macrophages for Rheumatoid arthritis treatment.

Route of administration	Drugs/agents	Carrier systems		Consequent			Reference
		Organic material	Inorganic material	Inflammatory cytokines		Polarization and apoptosis	
				Reduction	Increase		
	Clodronate	Chitosan	—	IL-8, IL-1β	—	—	Russo et al. (2016)
	TNF-siRNA	LPNs	—	TNF-α	—	—	Jansen et al. (2019)
	NOCCL	Acrylamide	—	TNF-α, IL-6	—	—	Park et al. (2017), Yeo et al. (2019)
Intraperitoneal injection	IL-10 plasmid DNA	Tuftsin-alginate NPs	—	TNF-α, IL-1β, IL-6	—	Polarization	Jain et al. (2015)
	—	—	Au25Sv5	TNF-α, IL-1, IL-6	—	—	Yuan et al. (2019a)
	—	—	Au29GSH7	TNF-α, IL-1β, IL-6	—	—	Gao et al. (2019)
Oral administration	Chloroquine	SLN	—	TNF-α	—	—	Bhalekar et al. (2016)
Diagnostic nanomaterials	—	&MFNPs	—	—	—	—	Periyathambi et al. (2017)

&Endogenous biomimetic materials.
[a]Active targeting.
FA-PPLNPs, folic acid–polyethylene glycol–poly (lactic-co-glycolic acid)–poly (cyclohexane-1,4-diylacetone dimethylene ketal)–lipid nanoparticles.
HAS: human serum albumin nanoparticles.
Sta-R8-FA-PLPNs, stearic acid-octa-arginine and folic acid decorated poly (lactic-co-glycolic acid)-PK3–based lipid polymeric hybrid nanoparticles.
DS-5β-cholanic acid, dextran sulfate-5β-cholanic acid nanoparticles.
DS-micelle, dextran sulfate-graft-methotrexate conjugate.
FGCN, folate-conjugated pH-responsive glycol-chitosan nanoparticles.
Fe3 + @HA MOFs, Fe3+ metal-organic frameworks with surface hyaluronic acid modification.
Au-DEN-NPs, nanogold core dendrimer nanoparticles.
PLGA, poly (lactic-co-glycolic acid).
HA-SLNs, solid lipid nanoparticles coated with hyaluronic acid.
PEG-b-PPS, poly (ethylene glycol)-block-poly (propylene sulphide).
GDR, pH-sensitive galactosyl-dextran-retinal.
PAT, poly-γ-glutamic acid-grafted di-tert-butyl L-aspartate hydrochloride.
mPEG-PLGA, methoxy-poly (ethylene glycol)-poly (lactide-co-glycolide).
HA-PNPs, hyaluronic acid–coated acid-sensitive polymeric nanoparticles.
DEPE-PEG2000, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-2000].
MNP, macrophage-derived microvesicle-coated nanoparticle.
F-CNM, folic acid-cellobiose-coated nanomatrix.
LA-PEG-FA, α-lipoyl-ω-folic poly (ethylene glycol).
Rv-β3-FFC, Rvβ3-integrin–targeted perfluorocarbon nanocarriers.
FA-PPNPs, folate acid-polyethylene glycol-poly (lactide-co-glycolide acid)-PK3 nanoparticles.
FS14-NPs, polymer–lipidoid hybrid nanoparticles.
FA-PEG-liposome, folic acid-poly (ethylene glycol)-liposome.
tGC, thiolated glycol chitosan polymers.
CLAN, cationic lipid–assisted poly (ethylene glycol)-block-poly (lactic-co-glycolic acid) nanoparticle.
PLC-PEG-PLC, polycaprolactone-polyethylene glycol-polycaprolactone triblock copolymer.
NLCs, nanostructured lipid carriers.
NE, nano-emulsion.
MFC-MSNs, manganese ferrite and ceria nanoparticle–anchored mesoporous silica nanoparticles.
HAP-NPs, hyaluronic acid coated hydroxyapatite nanoparticles.
ND-ODA, octadecylamine-functionalized nanodiamond.
QRu-PLGA-DS, quadrilateral ruthenium-poly (lactic-co-glycolic acid)-dextran sulfate nanocomposite.
LPNs, lipid–polymer hybrid nanoparticles.
MFNPs, magnetic fibrin nanoparticles.

preparations from the plant *Tripterygium*) has been applied to the treatment for RA. The mechanisms and characteristics of action of TCM on RA are similar to those of DMARDs; however, TCM requires a long period of therapy to alleviate symptoms of RA (Zhang et al., 2010).

There are numerous problems in the pharmacological treatment of RA. Long-term use of nonsteroidal anti-inflammatory drugs (NSAIDs), DMARDs, and GCs has serious adverse effects on gastrointestinal, cardiovascular, liver, and kidney functions (Oray et al., 2016; Schett et al., 2016; Buttgereit, 2020). Although biological agents are associated with a rapid onset of effect, they are limited by their high cost, which leads to poor patient compliance (Dalal et al., 2019). The use of TCM compounds in clinical practice is linked to definite therapeutic effects and a low incidence of side effects. Through the compatibility of medicines, TCM compounds can increase the effectiveness of the treatment and reduce the toxicity. However, the large differences in the composition and dosage of different formulations complicate their application. The compositions are often complex and occasionally have ill-defined active ingredients; moreover, the efficacy of these compounds is not supported by robust scientific research data (Burmester and Pope, 2017;

Xing et al., 2020). Therefore, it is important to overcome the disadvantages of these agents in the treatment of RA. Recently, research has shown that various nanomaterials can be used to carry anti-RA drugs by manipulating macrophages. This approach improves the solubility and bioavailability of the drug, avoids dose escalation, and enhances patient compliance (Dolati et al., 2016; Xiao et al., 2019). In this review, the manipulation strategies and anti-RA effects of nanomaterials on macrophages for the treatment of RA are analyzed, and the prospect of clinical application is also discussed.

STRATEGIES FOR THE MANIPULATION OF MACROPHAGES BY NANOMATERIALS

The routes of nanomaterial administration for the manipulation of macrophages in the treatment of RA mainly include intravenous injection, oral administration, percutaneous absorption, intraperitoneal injection, and intra-articular injection. In terms of the control strategy, the intravenous injection is primarily an active strategy, whereas the oral and percutaneous administrations are mainly passive strategies. Regarding the types of nanomaterials, inorganic materials are typically administered through intra-articular injection, while organic materials are mainly delivered through other routes. Based on the pathological role of macrophages in RA, the use of nanomaterials for the manipulation of macrophages mainly focuses on the early diagnosis and therapy of RA (**Table 1**).

Manipulation of Macrophages by Intravenous Injection of Nanomaterials

Intravenous injection is the most commonly used method of administration in the clinic. It offers some advantages compared with other administration routes, such as rapid onset of effect, no first-pass effect, and absence of effects on the digestive system (Anselmo and Mitragotri, 2016). Thus far, the pathogenesis of RA remains unclear, and long-term clinical treatment is required for this disease. The pharmacological treatment of RA often involves oral administration of drugs, such as GCs, NSAIDs, and disease-modifying drugs (chemical therapy, TCM, natural cures, etc.). Nevertheless, this approach is limited by the difficulty of reaching the diseased joints and the occurrence of systemic side effects (Abbasi et al., 2019). According to statistics, 37.8% of patients discontinued treatment due to serious adverse effects. Therefore, the development of novel drug delivery systems to transport therapeutic drugs that can specifically target the synovial macrophages has become a research hot spot in the field of RA (Littlejohn and Monrad, 2018).

Nanodrug delivery systems are characterized by small particles, a large specific surface area, and a strong adsorption property. These features extend the half-life of drugs *in vivo*, thereby prolonging the duration of action and minimizing the frequency of administration (Dolati et al., 2016). Simultaneously, after being modified by chitosan, poly (ethylene glycol) (PEG), and d-α-tocopheryl polyethylene glycol 1,000 succinate or prepared from endogenous biomimetic materials, nanodrug delivery systems can selectively target inflammatory tissues by passive or active targeting, releasing drugs through a response to endogenous (pH, redox, enzymes, etc.) or exogenous (temperature, light, electric field, magnetic field, etc.) stimuli. This selective targeting improves the therapeutic effects, lowers toxicity, and reduces the incidence of side effects (Liu et al., 2020).

Passive Targeting Strategy

The increase in vascular permeability and macrophage infiltration are the main pathological characteristics of RA, which can provide favorable conditions and target cells for nanodrug delivery systems. Persistent inflammation increases vascular permeability, which allows the nanodrug carrier to selectively accumulate and release drugs in the synovial tissue through the "ELVIS" (Extravasation through Leaky Vasculature and the subsequent Inflammatory cell–mediated Sequestration) effect, which is similar to the enhanced permeability and retention effect observed in the treatment of tumors (Kumari et al., 2016). Particle size is the key factor affecting the passive targeting strategy. This is because nanodrug carriers with a size of >200 nm and <10 nm can be eliminated by the spleen and the kidney, respectively. Thus, only nanodrug carriers with a size in the range of 100–200 nm could avoid uptake by the mononuclear phagocyte system and the reticuloendothelial system (RES), thus remaining longer in circulation in patients with RA (Kang et al., 2020) (**Figure 2**).

Inorganic Nanomaterials

Inorganic nanomaterials, such as an exogenous substance, are required to penetrate the various biological barriers *in vivo* prior to reaching the inflammatory synovial tissue of the diseased joint. Typically, after intravenous injection, nanomedicines can be easily cleared from the blood circulation through both the RES and the mononuclear phagocyte system (Yoo et al., 2010). Numerous researchers have reported that modification using hydrophilic materials can improve the surface hydrophilicity and steric hindrance of inorganic nanomaterials. These effects help to "stealth" the nanomedicines and prolong their time in circulation, providing the possibility of enrichment at sites of inflammation *via* the "ELVIS" effect (Hu et al., 2018).

PEG Modification. PEG is a solid hydrophilic polymer. It has been demonstrated that PEGylated nanomedicines accumulate more on inflamed synovium and are less eliminated in the spleen and the liver. In a recent study conducted by Heo et al., dexamethasone palmitate (DXP), a lipophilic prodrug of dexamethasone (Dex), and DSPE-PEG2000 were used to prepare PEGylated DXP-NPs. The preparation of DXP-NPs was based on the hydrophobic interaction between the stearic acid chain of polyethylene glycol lipids and the palmitic acid chain of DXP. It was observed that the nanomedicine was highly effective because it prevented the crystallization of DXP and avoided low drug loading and destabilization of the suspension over time. Furthermore, the PEG on the surface of NPs can make them "invisible" to the mononuclear phagocyte system and the RES and allow longer circulation in blood vessels. DXP-NPs are characterized by high vascular permeability of inflammatory

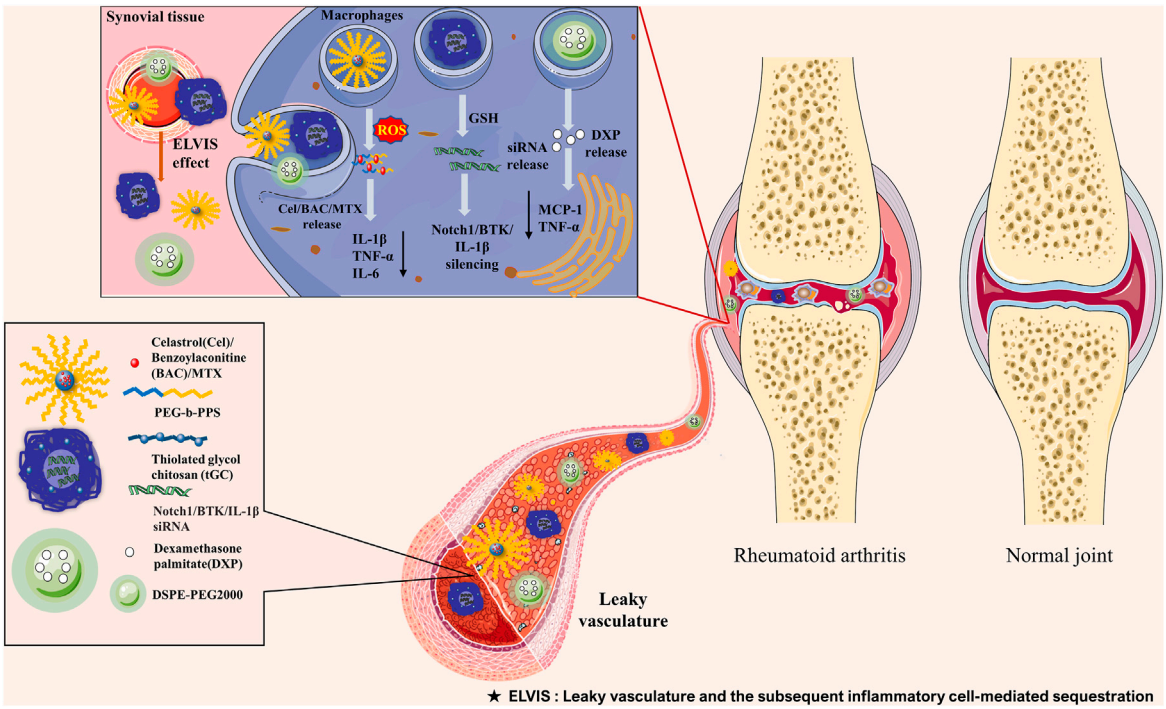

FIGURE 2 | Schematic illustration of the passive targeting delivery system for the management of rheumatoid arthritis by manipulating macrophages with nanocarriers encapsulating various therapeutic agents. Polymer nanoparticles, chitosan nanoparticles, and polymeric micelles have been used for the treatment of RA. Upon intravenous administration, nanoparticles accumulate in the inflamed joints *via* the ELVIS effect. Subsequently, these nanoparticles are taken up by activated macrophages and selectively deliver Cel, BAC, MTX, DXP, and Notch1/BTK/IL-1β siRNA through pH-responsive, redox-responsive, and ROS-responsive approaches. This process reduces the release of MCP-1, TNF-α, IL-1β, and IL-6, thus alleviating the progression of RA. BAC, benzoylaconitine; BTK, Bruton's tyrosine kinase; Cel, celastrol; DXP, dexamethasone palmitate; ELVIS, Extravasation through Leaky Vasculature and the subsequent Inflammatory cell–mediated Sequestration; GSH, glutathione; IL-1β, interleukin-1β; IL-6, interleukin-6; MCP-1, monocyte chemoattractant protein-1; MTX, methotrexate; RA, rheumatoid arthritis; ROS, reactive oxygen species; siRNA, small interfering RNA; TNF-α, tumor necrosis factor α.

joints, which can passively diffuse and accumulate in the lesion site. This accumulation led to the release of DXP, which inhibited the release of monocyte chemoattractant protein-1 (MCP-1) and TNF-α in macrophages (Lorscheider et al., 2019). Others synthesized diblock copolymer PEG-block-poly (propylene sulphide) *via* multistep chemical reaction. The blank micelle (B-PEPS) was prepared through self-assembly of a copolymer; subsequently, celastrol (Cel) was added to prepare Cel-loaded diblock copolymer nanomicelles (C-PEPS). The hydrophilic PEG block could increase the circulation time of nanomicelles, and the hydrophobic poly (propylene sulphide) block permits reactive oxygen species (ROS)-sensitive reactions. After reaching the inflammatory site through the "ELVIS" effect, the nanomedicine rapidly released Cel *via* the ROS-responsive approach, which prevented the cleavage and activation of nuclear factor-κB (NF-κB) and Notch1. This process suppressed M1 macrophage activation and the release of pro-inflammatory cytokines (e.g., TNF-α, IL-6, and IL-1β), whereas it enhanced the release of anti-inflammatory cytokines [e.g., TGF-β1 and macrophage colony-stimulating factor (M-CSF)] (An et al., 2020). Another monomer of TCM, benzoylaconitine (BAC), was also prepared in PEG-modified nanomedicine for the manipulation of macrophages in the treatment of RA. Researchers designed the methoxy-PEG-poly (lactide-co-glycolide) (mPEG-PLGA) copolymer through ring-opening polymerization and dissolved it in dimethyl sulfoxide and BAC in methanol. Finally, the mPEG-PLGA and BAC solutions were mixed to prepare BAC-loaded mPEG-PLGA NPs (NP/BAC). Through the "ELVIS" effect, the nanomedicine aggregated in the inflammatory joints and released BAC to reduce the overexpression of NF-κB p65, block the production of pro-inflammatory cytokines, and alleviate the development of inflammation (Gai et al., 2020). Small interfering RNA (siRNA)-mediated gene silencing has been used in the treatment of autoimmune diseases, such as RA. However, due to its poor stability and low permeability, chemical modification is required to overcome these limitations (Yu et al., 2020). Bruton's tyrosine kinase (BTK) is an important macrophage kinase which can promote the polarization of pro-inflammatory macrophages. Moreover, downregulating the expression of BTK in macrophages can reduce inflammatory cytokines, inhibit the development of RA, and alleviate symptoms of arthritis (Rip et al., 2018; Zhao et al., 2019). More recent studies conducted by Zhao et al. employed mPEG5K-b-PLGA11K and cationic liposome (DOTAP) to prepare cationic lipid–assisted NPs (CLANs) using a double-emulsion solvent evaporation method. Next, siBTK was encapsulated into CLANs to prepare

CLANsiBTK. The nanomedicine specifically targets the activated macrophages *via* the "ELVIS" effect to downregulate the expression of BTK, thereby reducing the release of inflammatory cytokines [e.g., TNF-α, IL-1β, and interferon-γ (IFN-γ)] (Zhao et al., 2019).

PEG Analogue Modification. PEGylated polymer or aqueous amphiphilic block copolymers, such as poly (ethylene oxide)-poly (propylene oxide)-poly (ethylene oxide) (PEO-PPO-PEO), can effectively prolong the blood circulation time and achieve long-term effects as the matrix of NPs. Scholars modified NPs with amphiphilic PEG analog F127 (PEO-PPO-PEO) to manipulate macrophages for delivering siIL-1β. F127 is an amphiphilic triblock copolymer composed of PEO and PPO. When the concentration reaches a specific critical value, the PEO-PPO-PEO block copolymer can self-assemble into a core–shell micelle structure due to the different hydrophilicities of PEO and PPO in water. In this structure, the PPO and PEO segments form the core and the shell of the micelle, respectively. Researchers injected a mixture of spermidine lipid (S14) and F127 into acetate buffer to synthesize polymer–lipid mixed NPs (FS14-NP) using the nanoprecipitation method. The nanomedicine selectively accumulated in the diseased joints through the "ELVIS" effect and was endocytosed through the natural phagocytosis of activated macrophages, delivering siIL-1β to suppress ankle swelling, bone erosion, and cartilage destruction (Song et al., 2019). Janakiraman et al. (2019) also developed PLGA NPs delivering MTX and minocycline. PEG analog d-α-tocopheryl polyethylene glycol 1,000 succinate was used as an absorption promoter, stabilizer, solubilizer, and emulsifier to prolong the circulation time of the drug. Surfactant Span-80 was added to prevent the aggregation of particles. As expected, the NPs were absorbed by activated macrophages, releasing MTX and minocycline in inflamed joints. Owing to its good biocompatibility and biodegradability, PLGA can enhance the release of medicine, which inhibited pro-inflammatory cytokines. In addition, minocycline controls infections caused by Gram-positive and Gram-negative bacteria and effectively treats RA associated with severe infection.

Chitosan Modification. As a natural polysaccharide rich in amino groups, chitosan is widely used in the biomedical field owing to its good biocompatibility, bioactivity, lack of toxicity, and biodegradability. Recently, the study of amphiphilic chitosan derivatives has attracted growing attention. Amphiphilic chitosan–based copolymers (e.g., glycol chitosan), formed by the hydrophobic modification of chitosan, can be dissolved in any aqueous solution and are characterized by prolonged circulation time (Jiménez-Gómez and Cecilia, 2020). Kim et al. (2015) have encapsulated the siRNA targeting the Notch pathway into thiolated glycol chitosan polymer, which realized the long cycle characteristics *in vivo* and assembled explicitly in RA joints. Following phagocytosis by activated macrophages, disulfide bonds in nanomedicine are broken in the presence of reducing agents such as glutathione (r-glutamyl cysteingl + glycine; GSH) in the cytoplasm. This results in the release of siRNA to silence the Notch pathway, thereby delaying bone erosion and cartilage injury.

Endogenous Biomimetic Nanomaterials

Recent evidence suggests that PEGylated modified polymer NPs cause a robust immune response, which can accelerate the blood clearance effect (Mohamed et al., 2019). Thus, a natural particle-based biomimetic drug delivery system was created, which disguised NPs as autologous components to reduce their immunogenicity, escape immune clearance, prolong circulation time, increase accumulation, improve therapeutic efficiency, and reduce toxicity and side effects (Jin et al., 2018). L-aspartic acid is an important amino acid with attractive properties for iron oxide NP functionalization, low cost, and high biocompatibility. It is widely used in the food, medical, and chemical industries; its application in medicine is particularly noteworthy (Salehiabar et al., 2018). In a study, triptolide (TPT), a monomer of TCM, was carried by poly-γ-glutamic acid–grafted di-tert-butyl L-aspartate hydrochloride, which was prepared *via* the amidation reaction of aspartic acid with poly-γ-glutamic acid. NPs concentrated in the diseased joints through the "ELVIS" effect were uptaken by activated macrophages and released TPT to reduce pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) (Zhang et al., 2018). Other researchers also used macrophage-derived microvesicles combined with nanocarriers to prepare macrophage-derived microvesicle-coated NPs (MNPs). NPs can be recognized as endogenous vesicles by connecting with natural cell membranes, reducing the elimination of MNPs by the host, and prolonging their circulation. They can also simulate macrophages through CD44 or macrophage antigen 1 (Mac-1) recognition, adhere to the endothelium to target the inflammatory site of RA for the transportation of drugs, and mimic a macrophage to combine with M-CSF and NF-κB ligand–receptor activator (RANKL) for the inhibition of osteoclastogenesis (Li et al., 2019).

Active Targeting Strategy

Direct Manipulation Strategy

Although the nanodrug delivery system can passively deliver drugs to RA lesion areas through the "ELVIS" effect, it cannot be completely ingested by macrophages. It can also bind with other nontarget cells, which has a limited effect on the manipulation of macrophages in RA (Gaspar et al., 2019). Active targeting strategies were developed to overcome shortcomings and enhance the aggregation of nanomedicines at the target site. The primary process of active targeting is achieved *via* surface modifications of nanomedicines with target-specific ligands (Shi and Lammers, 2019). Researchers used small-molecule ligands (i.e., folate, class A scavenger, and galactose) for the modification of nanodrugs to target a variety of specific receptors that are overexpressed on the surface of macrophages, such as folate receptors (FRs), class A scavenger receptors (SR-As), and galactose receptors (Pirmardvand Chegini et al., 2018); they constructed a nanodrug delivery system *via* active targeting of macrophages (**Figure 3**).

Receptor Targeting Strategy. Macrophages are a type of special immune cell with phagocytic function. Studies have shown that various membrane receptors can mediate the endocytosis of

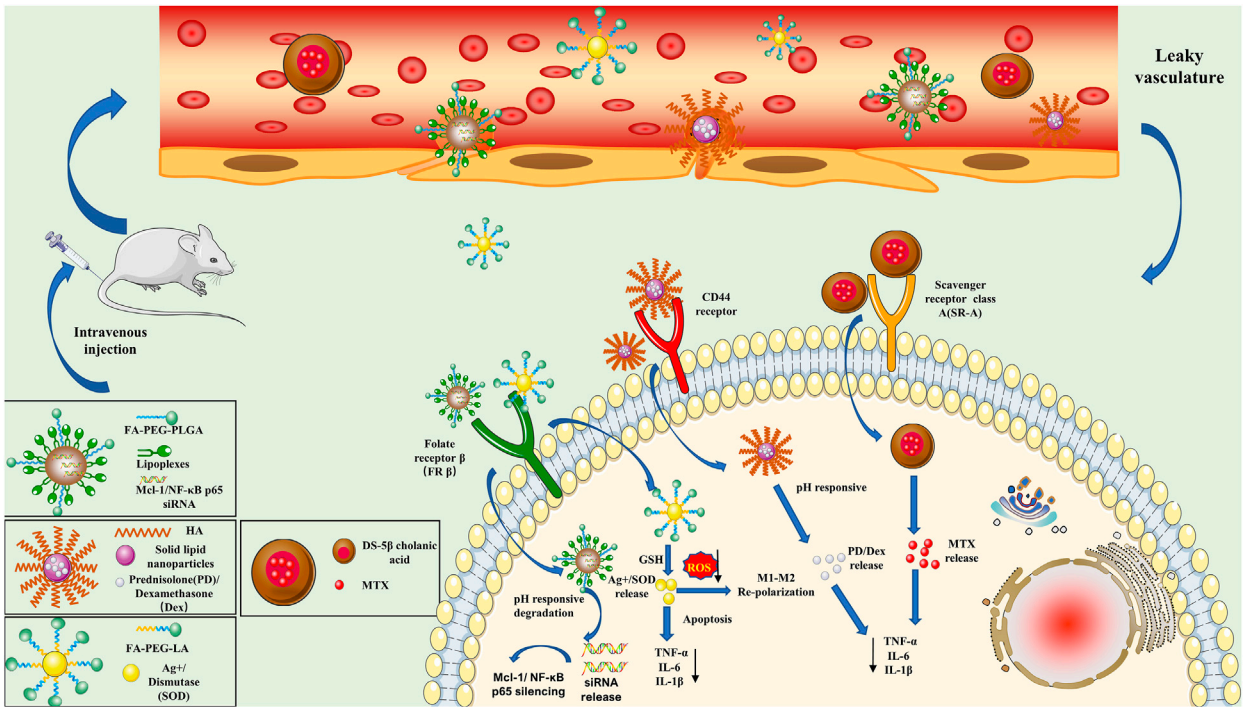

FIGURE 3 | Schematic illustration of the active targeting nanoparticles (NPs) approach in rheumatoid arthritis. (1) Mcl-1/NF-κB p65 siRNA-NPs are fabricated by encapsulating poly-siRNA into lipid–polymer hybrid NPs. Following systemic administration, NPs accumulate in the inflamed joints by taking advantage of the leaky blood vessels and selectively delivering Mcl-1/NF-κB p65 siRNA into activated macrophages through folate receptor–mediated endocytosis. (2) Ag+/SOD-loaded NPs dissolve and release Ag+/SOD in response to intracellular GSH, which synergistically induces apoptosis in M1 macrophages and scavenges ROS to cause the polarization of M1 macrophages to the M2 phenotype in inflamed synovial joints. (3) PD/Dex is wrapped in HA-coated solid lipid NPs to prepare NPs for pH-responsive drug release. HA coating allows NPs to enter macrophages through CD44 receptor–mediated endocytosis, thereby reducing the release of TNF-α, IL-1β, and IL-6. (4) Dextran sulfate acts as a ligand for macrophage scavenger receptor class A, which is overexpressed by activated macrophages. Nanoparticles enter activated macrophages to release MTX and inhibit the release of TNF-α, IL-1β, and IL-6. Dex, dexamethasone; FA-PEG-PLGA, folic acid-poly (ethylene glycol)-poly (lactide-co-glycolide); FA-PEG-LA, FA-PEG-lipoic acid; GSH, glutathione; HA, hyaluronan; IL-1β, interleukin-1β; IL-6, interleukin-6; Mcl-1, myeloid cell leukemia-1; MTX, methotrexate; NF-κB, nuclear factor-κB; PD, prednisolone; ROS, reactive oxygen species; siRNA, small interfering RNA; SOD, superoxide dismutase; TNF-α, tumor necrosis factor α.

specific ligands, such as FRs and SR-As. It was observed that the FR, SR, CD44 receptor, and galactose receptor are overexpressed on the surface of macrophages, mediating the endocytosis of folate, acetylated low-density lipoprotein, hyaluronan (HA), and galactose. The nanodrug carriers can manipulate macrophages through conjugation with cell surface–specific ligands, thus exerting their therapeutic effects (Pirmardvand Chegini et al., 2018; Tardito et al., 2019).

FR. The FR is a receptor on the cell surface that can be used as a target site for the treatment of RA. There are four common subtypes of FRs, namely, FR-α, FR-β, FR-γ, and FR-δ. FR-α is used for the targeted therapy of tumors. FR-β is overexpressed by activated macrophages in inflammatory synovial tissues and has been used for the targeted diagnosis and treatment of RA (Kumar et al., 2019). Duan et al. designed the PEGylated liposome targeting macrophages; siRNA/calcium phosphate NPs were loaded in its core and the antirheumatic drug MTX was loaded in the lipid shell. The nanomedicine possessed better transmembrane transport capacity and delivery characteristics than other siRNA nanocarriers (e.g., polyethyleneimine, dendrimers, and chitosan). Moreover, the PEGylated liposome combined with folic acid (FA) could specifically target activated macrophages *via* FRβ. This resulted in silencing the expression of NF-κB p65 (a classical inflammatory signaling pathway) to downregulate inflammatory cytokines and promote the polarization of M1 macrophages to M2 for the treatment of RA (Duan and Li, 2018). Zhao et al. (2017) also used FA as a ligand to deliver nanomedicines to activated macrophages in inflammatory joints. The difference is that the investigators used the acid-sensitive carrier polyketide poly (cyclohexane-1,4 diyl acetone dimethylene ketal) (PCADK) for acid-responsive targeted drug delivery. As a pH-responsive material, PCADK is stable in a neutral environment, prolonging its circulation time in plasma. In contrast, it rapidly degrades in the acidic environment to release drugs. However, PCADK also has shortcomings. Owing to the strong hydrophobicity, the hydrolysis half-life of the nanocarrier is longer, complicating its removal from the body (Wang et al., 2015; Zhao et al., 2017). Later, researchers synthesized a novel polyketal (PK3) by adding diols with strong hydrophilicity during the reaction, which overcame the limitations of PCADK (Guo et al., 2016). Zhao et al. added PK3,

FA-PEG-PLGA, egg phosphatidylcholine, stearic acid-octa-arginine (Sta-R8), and MTX to the mixed solvent of dichloromethane and acetone to prepare hybrid pH-responsive lipid polymer NPs (Sta-R8-FA-PLPNs/MTX). PEG-modified NPs selectively accumulate in inflammatory joints, penetrate the cell membrane through the effects of Sta-R8, and are subsequently phagocytosed by activated macrophages through FR-mediated endocytosis. PK3 can also act as a pH-sensitive switch that degrades under acidic conditions, thereby releasing MTX to inhibit pro-inflammatory cytokines (Zhao et al., 2018). The myeloid cell leukemia-1 (Mcl-1) protein, a member of the antiapoptotic B-cell lymphoma-2 (Bcl-2) family of proteins, was overexpressed in synovial macrophages of patients with RA. Mcl-1 protects macrophages from apoptosis by blocking the activation of the proapoptotic molecule Bax. NPs can induce apoptosis by silencing the expression of the Mcl-1 protein in activated macrophages to exert therapeutic effects in RA (Xiang et al., 2018). Therefore, Sun et al. (2019) also used the same strategy as Zhao et al. (2018) for the delivery of Mcl-1/siRNA to induce apoptosis and exert its anti-RA effects. Moreover, scholars incorporated MTX (an antirheumatic drug) into FA-conjugated glycol chitosan NPs to treat RA. The NPs could induce the mitochondrial membrane potential, increase the levels of nitric oxide (NO), reduce the antioxidant status, and induce apoptosis in macrophages (Kumar et al., 2020). In addition to the acid-sensitive drug release of NPs in macrophages, some scholars also used GSH for redox-responsive drug release. Researchers used FA-modified silver NPs to actively target M1 macrophages, leading to the release of Ag^{+} under the action of intracellular GSH. This caused apoptosis in M1 macrophages and ROS clearance so as to allow M1-to-M2 repolarization, thereby alleviating inflammation to achieve higher efficacy and biosafety (Yang et al., 2021). This study is the first to use bioactive nanomaterials (without drug loading) for the manipulation of macrophages in the treatment of RA, providing a new idea for treating RA with nanomaterials. Superoxide dismutase (SOD) is a metal enzyme widely distributed in animals, plants, and microorganisms which can catalyze the disproportionation reaction of superoxide radicals in organisms. It is a natural scavenger of O_2 in the body that plays a significant role in the immune system, which is used to treat RA and other diseases (Wang et al., 2018). Srivastava et al. (2018) added propylene sulfide to Pluronic F-127 using the emulsion ring-opening polymerization method to prepare a propylene sulfide nano-matrix. Subsequently, they used the propylene sulfide nano-matrix and fiber disaccharide solution as raw materials to prepare a cellobiose-coated nano-matrix (CNM). Targeting ligand FA was efficiently conjugated to SOD using the linking agent 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide to prepare F-SOD, the CNM was dispersed in the F-SOD solution to adsorb SOD, and the cellobiose-coated FA-SOD NPs (FECNM) were prepared. The porous coating of cellobiose in the NPs exerts a strong adsorption effect on the enzyme and could effectively adsorb SOD. Simultaneously, the addition of propylene sulfide can improve the oxidation resistance of NPs; the oxidant is converted into peroxide, and the hydrophobicity is transformed into hydrophilicity. FECNM selectively accumulates in inflammatory joints through the "ELVIS" effect and targets activated macrophages *via* FRβ, delivering SOD to improve the antioxidant response of macrophages and reduce pro-inflammatory cytokines. The NPs can also be used as an enzyme library of acid-unstable enzymes in the controlled form for the efficient treatment of RA.

CD44 Receptor. CD44 is a type of adhesion receptor widely distributed in epithelial cells, activated lymphocytes, and tumor cells. It was found that the expression of CD44 in the inflammatory environment is increased in macrophages, fibroblasts, and lining cells. HA is a natural polysaccharide widely used in drug delivery systems or tissue engineering as the ligand for the CD44 receptor (Jiao et al., 2016; Pandey et al., 2017). Researchers have prepared HA-solid lipid NPs/prednisolone (HA-SLNs/PD) by wrapping GC PD in SLNs coated with HA. SLNs possess high biocompatibility, physical stability, and drug loading and the ability to protect unstable drugs from degradation. The inclusion of PD in SLNs can enhance the accumulation of drugs in arthritic joints, prolong circulation time in the blood, and reduce severe adverse effects. HA-modified NPs can enter the target cells through CD44 receptor–mediated endocytosis, thereby reducing joint swelling, bone erosion, and serum inflammatory cytokines in experimental animals with RA (Zhou et al., 2018). Other researchers also developed HA-modified NPs for delivering Dex. However, in terms of drug release, researchers mainly used the pH-sensitive polyketone PCADK as the acid-sensitive carrier to release Dex, thereby reducing the levels of inflammatory cytokines (Yu et al., 2019).

SR. SR is a macrophage surface glycoprotein that can effectively mediate the uptake of oxidized and acetylated low-density lipoproteins. At present, SR has been widely used in the treatment of atherosclerosis. Studies have revealed that it also plays a crucial role in RA. Researchers conjugated hydrophobic 5β-cholanic acid to a hydrophilic dextran sulfate (DS) skeleton to synthesize the amphiphilic DS derivative, which could self-assemble in the aqueous status. Next, MTX was loaded into DS NPs to prepare MTX-loaded DS NPs by dialysis. As the ligand for the SR-A receptor, DS has been utilized as a drug cargo to bind to the SR-A receptor on the activated macrophages. The study demonstrated that the MTX-loaded DS NPs could accumulate in the inflammatory site through SR-A–mediated endocytosis, releasing MTX to inhibit TNF-α, IL-1β, and IL-6 in RA (Heo et al., 2017). Other scholars have synthesized the macrophage-targeted DS–MTX conjugate (DS-*graft*-MTX) and the untargeted dextran–MTX prodrug (De-*graft*-MTX) to determine differences in the treatment of RA. Notably, the diameters of these two kinds of MTX micelles are < 100 nm to avoid the rapid elimination by the RES and to selectively concentrate on the inflammatory joints of RA through the "ELVIS" effect. As expected, the degree of accumulation and the anti-arthritic effect of DS-*graft*-MTX in RA were markedly

higher than those of De-*graft*-MTX (Yang et al., 2017). Based on these findings, active targeting of macrophages by receptors has substantial clinical advantages and application prospects in the treatment of RA.

Galactose Receptor. The galactose receptor is a C2 type lectin, which is also overexpressed on macrophages, monocytes, dendritic cells, hepatocytes, and other cells. It can mediate the binding, phagocytosis, and clearance of microorganisms by macrophages. Studies have found that this receptor can specifically recognize galactose, glucose, and their conjugates to mediate cellular phagocytosis and remove foreign bodies *in vivo*. Li et al. (2020) formed pH-sensitive galactose-based dextran-retinal (GDR) NPs, using a dual-loading strategy to combine all-trans-retinal (all-trans retinoic acid prodrug) with a dextran backbone through a pH-sensitive hydrazone bond; the NPs were modified with galactose. After self-assembly, TPT was encapsulated into the hydrophobic core by dialysis to prepare the dual drug–loaded NPs (galactose-based dextran-retinal-TPT). They determined that the NPs accumulated in the RA joints through the "ELVIS" effect and were subsequently uptaken by galactose receptor–mediated endocytosis to release TPT and all-trans-retinal, in turn suppressing the production of pro-inflammatory cytokines in macrophages. Interestingly, all-trans-retinal is oxidized to all-trans retinoic acid, which can reduce the infiltration of macrophages and inhibit the differentiation of osteoclasts; both play a synergistic role in the treatment of RA.

Non-Receptor Targeting Strategy. In addition to the active manipulation of macrophages *via* linking with macrophage receptors through ligands, studies have reported some non-receptor manipulation strategies for drug-loaded NPs. For example, albumin is the most abundant protein in the blood and is characterized by good stability, biocompatibility, and biodegradability and a high drug encapsulation rate. It was used as a drug delivery carrier material in the 1990s, widely employed in the treatment of cancer, RA, diabetes, and other diseases. Human albumin NPs are non-immunogenic, thereby avoiding recognition by the RES and prolonging their circulation time in the bloodstream to exert their therapeutic effects in RA joints (Tan and Ho, 2018; Liu et al., 2019; Lamichhane and Lee, 2020). Gong et al. (2019) dissolved Cel and soybean oil in methylene chloride to provide an organic phase and added HS15 to 20% human serum albumin (HSA) dispersed in distilled water to achieve an aqueous phase. Cel-HSA-HS15 NPs are prepared by adding the aqueous phase to the organic phase. Because of the "ELVIS" effect and the targeting ability of albumin, NPs can accumulate in the site of inflammation and are subsequently phagocytosed by the activated macrophages *via* macropinocytosis and clathrin-mediated endocytic pathways. The anti-arthritis drug Cel is released, manipulating them to inhibit pro-inflammatory cytokines (e.g., TNF-α and IL-1β). Of note, the drug-loaded NPs can also improve RA-related lung diseases. Yan et al. (2019) also used albumin as a nanomaterial for the delivery of PD and the monomer curcumin (CU) *via* a dual drug delivery strategy to manipulate macrophages in the treatment of RA. First, PD and CU were dissolved in acetone. Oleic acid (OA) was added to prepare PD-OA and CU-OA. PD-OA, CU-OA, purified yolk lecithin (E80), and cholesterol were dissolved in chloroform. Finally, HSA was added to prepare dual drug–loaded albumin NPs (N-PD/CU) by forming new disulfide bonds in the free sulfhydryl groups of albumin using the high-pressure homogenization method. These drug-loaded NPs can compensate for the poor bioavailability of PD and CU. The disulfide bond of NPs can rapidly release PD and CU in the presence of GSH. Interestingly, PD in NPs can reduce pro-inflammatory cytokines, while CU can increase the anti-inflammatory cytokine IL-10. Both play a synergistic anti-RA role by regulating the balance of pro- and anti-inflammatory cytokines. In the two studies above regarding albumin drug-loaded NPs, the researchers proposed that albumin NPs can accumulate in RA lesion sites through the "ELVIS" effect due to their non-immunogenic characteristics and can subsequently be phagocytosed by the activated macrophages, resulting in the manipulation of these cells. This strategy appears to be a passive targeting method. Nevertheless, Liu et al. investigated the drug delivery system based on albumin and suggested that the use of these drug-loaded NPs was an active targeting strategy. Researchers have confirmed that albumin nanocarriers can accumulate in RA synovial tissue due to the secreted protein acidic and rich in cysteine (SPARC). SPARC is a member of the extracellular matrix that is overexpressed in the synovial membrane of patients with RA and mice with collagen-induced arthritis. It has a strong intrinsic affinity for albumin, which can improve the enrichment of albumin carrier drugs in the RA synovial membrane and realize the active targeting of drug-loaded albumin NPs (Nakazawa et al., 2020). The study also proved that HSA, as a target, binds to SPARC; this leads to the accumulation of drug-loaded human albumin NPs in the arthritis synovium and the release of drugs under acidic conditions (Liu et al., 2019).

Indirect Manipulation Strategy

Indirect manipulation strategies have also been used in the treatment of RA. The NPs prepared by Zhou et al. do not directly manipulate macrophages by delivering drugs. Instead, they indirectly suppress inflammation through their transfer to angiogenic vessels to generate endothelial NO through a local nitrosative response. In their test, the researchers saponified fumagillin dicyclohexylamine salt to fumagillol. They subsequently esterified the product with 1-palmitoyl-2-azelaoyl-sn-glycero-3-phosphocholine to prepare the Sn-2 phospholipase labile fumagillin prodrug (Fum-PD). Finally, the Fum-PD was wrapped in Rvβ3 integrin–targeted perfluorocarbon NPs to prepare Rvβ3-targeted fumagillin prodrug–loaded perfluorocarbon (Fum-PD-FFC) NPs. Following its entry into endothelial cells, Fum-PD-FFC was cut by phospholipase at the Sn-2 site to release the active drug. Fum-PD induced the release of NO, which in turn activates AMP-activated protein kinase (AMPK), inhibits mammalian target of rapamycin (mTOR), enhances autophagy flux, and ultimately suppresses the NF-κB signaling pathway and the release of inflammatory cytokines (Zhou et al., 2014).

Inorganic Nanomaterials

In recent years, the development of inorganic nanomaterials has been rapid. These materials offer the advantages of easy structure adjustment and modification, superior chemical stability, good biological safety, and high drug-loading rate compared with the organic polymer nanomaterials widely used in targeted drug delivery, imaging diagnosis, and collaborative drug therapy. Researchers engineered gold (Au) nanomaterials to manipulate macrophages for the treatment of RA through intravenous administration. The results showed that Au-NPs could enter macrophages *via* a receptor-mediated, clathrin-dependent endocytosis pathway and inhibit thioredoxin reductase (TrxR), which is involved in the redox activity of macrophages. Inhibition of TrxR can induce oxidative stress and promote apoptosis in macrophages. The uptake of TrxR is greater than that of auranofin, which is transported into cells using the sulfhydryl shuttle model. Simultaneously, the surface of Au-NPs can be modified with a Au–sulfur (Au–S) bond *via* chemical grafting or electrostatic coating, which endows nanomaterials with multiple biological functions (James et al., 2015). In the study conducted by Pandey et al., the Au-NP was modified with a thiolated dendritic polymer to produce nanogold core dendrimer NPs (Au-DEN-NPs). The hydroxyl groups on the surface of the NPs were conjugated with MTX, and near-infrared active bioactive IR780 was encapsulated to offer a photothermal benefit. MTX is an FA analog with similar physical, chemical, and structural properties to those of FA. Therefore, researchers used MTX instead of FA as the target ligand to achieve selective localization through upregulated FA receptors in arthritis tissue. The near-infrared irradiation increased the temperature of the exposed environment and mediated the release of MTX. Simultaneously, the combination of MTX and ROS produced by IR-780 near-infrared laser irradiation exerts a synergistic effect to inhibit pro-inflammatory cytokines (Pandey et al., 2019). Metal-organic frameworks (MOFs) are organic–inorganic hybrid materials with intramolecular pores assembled from organic ligands, metal ions, or clusters through coordination bonds. As new nanomaterials, MOFs are characterized by high drug-loading capacity, simple preparation, and good biodegradability and are widely used in various fields (Ibrahim et al., 2017). Guo et al. (2021) encapsulated MTX into HA-modified MOFs to prepare MTX-loaded MOFs, which markedly accumulated in arthritis joints. Researchers used tannic acid as a linker in the drug-loaded NPs for conjugation with MTX through an ester bond to improve the stability of NPs. By interfering with the expression of TNF-α, IL-1β, and IL-6 in macrophages, NPs can alleviate inflammation and bone destruction.

Manipulation of Macrophages by Nanomaterials Administered Through Percutaneous Absorption

Percutaneous absorption represents an extremely attractive and innovative route of administration. It offers numerous advantages compared with oral and intravenous administration, namely, patient convenience, avoidance of first-pass metabolism and gastrointestinal tract irritants, maintenance of constant target drug concentration, and reductions in the frequency of administration and side effects (Qindeel et al., 2020b). However, due to the barrier function of the stratum corneum, penetration of the skin by traditional percutaneous absorption products may be difficult, thereby limiting their clinical use. Nanomedicine attracts considerable attention because of its advantages (i.e., improving chemical stability and promoting percutaneous absorption) and has thus become an ideal transdermal drug delivery method (Seah and Teo, 2018). Researchers developed polycaprolactone-PEG-polycaprolactone–based nanomicelles for the transdermal delivery of MTX. Subsequently, the nanomicelles were loaded into a carbopol 934–based hydrogel with eucalyptus oil to prepare an MTX nanomicelle–loaded hydrogel. As an enhancer of penetration, eucalyptus oil can improve the skin permeability and release of MTX. This allows MTX nanomicelles to selectively accumulate in inflammatory joints and be internalized by activated macrophages through clathrin- and scavenger receptor–dependent endocytic pathways (Qindeel et al., 2020a). In another study, researchers developed a hydrogel with co-incorporated chemical enhancers (CEs) and lipid nanocarriers for the efficient transdermal delivery of MTX (MTX-nanostructured lipid carrier gel). Surfactant Kolliphor® P188 was added to reduce the particle size and improve the encapsulation efficiency. The results confirmed that the nano-size of nanostructured lipid carriers could maintain close contact with keratinocytes. The synergistic effect of the CE and lipid mixture can enhance flux and facilitate percutaneous absorption (Garg et al., 2016). In addition, Gokhale et al. prepared quercetin (QCT)-loaded nanoemulsion (NE)-based gel (QCT-NE gel) for the treatment of RA. QCT-NE, prepared by spontaneous emulsification techniques, was dispersed in a carbomer 940 gel matrix to produce a QCT-NE gel. Next, Tween 20 and PEG-400 were added to increase the solubilization ability and the permeability coefficient of NE. Owing to its small droplet size, NE significantly increases the permeation rate since the nano-sized droplets can transfer the drug through the skin barrier and rapidly move into the stratum corneum. Furthermore, water in the gel system hydrates the skin, leading to cell expansion in the stratum corneum and broadening the drug channel, thereby improving cumulative penetration. Following the transdermal administration of NPs, QCT was released through skin-specific accumulation in the arthritis site to inhibit the release of pro-inflammatory cytokines from activated macrophages (Gokhale et al., 2019). At present, there are few studies on percutaneous absorption drug delivery pathways for the treatment of RA. Due to the skin barrier function, penetration and absorption through the skin may be challenging for most drug molecules. Mice are often selected for animal experiments in research concerning the percutaneous absorption drugs. However, because of the thin layer of the mouse skin and the differing

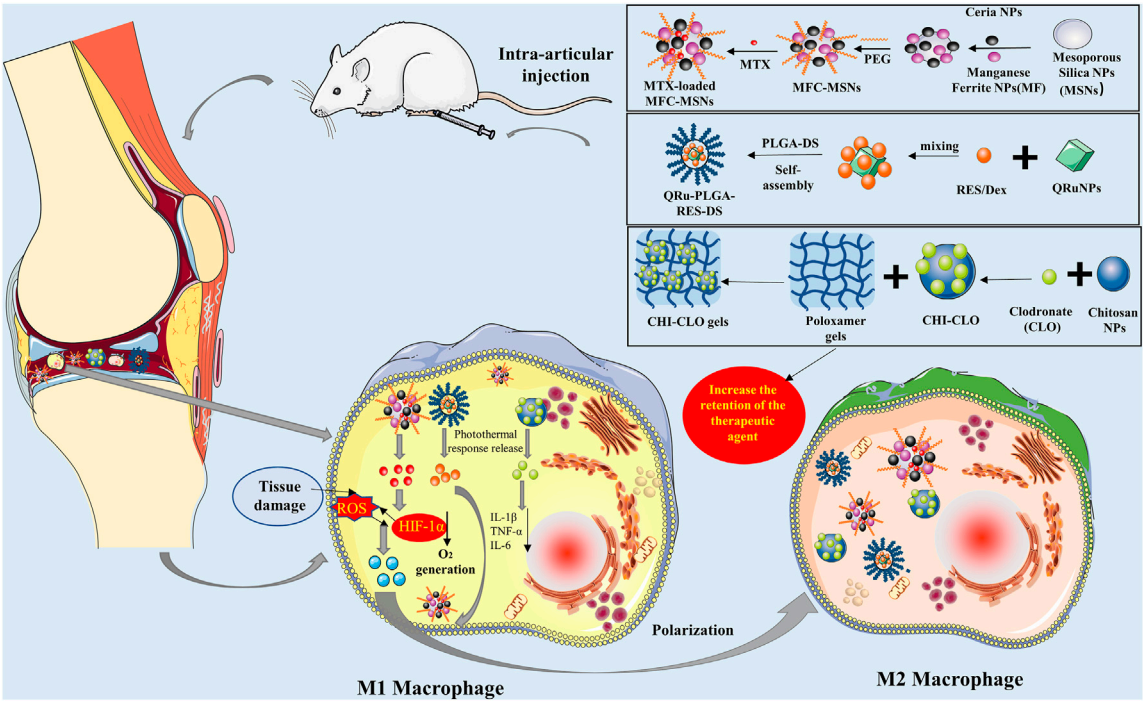

FIGURE 4 | Preparation and application of MTX-loaded MFC-MSN nanoparticles (NPs), QRu-PLGA-RES-DS NPs, CHI-CLO gels, and intra-articular injection of NPs into an RA model mouse. (1) MFC-MSNs synergistically scavenge ROS and produce O_2, leading to the polarization of pro-inflammatory M1 macrophages to the anti-inflammatory M2 phenotype in hypoxic and inflamed synovial joints. (2) As photothermal agents, the Ru NPs control the precise release of the RES through the photothermal effect and achieve high-efficiency polarization of M2 type macrophages for treating RA. (3) CHI-CLO NPs were added into the poloxamer gel matrix for intra-articular administration, which increased CLO retention in the joint, improved the therapeutic effect, reduced the side effects, and inhibited the release of IL-1β, TNF-α, and IL-6 in macrophages. CHI-CLO, chitosan-clodronate; IL-1β, interleukin-1β; IL-6, interleukin-6; MTX, methotrexate; MFC-MSNs, manganese ferrite and ceria nanoparticle–anchored mesoporous silica nanoparticles; PEG, poly (ethylene glycol); QRu-PLGA-RES-DS, quadrilateral ruthenium-poly (lactic-co-glycolic acid)-resveratrol-dextran sulfate; RA, rheumatoid arthritis; ROS, reactive oxygen species; Ru, ruthenium; TNF-α, tumor necrosis factor α.

characteristics from those of humans, animal models may not yield robust results.

Manipulation of Macrophages by Nanomaterials Administered Through Intra-Articular Injection

Intra-articular injection is currently a common method used in the treatment of RA; it changed the distribution pattern by directly delivering the drug in the synovial joint. This approach avoids physiological barriers during transport, ameliorating the dosage and safety profile of drugs (Rai and Pham, 2018). However, due to the fast metabolism of the joint cavity, the injected drug is rapidly cleared, resulting in a short retention time and short-acting effect. Thus, frequent injections are required to achieve therapeutic effects, leading to local pain, swelling, and infection. Owing to its targeting properties, the nanodrug delivery system can be selectively adsorbed on inflammatory synovial tissue, overcoming this defect (Rahman et al., 2017). The carriers used for intra-articular injection are mostly inorganic materials (**Figure 4**). Kim et al. synthesized manganese ferrite NPs *via* thermal decomposition of manganese oleate and iron oleate complex and dissolved cerium (III) acetate and oleylamine in xylene to synthesize ceria NPs. Finally, BMPA-capped manganese ferrite NPs were synthesized by dissolving BMPA, citric acid, and capped manganese ferrite NPs in a mixture of chloroform and N, N-dimethylformamide. Similarly, BMPA-capped ceria NPs were synthesized using the same method. Large pore-sized mesoporous silica NPs and MTX were added to these two NPs to prepare manganese ferrite and ceria NP–anchored mesoporous silica NPs. The NPs were directly injected into the articular cavity to manipulate macrophages through phagocytosis. Through the synergistic effect of manganese oleate, cerium, and MTX, these NPs inhibit the expression of hypoxia-inducible factor (HIF-1α), increase ROS clearance, release O_2, and induce the transformation of M1 macrophages to M2 macrophages (Kim et al., 2019). A multifunctional nanotherapeutic system based on another inorganic material, ruthenium (Ru), is also used to manipulate macrophages for the treatment of RA. Chen et al. (2019) formed QRu-PLGA-RES NPs by mixing the quadrilateral ruthenium (QRu) NPs, PLGA, and resveratrol; QRu and the heat-sensitive molecule PLGA were in the core and the shell of the NPs, respectively. Owing to the high affinity of DS for SR, NPs can be engulfed by activated macrophages to release resveratrol in the presence of exogenous light and induce the polarization of M1 macrophages to M2 macrophages. Recently, scholars used surface-modified nanodiamond (ND)

as a nanocarrier for the delivery of Dex in the treatment of RA. In their test, researchers reacted ND-COOH with thionyl chloride in N, N-dimethylformamide to synthesize ND-COCl, which was reacted with octadecylamine (ODA) to replace the -Cl group with a -nh2 group; finally, Dex was added to prepare ND-ODA-Dex. The surface modification of ODA can significantly increase the adsorption of Dex. ND-ODA-Dex can be engulfed by activated macrophages after intra-articular injection, thereby inhibiting the release of pro-inflammatory cytokines and promoting the polarization of M1 macrophages to M2 macrophages. Researchers also found that even in the absence of loaded drugs, ND-ODA also exerts a particular anti-inflammatory effect (Pentecost et al., 2017). Hydroxyapatite (HAP) is the main inorganic component of vertebrate bones and teeth, with excellent biocompatibility, bioactivity, and safety profile. Moreover, HAP is easily hydrolyzed under acidic conditions, enabling the use of nano-sized hydroxyapatite as a carrier for drug delivery (Ramesh et al., 2018). Pandey et al. (2021) incorporated MTX and teriflunomide into HA-coated HAP NPs for the treatment of RA. Modified HA can improve the encapsulation efficiency, hydrophilicity, and macrophage targeting of NPs. More importantly, HA can prolong the residence time and improve the timeliness of the drug because of its viscoelastic properties. As expected, the dual drug–loaded inorganic NPs can inhibit pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) and increase osteoblast and chondrocyte activity. In addition to inorganic material, researchers also use organic material nanocarriers to deliver drugs into the joint cavity for the manipulation of macrophages in the treatment of RA. Yeo et al. synthesized NO-scavenging polymer nano-gels for the efficient transdermal delivery of a NO-cleavable cross-linker, which reduces inflammation by scavenging NO *in vivo* (Park et al., 2017; Yeo et al., 2019). Jansen et al. (2019) also used organic nanomaterials to deliver therapeutic drugs through intra-articular injection. However, unlike other researchers, they targeted TNF-siRNA delivery, which results in the silencing of TNF in macrophages for the treatment of RA. Clodronate (CLO) is a first-generation bisphosphonate often used in patients with bone loss. It can inhibit the synthesis of inflammatory mediators and cytokines and induce apoptosis in macrophages. Russo et al. (2016) encapsulated CLO into chitosan NPs to prepare chitosan-CLO NPs, which were subsequently introduced into a poloxamer gel. Because of the targeting and retention in the inflamed region, the therapeutic effect of chitosan-CLO gel was markedly enhanced compared with that of pure CLO.

Manipulation of Macrophages by Nanomaterials Administered Through Intraperitoneal Injection

Owing to its easiness and rapidity, intraperitoneal injection is a widely used administration method in experimental animal research. Due to the large area of the peritoneal cavity, dense blood vessels and lymphatics have a strong absorption capacity, promoting the absorption of liquids. Thus, considering the damage caused to the abdominal blood vessels, intraperitoneal injection can lead to cumulative irritation; thus, it is rarely used in clinical practice. Occasionally, it can be used for special treatments in animal experiments (Yahyaei et al., 2019). As one of the traditional treatments for arthritis, Au reagents have valuable properties (e.g., biocompatibility and superficial modification), which inhibit the infiltration of mononuclear macrophages into synovial tissue. Moreover, Au can selectively accumulate in inflammatory synovial tissues and form Au-rich sediments. Therefore, scholars have suggested that macrophages can be an essential target for Au reagents. On account of their direct anti-arthritic effects, Au clusters can be efficiently used as a nanodrug even without drug loading (Khan and Khan, 2018; Yuan et al., 2019b). The strong positive charge of the Sv peptide can assist Au clusters in penetrating the cell membrane. Therefore, Yuan et al. anchored the Au clusters with the Sv peptide *via* strong Au–S bonds in aqueous solution under mild conditions to prepare Au25Sv9. It was found that Au25Sv9 selectively gathered in synovial tissues of animals with arthritis after intraperitoneal injection. This effect inhibited the receptor activator of RANKL and indirectly reduced the generation of osteoclasts and bone erosion. Of note, Au25Sv9 can effectively inhibit the activation of NF-κB induced by RANKL, directly inhibit inflammation-induced differentiation of osteoclasts, and block inflammatory bone destruction (Yuan et al., 2019a). The same research group also evaluated the effect of GSH as the template for ultrasmall Au nanoclusters to manipulate macrophages in the treatment of RA. The GSH molecules combine with these 29 Au atoms through the Au–S bond to form $Au_{29}GSH_{27}$. GSH, as a natural peptide with a small hydrodynamic diameter, is a suitable surface ligand of the Au cluster to improve pharmacokinetic characteristics *in vivo* (Gao et al., 2019). Jain et al. (2015) used peritoneal macrophages as drug-loaded carriers, acting as Trojan horse vectors to transport IL-10 plasmid DNA to the inflamed joint. The surface of the nanocarrier was modified with the four-amino acid peptide tuftsin, which promotes phagocytosis by binding with Fc and neuropilin-1 receptors on macrophages. As the peritoneal macrophages migrate to the site of arthritis, the drug-loaded NPs are also transported to promote the polarization of M1 macrophages to M2 macrophages, thereby reducing inflammation.

Manipulation of Macrophages by Nanomaterials Administered Orally

Chloroquine (CQ), an established antimalarial drug, exerts a specific immunoregulatory effect. Recently, CQ was used to treat immune-related diseases, such as RA, systemic lupus erythematosus, and solar erythema, and is thought to inhibit the expression of TNF-α in synovial tissue. Researchers encapsulated CQ into SLN, which was uptaken by the intestinal lymphatic region. Bhalekar et al. validated that the levels of TNF-α at the site of inflammation were reduced following the oral administration of CQ-SLN. In this study, they mixed Compritol 888 ATO with CQ to obtain a precise drug–lipid mixture and added span80 to prepare CQ-loaded SLN

(CQ-SLN). It was found that an increase in span80 led to an increase in the particle size and entrapment efficiency. NPs enter the systemic circulation through clathrin- and caveolae-mediated endocytosis in the intestinal lymphatic region, specifically accumulating in the arthritis joint and reducing the progression of the disease (Bhalekar et al., 2016). However, few studies have investigated the manipulation of arthritis synovial macrophages through oral drug-loaded NPs for the treatment of RA. The new delivery strategy based on the lymphatic uptake of drug-loaded NPs through oral administration provides novel insights and directions for researchers and clinicians.

Manipulation of Macrophages by Nanomaterials for the Diagnosis of RA

In patients with RA, the pathological damage to the structure of the joint cannot be reversed. Hence, early diagnosis and treatment of RA are extremely important, as they can delay and prevent its development and reduce damage to the joint. Recently, nanomaterials were used to manipulate macrophages for the early diagnosis of RA. The protein fibrin is a natural matrix for cell attachment, proliferation, and extracellular matrix formation during wound healing and bone formation. More importantly, fibrin NPs exhibit good biocompatibility, immunocompatibility, hemocompatibility, and biodegradability (Weisel and Litvinov, 2017). Periyathambi et al. used goat blood as a precursor to prepare fibrin. The fibrin was dissolved in NaOH, and the iron solution was added under vigorous stirring to form magnetic fibrin NPs (MFNPs). Subsequently, FA and MFNPs were combined to prepare FA-MFNPs by ethyl-3-(3-dimethylaminopropyl)carbodiimide/N-hydroxysuccinimide reaction. FA was modified to target FR-β on the membrane of macrophages. The results suggested that FA-MFNPs can be used as magnetic resonance imaging contrast agents to detect activated macrophages in the synovial tissue of joints, indicating early RA (Periyathambi et al., 2017). However, the researchers used goat-derived fibrin, which may not have immunogenicity. Thus, further experimental research is warranted to determine the usefulness of fibrin in this setting.

EFFECTS OF MACROPHAGE MANIPULATION BY NANOMATERIALS

Inflammatory Cytokines

In the pathogenesis of RA, a complex network is formed by the mutual regulation of various cytokines. Cytokines are small molecular proteins that act as important mediators of intercellular communication. They play a crucial role in response to various stimuli throughout the inflammatory process. The imbalance between pro- and anti-inflammatory cytokines is deemed responsible for the development of RA. Both the overexpression of pro-inflammatory cytokines and the insufficient formation of anti-inflammatory cytokines can easily lead to RA (Mateen et al., 2016; Chen Z. et al., 2019). Pro-inflammatory cytokines (IL-1, IL-6, IL-17, IL-8, IL-1β, etc.) are the main factors linked to RA. Thus, the inhibition of pro-inflammatory cytokines has been suggested as the primary approach to treating RA (Alam et al., 2017). GCs (PD and Dex), antirheumatic drugs (MTX), Chinese medicine monomers (CEL, TPT, and BAC), SOD, tacrolimus, CLO, QCT, CQ, and the NO-cleavable cross-linker are delivered by carrier nanomaterials for the manipulation of macrophages to reduce pro-inflammatory cytokines.

Macrophage Polarization

Macrophages are a group of highly heterogeneous cells. According to their polarization state, macrophages are divided into M1 macrophages and M2 macrophages. Macrophages are highly plastic, and their polarization is affected by a variety of cytokines in the microenvironment. The activated macrophages are termed M1 macrophages, which can induce T helper 1 cell activation, promote inflammation, and accelerate the elimination of intracellular pathogens. Uncontrolled over-activation of M1 macrophages will cause excessive inflammation and tissue damage (Sun et al., 2017; Tardito et al., 2019). Macrophages can be polarized into M2 macrophages by T helper 2 cell cytokines, such as IL-4 and IL-13. In contrast, as anti-inflammatory factors, M2 macrophages inhibit inflammation, thereby promoting tissue repair (Quero et al., 2017; Funes et al., 2018). Therefore, the induction of M1 macrophages to M2 macrophages has gradually attracted attention in the treatment of RA. Ag+, NF-κB p65 siRNA, MTX, RES, and IL-10 plasmid DNA can be delivered to induce polarization of M1 macrophages into M2 macrophages for the treatment of RA.

Macrophage Apoptosis

Apoptosis refers to the autonomous and orderly death of cells controlled by genes to maintain the stability of the internal environment, which is of great significance to multicellular organisms. In biological development, apoptosis enables the elimination of harmful cells (e.g., neoplastic and senescent cells) and ensures the development of tissues and the balance of the internal environment (Pistritto et al., 2016; Yang et al., 2020). Lack of apoptosis may lead to increased numbers of macrophages in RA. Therefore, inhibiting the activation and promoting the apoptosis of macrophages may be an effective method for the treatment of RA (Henc et al., 2017; Yang et al., 2021). The delivery of Mcl-1 siRNA, Cel, and MTX discussed in this review can inhibit the activation of macrophages, induce macrophage apoptosis, and indirectly result in a superior anti-arthritic activity.

BIOSAFETY OF NANOMATERIALS

A variety of nanomaterial-based nanocarriers have been used for the macrophage manipulation in the treatment of RA. Typical nanocarriers were constructed using the inorganic materials, organic materials, or endogenous biomimetic materials which possess different sizes, structures, and surface characteristics. These nanocarriers can promote the specific accumulation of drugs in the inflammation site to enhance the anti-RA effects of

different drugs. Recently, the biosafety of nanomaterials has attracted much attention from pharmacologists as an important impact factor of nanomedicine, which requires that the nanosystem possess high macrophage selectivity in the inflammatory location of RA with low toxicity. By far, there are no approved assessment criteria on the biosafety of nanomaterials. The evaluated biosafety results of nanomaterials are always obtained based on the *in vitro* or *in vivo* animal study; however, it is difficult to infer the security of nanomaterials in the body (Su et al., 2018).

Size is the most important parameter in toxicity evaluation of nanocarriers. Compared with large-size nanoparticles, nanoparticles with a smaller size might possess lower security, because of which they can easily penetrate the skin and reach various organs such as the lungs and the brain. Other factors such as the shape and surface charge of nanocarriers and the route of administration also affect the biosafety of nanomaterials (Bianco et al., 2017). For example, positively charged or cationic nanocarriers possess greater toxicity than other nanocarriers, and intravenous administration of nanocarriers has more side effects than oral administration.

Nanomaterials produced from biological materials always have no obvious toxicity for the body; however, it is also not easy to eliminate from the body when it acts as a nanocarrier because of the extensive tissue distribution, which therefore might result in *in vivo* toxicity (Zielińska et al., 2020). Most of the inorganic or organic nanocarriers (such as liposomes, micelles, dendrimers, mesoporous silica nanoparticles, gold nanocarriers, super paramagnetic iron oxide nanoparticles, etc.) are obtained by chemical synthesis. Thus, their cell compatibility, blood compatibility, and good immune compatibility remain to be further investigated. Interestingly, the endogenous biomimetic nanomaterials possess excellent biocompatibility, which can greatly reduce the immunogenicity of the nanocarriers, thus improving the biological safety of the produced nanomedicine (Hossen et al., 2019).

By far, the biosafety evaluation of nanocarriers is still a challenge that needs to be further explored. In addition, the technology of the extraction and separation of cell membranes or membrane-like structures to obtain the endogenous biomimetic nanomaterials still needs to be verified in clinical settings. Together, the endogenous biomimetic nanomaterials possess higher biological security than other nanomaterials and might act as the ideal nanocarrier with drug encapsulation to manipulate macrophages for RA treatment.

CONCLUSION

The pathogenesis of RA is complicated and, currently, there is no treatment that can completely cure the disease. NSAIDs, DMARDs, GCs, biological agents, and other treatments can only relieve pain, prevent disease relapse, reduce articular damage, and improve physical function and quality of life. Nanomaterials have been widely used in the treatment of RA owing to their ability to improve the bioavailability of drugs and promote clinical efficacy. In recent years, studies have found that the abnormal metabolism of macrophages is involved in the pathogenesis and development of RA. Therefore, the use of nanomaterials to target synovial macrophages, induce macrophage polarization and apoptosis, inhibit the production of pro-inflammatory cytokines, and regulate the function of macrophages to treat autoimmune diseases (e.g., RA) has become a research hot spot. Although targeted agents have achieved success in the treatment of RA, various targeted carriers are characterized by multiple limitations, such as minor drug loading, poor stability, immature preparation techniques, lack of pharmacokinetic models, immature quality of evaluation indexes, insufficient effectiveness, and a poor safety profile. The distribution of carriers in non-targeted tissues may lead to toxicity. Surface PEGylation of nanomedicines can avoid recognition by the RES, thereby extending their circulation time in the blood and increasing their accumulation in inflammatory sites. However, it is terrible for the infiltration of nanomedicine in tissues, internalization, and lysosome escape. Most procedures for the preparation of nanomedicines are complicated, and the clinical cost is high. Although exogenous biomaterials are nontoxic to the human body, they may also lead to immune rejection. Future research should focus on overcoming these shortcomings. This article mainly presents the progress achieved in the research on nanomaterials targeting macrophages for the treatment of RA, including the role of macrophages, the shortcomings of current drugs and the advantages of nanomedicines, and the methods and effects of the manipulation of macrophages by nanomaterials. This review provides new directions for the targeting of macrophages in the treatment of RA.

AUTHOR CONTRIBUTIONS

J-XL and WC conceived the idea and designed the experiment; SL collected and analyzed the literature; SL prepared the manuscript; J-XL and JS revised the manuscript; and J-XL, WC, and JS reviewed the manuscript.

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REVIEW
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The Advancing of Selenium Nanoparticles Against Infectious Diseases

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Infectious diseases, caused by the direct exposure of cellular or acellular pathogens, are found to be closely associated with multiple inflammation and immune responses, keeping one of the top threats to human health. As an indispensable trace element, Selenium (Se) plays important roles in antioxidant defence and redox state regulation along with a variety of specific metabolic pathways. In recent decades, with the development of novel nanotechnology, Selenium nanoparticles (Se NPs) emerged as a promising agent for biomedical uses due to their low toxicity, degradability and high bioavailability. Taking the advantages of the strong ability to trigger apoptosis or autophagy by regulating reactive oxygen species (ROS), Se NPs have been widely used for direct anticancer treatments and pathogen killing/clearance in host cells. With excellent stability and drug encapsulation capacity, Se NPs are now serving as a kind of powerful nano-carriers for anti-cancer, anti-inflammation and anti-infection treatments. Notably, Se NPs are also found to play critical roles in immunity regulations, such as macrophage and T effector cell activation, which thus provides new possibilities to achieve novel nano-immune synergetic strategy for anti-cancer and anti-infection therapies. In this review, we summarized the progress of preparation methods for Se NPs, followed by the advances of their biological functions and mechanisms for biomedical uses, especially in the field of anti-infection treatments. Moreover, we further provide some prospects of Se NPs in anti-infectious diseases, which would be helpful for facilitating their future research progress for anti-infection therapy.

Keywords: selenium nanoparticles, infectious diseases, pathogens, anti-infection therapy, nano-immune synergetic strategy

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INTRODUCTION

Infectious diseases, induced by deadly pathogens like Covid-2019, *Mycobacterium tuberculosis* and *Staphylococcus aureus* (*S. aureus*), are still major threat to public health with high infectivity and mortality worldwide. Current chemotherapeutic methods have contributed largely to the control of infectious diseases, however, the frequent use of antibiotics with low targeting effects always lead to low treatment efficiency and promoted drug resistance (Ji et al., 2016; Huang et al., 2017). Moreover, the biofilms of multidrug resistant bacteria are always resistant to antibiotics, which results in the need of more powerful therapies (Hoiby et al., 2010; Hoiby et al., 2011). Thus, how to enhance the efficiency of current therapeutics against infectious diseases becomes an emerging urgent issue to global public health.

Selenium is a crucial trace element for maintaining human health through the selenoproteins, antioxidant defense, cell signal transduction, immune regulation and other metabolic processes (Labunskyy et al., 2014). Previous studies have reported that the deficiency of selenium is closely associated with the high morbidity of cancer, infectious diseases, and cardiovascular diseases (Rayman, 2012; Hatfield et al., 2014; Liu et al., 2017). The organic and inorganic selenium compounds that are widely served as food additives. However, with a relative narrow safety at the therapeutic dosage, excessive Se intake can result in unexpected toxic effects (Rayman, 2012).

In the past decade, prompted by the rapid nanotechnology developments, selenium nanoparticles (Se NPs) have attracted extensive attention from researchers in biomedical fields due to their exclusive physical, chemical and biological properties (Skalickova et al., 2017; Menon et al., 2018). Compared with traditional organic and inorganic selenium compounds, Se NPs show numerous advantages including low toxicity, high degradability, excellent anticancer, antimicrobial and antiviral activities (Wadhwani et al., 2016; Hosnedlova et al., 2018). Furthermore, in order to kill cancer cells more efficiently, Se NPs can be furnished as delivery carriers to encapsulate drug or biomacromolecules for chemotherapy (Maiyo and Singh, 2017; Guan et al., 2018). Khurana et al. have reviewed the recent progress and potential therapeutic benefits of Se NPs in various oxidative stress and inflammation mediated disorders like arthritis, cancer, diabetes and nephropathy, as well as the discussions of the significance for the pharmacological activity of Se NPs (Khurana et al., 2019). However, the reviews and discussions for the synthesis of Se NPs, and their anti-infection applications remain to be further emphasized.

More interestingly, Se NPs are also capable of targeting macrophages and regulating macrophage polarization to initiate innate immunity for antimicrobial inhibition by regulating the production of cytokines (Pi et al., 2020). Se NPs could also act as an immunomodulatory agents to inhibit tumor growth by enhancing anti-tumor immune responses, such as regulating tumor-associated macrophages and activating specific T cells (Gautam et al., 2017; Hu et al., 2019). These immunological functions further indicate the potential use of Se NPs as immunomodulatory agents for pathogen defense, thus contribute to the immune therapy of infectious diseases. In this review, we summarize the methods of synthesis and bio-activity of Se NPs, followed by the recent progress of Se NPs for anti-infection treatments, which are expected to facilitate their future research progress for anti-infection therapy.

SYNTHESIS OF SELENIUM NANOPARTICLES

Compared with traditional organic or inorganic selenium compounds, the chemical structure of Se NPs is more complicated. Numerous factors should be taken into consideration when the Se NPs are designed and synthesized for biomedical application, including size, shape, composition, surface property and dispersion. Thus, it is of vital importance to develop novel Se NPs with controllable size distribution, functional agents, morphological characteristics and surface properties.

Physical, chemical and biological techniques are three most widely used approaches for the synthesis of Se NPs. With vitamin C, sodium sulfite, sodium thiosulfate and hydrazine as commonly reducing agents, Se NPs are always prepared by chemical reduction method, which is considered as the most convenience method for Se NPs preparation. In addition, Hydrothermal method, template method, laser ablation method, and biosynthesis method are also successfully applied for Se NPs preparation.

Hydrothermal Method for Selenium Nanoparticles Preparation

The hydrothermal synthesis was developed by Niu et al. With some advantages such as low cost, simple and efficient preparation for the synthesis of crystalline selenium nanostructures (Niu et al., 2012). Typically, an optically polished bulk glass made of GeSe3 is placed in a container filled with deionized water. The sample is hydrolyzed at the reaction temperature, with the release of Se atoms and fragments from GeSe3 in the solution where they form a colloidal suspension of amorphous selenium. Then, Se nanospheres can form a more stable hexagonal crystalline phase and the polycrystalline (t-Se) nanospheres can be observed *via* dissolution recrystallization. Se NPs with different diameters ranging from 10 to 1,000 nm can be obtained using this hydrothermal method.

Using similar hydrothermal method, Shar et al. also prepared Se NPs using sodium selenite as a precursor and L-ascorbic acid as reducing and stabilizing agent, which showed very good hexagonal shape with a clean and smooth surface and revealed very narrow size distribution ranging from 100 to 200 nm (Shar et al., 2019). Shin et al. also demonstrated the reduction of sodium selenite to form elemental selenium nanoparticles using cellulose nanocrystal (CNXL) as a reducing and structure-directing agent under hydrothermal conditions, which prepared Se NPs of 10–20 nm in diameter (Shin et al., 2007). These works suggested that hydrothermal synthesis of Se NPs able to produce functional Se NPs with different shapes and diameters, however, most of the prepared Se NPs using this method was aimed for industry application.

Selenium Nanoparticles Prepared by Template Method

Se NPs can be formed from selenium element in the guidence of some chemical templates as stabilizers. PEG200 was introduced as a kind of template and surface decorator for Se NPs synthesis (Zheng et al., 2012). Firstly, gray Se was dissolved in PEG200 solution at 210–220°C for 15–20 min, followed the addtion of water at a ratio of 1:1. The solution was centrifuged at 10,000 rpm for 10 min and then washed with Milli-Q water for five times to clear the excess PEG. The as-prepared PEG-Se NPs displayed

monodisperse and homogeneous spherical structures with an average diameter of about 5 nm.

Based on chemical reductions, template method is most widely used in the preparation of Se NPs. Using different templates, Se NPs with different size, shapes, and surface properties can be obtained. Hu et al. introduced sulfate polysaccharidesv (SPS) as template to prepare Se NPs using a one-step method (Hu et al., 2020). Polysaccharides were dissolved in DMSO and stirred at room temperature overnight to improve their solubility. Then, sulfur trioxide-trimethylamine (STMA) was added to prepare the SPS at a certain temperature. The resultant solution was cooled to room temperature, neutralized with sodium hydroxide and dialyzed against distilled water for 5 days. Sodium selenite and ascorbic acid aqueous solution with 4:1 molar ratio were added to the prepared SPS solution at room temperature. The reaction product was dialyzed against distilled water and freeze-dried to obtain size controlled stable SPS-Se NPs, with diameter ranging from 54.35 to 123.04 nm.

As the mostly widely used method, the reduction mechanism of sodium selenite by ascorbic acid is listed as below:

$$H_2SeO_3 + 2C_6H_8—Se\downarrow+2C_6H_6O_6+3H_2O$$

During the formation of Selenium element, the presence of some templates, also recognized as stabilizers, would lead these Selenium element to form nano-sized particles in aqueous solutions. A lot of chemicals can be used as templates for Se NPs preparation, such as chitosan (Bai et al., 2017), folate (Pi et al., 2013), hyaluronic acid (Zou et al., 2019),polyethylenimine (PEI) (Li et al., 2016)and ferulic acid (Cui et al., 2018b). In terms of the low cost, convenient procedure and controllable size, these template methods thus offer novel design of Se NPs with high efficacy.

Laser Ablation Method for Selenium Nanoparticles Preparation

Using laser to irradiate the pure selenium pellet in the bottom of microcentrifuge tube, Guisbiers et al. have successfully prepared Se NPs to inhibit the bacteria growth (Guisbiers et al., 2016). With quick pulse duration and high repetition rate, the laser beam was focused on the surface of the selenium pellet. The irradiation time was fixed at 15 min with a wavelength of 355 nm to produce a more stable colloidal solution. Meanwhile, the conical shape of the cuvette helps to reduce the amount of water required in the vessel, remaining enough height of water above the target to prevent evaporation during irradiation. Compared with other methods, laser ablation reveals many advantages for synthesis of Se NPs, including the reduction of contamination with chemical reagents, low cost for equipment and easy collection of the produced nanoparticles.

Using the similar pulsed laser ablation method, 248 and 532 nm lasers were used to produce Se NPs with different size (Altuwirqi et al., 2020). Menazea et al. synthesized polyvinyl alcohol/chitosan doped selenium nanoparticles *via* one step laser ablation route, which significantly improved the antibacterial activity of their pure blend (Menazea et al., 2020). Additionally, Guisbiers et al. firstly introduced the synthesis of Se NPs by femtosecond pulsed laser ablation at 800 nm in de-ionized water (Guisbiers et al., 2017). The obtained Se NPs have been successfully used to inhibit the formation of Candida albicans biofilms as they can easily adhere on the biofilm, then penetrate into the pathogen, and consequently damage the cell structure by substituting with sulfur. These works further indicated that laser ablation method was very suitable for the preparation of anti-bacterial Se NPs.

Biosynthesis of Selenium Nanoparticles

Physical and chemical methods have widely developed and used in Se NPs synthesis. However, these methods always require specific environments (such as temperature and laser) and unwanted chemical reagents, which may result in environmental pollution and unwanted toxicity of produced Se NPs (Wadhwani et al., 2016). With growing global environment awareness, the development of environmentally friendly green synthesis has received widespread attention in biomedical fields. Nowadays, a large number of plants are reported for Se NPs synthesis, such as hawthorn fruit (Cui et al., 2018a), lemon leaf (Prasad et al., 2013), *Hibiscus sabdariffa* (roselle plant) leaf (Fan et al., 2020), *Clausena dentata* leaf extract (Sowndarya et al., 2017), *Theobroma cacao* L. bean shell extract (Mellinas et al., 2019), gallic acid (GA) from various fruits and plants (Zhou et al., 2016), and so on.

Furthermore, many bacteria and fungi are also found to synthesize Se NPs based on their ability to reduce selenite to elemental selenium, including Rhodococcus aetherivorans BCP1 (Presentato et al., 2018),*Azoarcus* (Fernandez-Llamosas et al., 2016), *Acinetobacter* (Wadhwani et al., 2018), *Enterococcus faecalis* (Shoeibi and Mashreghi, 2017), *Streptomyces* sp. ES2-5 (Tan et al., 2016), *Pseudomonas aeruginosa* ATCC 27853 (Kora and Rastogi, 2016), Edible *Lentinula edodes* (Vetchinkina et al., 2013). Combining the ability of reduction from plants and microbes with eco-friendly biosynthetic techniques, biosynthesis of Se NPs have presented non-toxicity, lower price, higher stability and biocompatibility than physical and chemical methods. Such biogenic Se NPs are displaying great potential for anti-bacterial and anti-cancer treatments.

BASIC BIOLOGICAL FUNCTIONS OF SELENIUM NANOPARTICLES

As a kind of novel nanomaterials, Se NPs not only display excellent physical and chemical properties, but also exert powerful biological activities for anti-cancer, anti-infection treatments. Herein, we summarized the basic biological functions of Se NPs, such as induce apoptosis and autophagy, act as drug delivery system and protect chemotherapy induced side effects (**Figure 1**), as well as their immunomodulation effects (**Figure 2**).

Selenium Nanoparticles Induced Cell Apoptosis

Apoptosis, an active mode of cell death, is a genetically regulated suicide mechanism that plays an important role in the

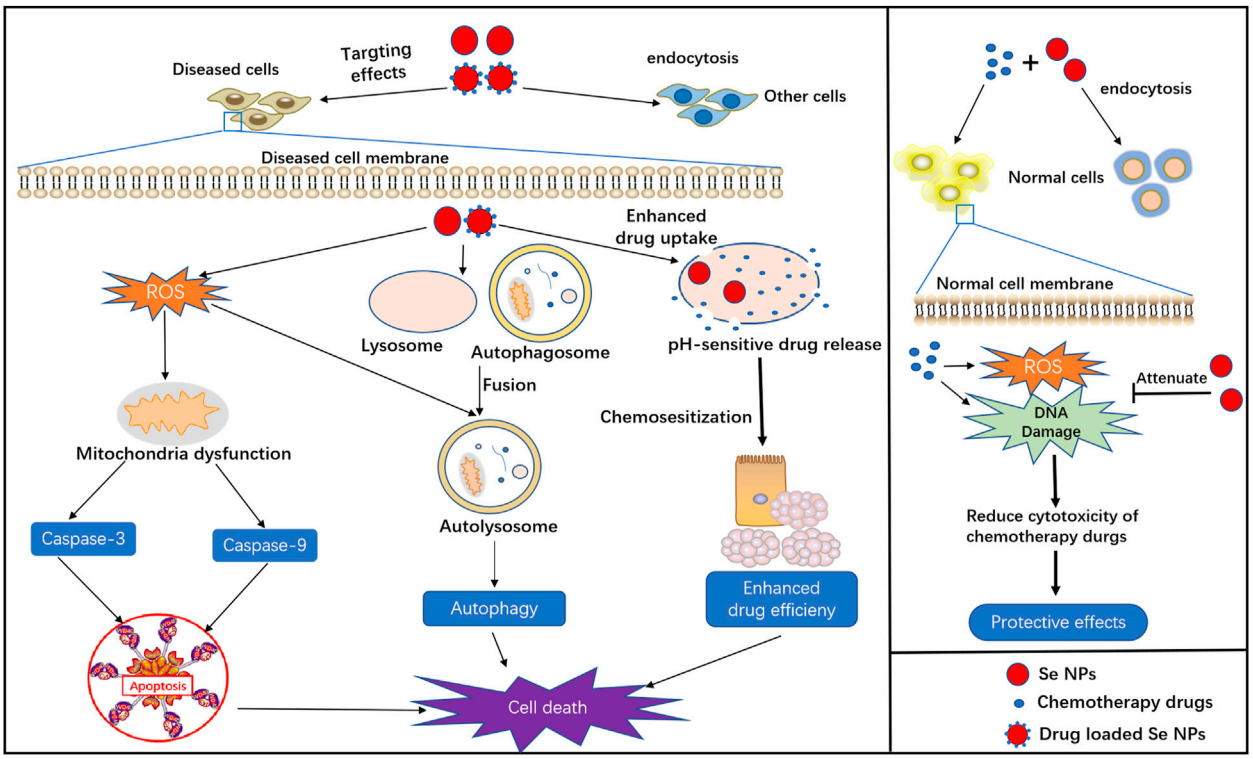

FIGURE 1 | Basic biological functions of Se NPs, including apoptosis and autophagy induction, drug delivery, chemosensitization and protective effects in chemotherapy.

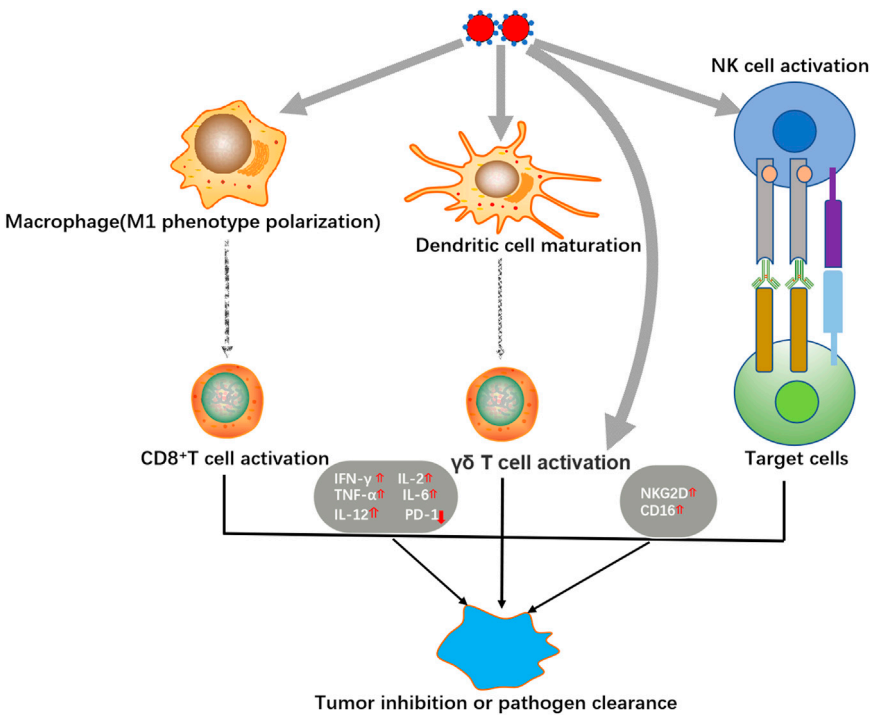

FIGURE 2 | Immunomodulation activities of Se NPs.

development and defense of muticelluar organisms (Zhang et al., 2013). Many studies have reported that ROS formation is involved in the process of apoptosis and closely related to the electron respiratory chain of mitochondria. Generally, Se NPs are found to produce excess ROS for apoptosis induction through the blockage of electron respiratory chain. Therefore, the up-regulated intracellular ROS generation induced by Se NPs are capable of inducing cell apoptosis and cell cycle arrest, which is helpful for facilitating cancer cell death. Furthermore, apoptosis can also manifest killing effects towards cancer cells through extrinsic or intrinsic pathways (Menon et al., 2018). The extrinsic pathway can be initiated by attachment of a pro-apoptotic ligand to death receptor, which triggers caspase 8-mediated apoptosis. Whereas, the intrinsic pathway is activated within the cell through caspase 9 due to DNA damage or cell oxidative stress. Thus, excessive ROS production that damages the DNA and induces apoptosis is always responsible for the cytotoxic effects of Se NPs(Wang Y. et al., 2015).

The most widely application of Se NPs induced apoptosis is to kill cancer cells with the participation of some important signaling events, such as ROS generation, anti-apoptotic gene down-regulation, pro-apoptotic gene up-regulation and caspases activation (Nie et al., 2016; Bidkar et al., 2017; Cui et al., 2018b). Internalized Se NPs can quickly and apparently initiate extrinsic signaling pathways such as DISC/caspase-8/caspase-3 signaling, to promote the most common form of physiological cell death-apoptosis. Meanwhile, through the cell cycle analysis, Se NPs are also found to induce apoptosis with the involvement of G2/M phase arrest in a dose-dependent manner. Moreover, overproduction of ROS could lead to mitochondria dysfunction, such as the disruption of mitochondria membrane potential (MMP), which may contribute to the activation of mitochondrial apoptosis pathway. The overproduced ROS could always increase cytochrome c to induce the activation of caspase-9, which acts as the vital pro-apoptotic protein and promotes the downstream caspase-3 activation, leading to the intrinsic apoptosis. Such strong ability to kill cancer cells with low cytotoxicity against normal cells by Se NPs, would contribute to the inhibition of *in vivo* tumor growth (Xia et al., 2018; Song et al., 2020).

Li et al. investigated the anti-cancer effects and mechanisms of Galangin functionalized Se NPs(Se@Ga) on human hepatoma HepG2 cells (Li et al., 2018b). With relatively high cytotoxicity and anti-proliferative effects, Se@Ga could effectively inhibit HepG2 cell proliferation, which was associated with apoptosis induced by ROS generation. These results provide valuable strategies on anticancer treatment for exploring the mechanism of Se NPs induced apoptosis in HepG2 cells. And using transferrin (Tf)-conjugated Se NPs loading with doxorubicin, Huang et al. demonstrated the strong ability of Tf-Se NPs to activate caspase-3, caspase-8, caspase-9 associated apoptosis (Huang et al., 2013; Wang Y. et al., 2015). Importantly, some anti-apoptotic genes of Bcl-xl and ERK were suppressed, while pro-apoptotic genes of p38, p53 and Bad were up-regulated. Wang et al. found that selenium-substituted hydroxyapatite (HA) nanoparticles could inhibit Cdk1 protein expression, arrest the cell cycle at the S-G2/M phase, and accelerate the DNA damage of cancer cells, leading to tumor necrosis (Yanhua et al., 2016). Additionally, when entering into HCC cells, these prepared Se NPs could be dissolved by lysozyme to release lots of calcium ions that could destroy the cell membrane and facilitate the ROS generation induced cell death.

And except for the ability to kill cancer cells, apoptosis is also a common pathway for the destruction of intracellular bacteria, which is very important for the host immunity defense against bacterial infection (Behar and Briken, 2019). During microbial infection, apoptotic cell death is generally beneficial for the host and detrimental for the pathogen, as it can avoid the pathogens to exit the host cells for further disperse. However, some pathogens have tremendously stable cell walls that are unlikely to be damaged by apoptosis, and some pathogens may also have the ability to inhibit host cell apoptosis as a critical way for their immune escape (Behar and Briken, 2019). Therefore, it would be a great idea to clear the intracellular bacterial pathogens by inducing apoptosis in infected cells, which provides the possibility to enhance host cell immunity by regulating apoptosis (Bewley et al., 2014; Cui et al., 2016; Lee K.-I. et al., 2020). Our recent work also demonstrated the ability of Se NPs to induce Mtb-infected macrophage apoptosis, which was helpful for the antimicrobial immunity and intracellular Mtb clearance and killings (Pi et al., 2020).

Selenium Nanoparticles Promoted Cell Autophagy

As a catabolic process, autophagy is necessary and beneficial for cell homeostasis as it could prevent the toxic protein aggregation, remove damaged organelles and provide cell and organism with bioenergetic substrates for survival (Doherty and Baehrecke, 2018). However, excessive autophagy that is correlated with cell apoptosis could consume the cellular organelles, causing irreversible disorder of functions in cells and even leading to cell death (Chen et al., 2018).

Autophagy can suppress or promote tumors depending on the developmental stage and tumor type. Modulating autophagy for cancer treatment is an attractive therapeutic strategy currently under intense investigation (Antunes et al., 2018). Apart from the promotion of cell survival, autophagy can also initiate the apoptotic signaling pathways to induce cancer cell death, which provide a novel idea in nanoparticle-induced cytotoxicity. Se NPs have been found to regulate autophagy in different type of cancer cells, which are always associated with cancer cell apoptosis. The intracellular autophagy initiator Beclin-1 was found to be significantly up-regulated to increase LC3-II expression and decrease p62 expression in the first 24 h of Se NPs treatment, indicating that Se NPs could facilitate the formation of autophagosome and promote the process of autophagy by regulating autophagy proteins. Besides, autophagy may synergistically promote apoptosis to induce cancer cell death in a time and dose manner upon Se NPs treatment. Excessive activation of autophagy could lead to mitochondrial dysfunction, which would eventually induce apoptosis of tumor cells (Huang et al., 2018; Huang et al., 2019). However, some studies have also demonstrated that Se

NPs play a role in inhibiting autophagy to reduce the resistance of tumor cells. The levels of p62 and Beclin-1 was significantly increased after treatment with Se NPs for 12 h, suggesting that the early phase of autophagy was activated but the late phase of autophagy was blocked. These results indicated that autophagy related to the self-production mechanisms of tumor cells was inhibited by Se NPs. Moreover, though the evaluation of lysosomal acidity, Se NPs were found to reduce the fusion between autophagosomes and lysosome or suppressed the degradation of lysosomes, which ultimately inhibited the late stage of autophagy (Cui et al., 2019). Such autophagy promoting or blocking mechanisms in different cell models induced by Se NPs further confirmed the anticancer strategy by regulating cancer cell autophagy.

It's also important to note that autophagy can regulate immunological functions that influencing pathogen infection and pathogen survive in host cells. The host cells tend to kill the intracellular pathogens by autophagy pathways, but some bacteria have developed diverse strategies to avoid autophagy by interfering with autophagy signaling or the autophagy machinery (Huang and Brumell, 2014). And in some cases, pathogens can even exploit autophagy for their growth (Huang and Brumell, 2014). Thus, how to regulate host cell autophagy for intracellular pathogen clearance remains a big challenge. Some interesting ideas to kill intracellular pathogens by inducing autophagy has presented the attractive prospect of autophagy-associated anti-bacterial strategy (Tindwa et al., 2015; Biering et al., 2017; Lee H. J. et al., 2020). We have also demonstrated the possibility of using Se NPs to promote autophagy in Mtb-infected macrophages, which lead to the enhanced intracellular Mtb inhibition, thus providing novel method for intracellular Mtb clearance (Pi et al., 2020).

Drug Delivery by Selenium Nanoparticles

Taking the advantages of targeted drug delivery and controlled drug release, functional nanosystems provide novel therapeutic strategies for disease treatment. With low toxicity, high bioavailability and biocompatibility, Se NPs can be conjugated with different kind of agents for targeted drug delivery. Drugs can be loaded into Se NPs at high concentrations than their intrinsic solubility, which significantly enhanced their anticancer effects (Liu et al., 2012; Guan et al., 2018). Compared with individual agents, Se NPs showed higher selectivity to cancer cells with high bioactivity with increased drug solubility and targeting effects, and finally resulted in the increased drug efficiency and reduced side effects (Chen et al., 2008).

Xia et al. used galactose (GA) modified Se NPs as doxorubicin (DOX) delivery system with active tumor-targeting property (Xia Y. et al., 2019). And in order to reduce the side effects of chemotherapeutic drugs, Liu et al. prepared 5-fluorouracil surface-functionalized selenium nanoparticles (5FU-Se NPs), which significantly enhanced anticancer efficacy via the induction of caspase-dependent apoptosis (Liu et al., 2012). Zhang et al. have developed an injectable Se NPs nanosystem based on the thermosensitive hydrogel PLGA-PEG-PLGA to load sorafenib (SOR) as effective drug release library for both *in vitro* and *in vivo* tumor inhibition (Zheng et al., 2019). Our previous works also demonstrated the potential of GE11 peptide conjugated Se NPs as a delivery system to enhance the cancer targeting effects and solubility of oridonin, which dramatically enhanced the anticancer effects of Oridonin both *in vivo* and *in vitro* (Pi et al., 2017).

Additionally, the drug delivery capacity of Se NPs can also be applied to enhance the killing efficiency of drugs against bacteria. Liu et al. introduced ciprofloxacin loaded engineered selenium lipid nanocarriers as effective drug delivery system for preventing lung infections of interstitial lung disease (Liu et al., 2019). Our recent work also introduced the use of Se NPs as macrophage-targeted delivery system to enhance the intracellular Mtb killing efficiency of Isoniazid (Pi et al., 2020), indicating that Se NPs could also serve as drug delivery system for intracellular pathogen clearance.

Immunomodulation of Selenium Nanoparticles

Se NPs have been proved to exhibit strong immunomodulatory activity by regulating different immune cells or modifying some important immune-associated signaling events (**Figure 2**). With the rapid development of chimeric antigen receptor T-cell (CAR-T) therapy, immune therapy has emerged as a promising new treatment for malignant tumors (Neelapu et al., 2018). Taking the advantage to strengthen the anti-tumor cytotoxicity of immune cells, Se NPs have been proved to benefit immune therapy of tumor.

Wang et al. fabricated a novel immunogenic core-shell Au@Se NPs to activate anti-tumor immunity by synergetic manipulation of Se NPs-mediated chemotherapy and Au NSs-induced photothermal therapy (Wang J. et al., 2020). The *in vivo* results indicated that Au@ Se NPs not only generated the anti-tumor immune responses with excellent cancer killing effect under the presence of tumor-associated antigens, but also effectively transformed the tumor associated macrophages (TAMs) from M2 to M1 phenotype. These effects could further promote T cell activation for tumor rejection, which also contributed to phagocytosis of the distant tumor. Hu et al. introduced the effects of Se NPs to up-regulate the expression of cytotoxicity related molecules including NKG2D, CD16, and IFN-γ in $\gamma\delta$ T cells, meanwhile, downregulate PD-1 expression in $\gamma\delta$ T cells, which significantly enhance the cancer killing effects and *in vivo* tumor growth inhibition (Hu et al., 2019). These application of Se NPs for innate and acquired immunity modulation strongly suggest the potential use of Se NPs as immunoregulator against cancer.

Infectious diseases are always associated with multiple immunological responses, thus providing the possibility to treat infectious diseases by modulating immunity. Dietary chitosan-selenium nanoparticle (CTS-Se NP) have been proved to enhance immunity and disease resistance in zebrafish against bacterium *Aeromonas hydrophila* infection (Xia I. F. et al., 2019). With the stimulation of lipopolysaccharide (LPS) and concanavalin A (ConA), zebrafish splenocytes exhibited higher proliferation after treatment of CTS-Se NP. And the immune response of splenocytes against ConA was found to be associated with the up-regulation in IL-2 and IL-12 production. Our work also demonstrated the ability of Se NPs to inhibit Mtb-lysosome

escape, and promote the host antibacterial immunity to induce host cell apoptosis, autophagy, and M1 anti-bacterial polarization, which significantly enhanced the intracellular Mtb killing efficiency (Pi et al., 2020). These works collectively suggest that Se NPs could be served as novel immunomodulator against different bacteria infection, which therefore provides new possibilities for infectious diseases treatment.

Antimicrobial and Antiviral Activity of Selenium Nanoparticles

Se NPs have aroused widespread interest due to their powerful antimicrobial activity. With the enhanced release of selenium ions to destroy the bacteria structure, Se NPs can be used to prevent multidrug-resistant bacterial infections, which therefore shows promising potential as antibiotic alternative (Lin et al., 2019). Moreover, chitosan coupled Se NPs have possessed efficient dose-dependent inhibition against *C. albicans* biofilm, further confirming the fungicidal effects of Se NPs (Lara et al., 2018). Besides, biosynthesized Se NPs could effectively suppress the growth of type-1 dengue virus, indicating the antiviral property of Se NPs (Ramya et al., 2015). These works strongly suggest that Se NPs can be served as a reliable agent to directly anti-bacterial and anti-viral treatment, which we will further discuss in the following section.

Selenium Nanoparticles Induced Chemosensitization

Drug resistance is a major challenge for cancer and infectious disease therapies, which results in treatment failure. As a kind of novel drug delivery system, Se NPs not only enhance the targeting effects of drugs, but also present the strength to increase the drug sensitivity for anticancer and anti-infection treatment (Liu et al., 2012; Pi et al., 2017; Xia Y. et al., 2019; Zheng et al., 2019; Pi et al., 2020). Ahmed et al. showed that Se NPs could potentiate the cancer inhibition effects of 5-fluorouracil (FU)-encapsulated PLGA nanoparticles for enhanced chemo-sensitivity (Abd-Rabou et al., 2019). These functions were found to be associated with the regulation of antioxidant activity by selenium *via* glutathione peroxidases and thioredoxinreductases, which was considered to be one of the main mechanisms of Se NPs for cancer therapy. An *in vivo* study also indicated that administration of Se NPs along with Cyclophosphamide caused more significant reduction in tumor volume, packed cell volume, viable tumor cell count, and increased the survivability of the tumor-bearing hosts (Bhattacharjee et al., 2017). Such interesting ability of Se NPs thus provide novel possibility to use Se NPs for enhanced anti-infectious diseases treatment, although lots of works need to be done.

Protective Effects of Selenium Nanoparticles

With low toxicity and high bioavailability, Se NPs have the ability to protect normal cells from the cytotoxic effects of common chemotherapeutic agents. For instance, Rezvanfar et al. have found that Se NPs may be helpful to prevent cisplatin -induced gonadotoxicity through antioxidant capacity (Rezvanfar et al., 2013). With cisplatin and Se NPs treatment, serum testosterone, sperm quality, and spermatogenesis in rats were significantly improved. And cisplatin-induced free radical toxic stress and spermatic DNA damage were also reduced in male rats by Se NPs treatment, which significantly alleviated the toxicity of cisplatin. Li et al. also introduced the use of Se NPs to achieve enhanced antioxidant activity and antagonis against cisplatin-induced nephrotoxicity, which indicated the attractive potential of Se NPs in prevention of cisplatin-induced renal injury (Li et al., 2011). These works suggest that Se NPs could also act as a kind of protective agents in chemotherapy to reduce the side effects, which might provide new solutions for the current strong side effects of antibiotics against infectious diseases.

ANTI-INFECTION APPLICATION OF SELENIUM NANOPARTICLES

Taking the advantages of smaller size and higher surface area that can facilitate the reactions with biological molecules, Se NPs have also gained lots of attention for anti-infection treatment. The common mechanism of biofilm disruption is beneficial to inhibit the pathogen growth (Vallet-Regi et al., 2019). Besides, microbial resistance is generally correlated with the cell wall and cell membrane that form a rigid defensive barrier towards environmental aggression. The increased Se ions can not only disrupt the cell walls, but also destroy the integrity of cell membranes, which would result in intracellular homeostasis damage and microbial dysfunction, thereby leading to the death of microbial cells (Baptista et al., 2018). To further understand the emerging roles of Se NPs against infectious disease, we therefore summarized the anti-infection of Se NPs against pathogenic bacteria, fungi, virus and parasite (**Figure 3**).

Anti-Bacterial Activity of Selenium Nanoparticles

Bacteria are small organisms that can invade the body, although most of them are harmless, and some actually help to digest food, destroy disease-causing microbes, fight against cancer cells, and provid essential nutrients. However, a small fraction of harmful bacteria are capable of crowding out healthy bacteria, growing in sterile tissues and emiting toxins, which causes complicated bacterial infection diseases. Although most of the bacterial pathogens can be successfully controlled by current antibiotics, some extremely cunning bacteria or drug-resistant bacteria are still serious threatening to human health. As a kind of potent antibacterial agents, Se NPs can inhibit a panel of nosocomial infection caused by pathogenic bacteria (Hariharan et al., 2012), which introduces Se NPs a potential candidate as antibacterial agents or chemosensitizer for enhanced bacteria killing.

Broad-Spectrum Anti-Bacterial Effects of Selenium Nanoparticles

Selenium status may affect the function of cells in both adaptive and innate immunity, therefore shows the ability of selenium to control bacterial infection. Additionally, selenium compound has also been

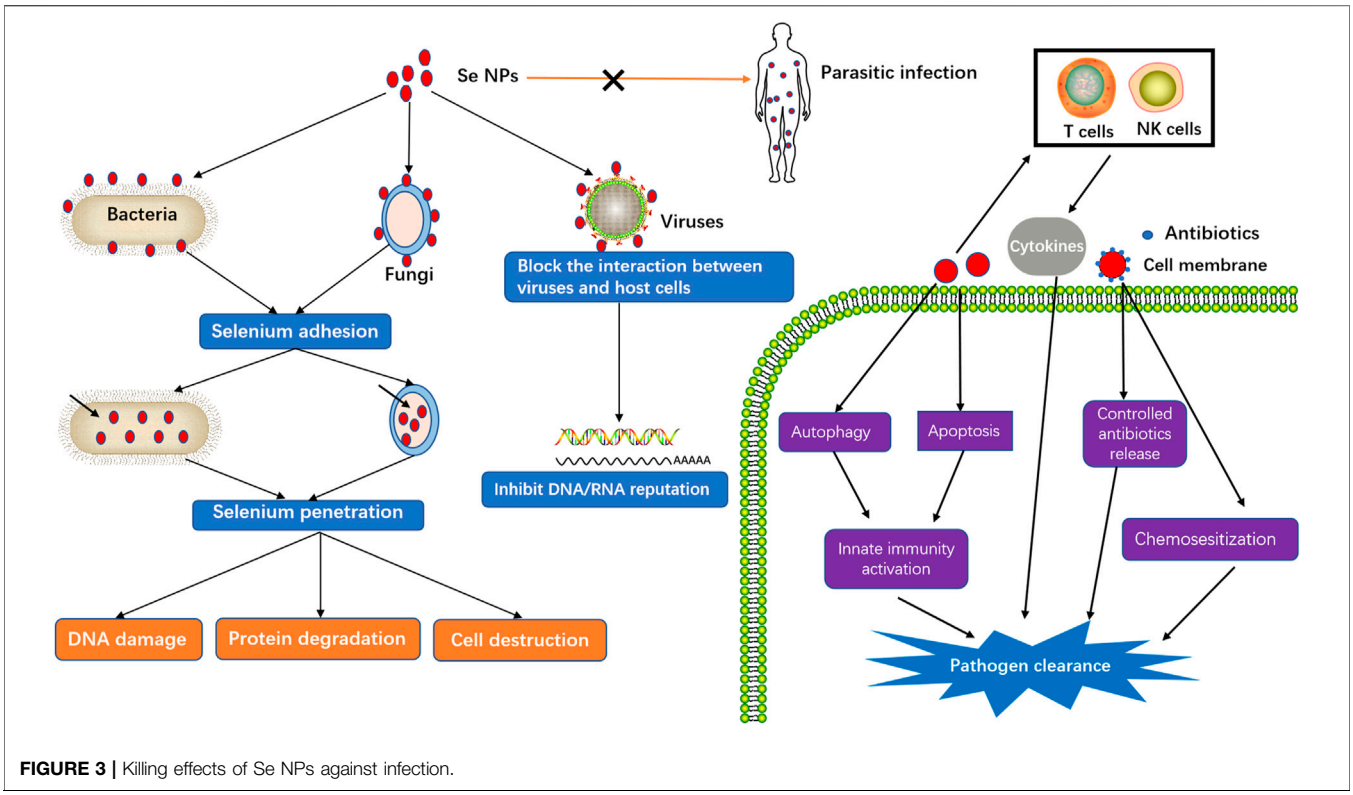

FIGURE 3 | Killing effects of Se NPs against infection.

found to show strong bacteria inhibition effects on different bacterial pathogens (Narayanan et al., 2018) (Pellissery et al., 2019). As a kind of novel selenium form with stronger biological activities than selenium compounds, Se NPs are also widely reported to show wide-spectrum antibacterial activity (Wang Q. et al., 2015; Guisbiers et al., 2016; Liu et al., 2016; Tran et al., 2016; Medina Cruz et al., 2018; Yazhiniprabha and Vaseeharan, 2019). Moreover, owing to the ability to adsorb proteins, restrain the biofilms, disrupt the membrane, regulate bacterial genes and reduce the free intracellular thiol, Se NPs have exhibited efficient antimicrobial properties *in vivo* and vitro studies.

Staphylococcus aureus (*S. aureus*), one of the typical Gram-positive bacteria, is the major antibiotic-resistant pathogen inducing skin infections, pneumonia, enteritis and other deadly diseases (Oliveira et al., 2018). It has been proved that *S. aureus* can be significantly killed by Se NPs in few hours, which introduced the possibility of Se NPs for direct Gram-positive bacteria killing (Tran and Webster, 2011). And additionally, the adherence on different surfaces and the biofilm formation of *S. aureus* could also be significantly inhibited by Se NPs (Cihalova et al., 2015; Sonkusre and Singh Cameotra, 2015). These results suggested that Se NPs might also be used for medical device coating to serve as an alternative approach for prevention of biofilm related infections.

Furthermore, Se NPs also showed strong antibacterial activity against Gram-negative such as *Escherichia coli* (*E. coli*). Biogenic Se NPs without any cytotoxicity exerted protective effects on intestinal barrier dysfunction caused by Enterotoxigenic E.*coli* K88, which played an essential role in promoting the growth of intestinal epithelial cells and maintaining intestinal microflora balance (Xu et al., 2018). These inhibition effects of Se NPs against E.*coli* might be closely associated with the ability of Se to reduce exopolysaccharide (EPS) synthesis, inhibit biofilm formation, and inactivate the mature *E. coli* biofilms (Nair et al., 2018).

Besides, some other Gram-positive bacteria, including *Staphylococcus epidermidis* (Tran et al., 2019), *Bacillus substilis* (Chandramohan et al., 2019), *Enterococcus faecalis*, *Streptococcus mutans* (Yazhiniprabha and Vaseeharan, 2019), and some other Gram-negative bacteria including *Pseudomonas aeruginosa* (Srivastava and Mukhopadhyay, 2015; Liu et al., 2019) can also be suppressed by Se NPs, which strongly suggested the wide-spectrum antibacterial activity of Se NPs.

Drug-Resistant Bacteria Inhibition by Selenium Nanoparticles

The threat of antimicrobial resistance is a worsening problem in recent decades not only in public health, but also in economic and social impacts, which requires the development of new drugs for more effective treatments. Nanomedicines have attracted increasing attentions for fighting against bacterial resistance to offer a chance of biofilm internalization, prolong antibiotic release, increase targeted delivery effects and improve systemic circulation of antibiotics (Eleraky et al., 2020). The strong ability of Se NPs to inhibit bacterial growth also provide new strategies against drug-resistant bacteria infections.

When combined with some antibacterial components, Se NPs would show more potent antibacterial activities. Lysozyme is a

biomolecule that has been widely distributed in humans, vertebrates, plants, bacteria and phages, which plays an important defensive role in the innate immune system and direct bacteria killings. Considering the natural antimicrobial effects of lysozyme, as well as the promising antimicrobial potential of Se NPs, Vahdati et al. investigated the interactions between Se NPs and lysozymes, and also determined their combined anti-bacterial effects (Vahdati and Tohidi Moghadam, 2020). Se NPs play an important role in inhibition of bacterial growth at very low concentrations of lysozyme, whereas very high amount of the lysozyme is required to inhibit bacterial growth individually. These results indicate the potentials to design Se NPs-based nanohybrid systems with synergistic antibacterial properties to overcome the emerging antibiotic resistance as well as to define fruitful applications in biomedicine and food safety.

Huang et al. reported that Se NPs exhibited strong antibacterial activity against eight bacterial species, including Gram-positive, Gram-negative, and drug-resistant strains (Huang et al., 2020). Furthermore, unlike the conventional antibiotic kanamycin, Se NPs did not readily induce resistance in *E. coli* or *S. aureus*, indicating the potential use of Se NPs to delay drug resistance. Lin et al. established a kind of novel Se NPs system for methicillin-resistant *Staphylococcus aureus* (MRSA) treatment combining the advantages of natural red blood cell membrane (RBCM) and bacteria-responsive gelatin nanoparticles (Lin et al., 2019). The obtained Se NPs were helpful for the escape of nanoparticles from the immune clearance and neutralization bacterial exotoxins. gelatin nanoparticles could be degraded by gelatinase in pathogen-infected areas *in situ* for controlled Se NPs release, which could induce robust ROS generation to destroy bacterial cell membrane effectively.

These works suggest that Se NPs might be a promising antibiotic alternative to combat different kinds of bacteria, including the threatening multidrug-resistant bacteria. The strong effects of Se NPs to destroy cell wall structures for direct bacteria killing or induce anti-bacterial metabolites for intracellular bacteria killing have been well understood. However, some critical activity of Se NPs, such as drug delivery, chemosensitization or immunomodulation remain to be further investigated for anti-bacteria treatment.

Intracellular Mycobacterium Tuberculosis Clearance by Selenium Nanoparticles

Tuberculosis (TB), caused *Mycobacterium tuberculosis* (Mtb), has become one of the top killers among infectious diseases. In recent decades, the occurrence of drug-resistant TB cases become an emerging issue, which requires the development of new alternative treatments beyond the current antibiotics or novel techniques to enhance the efficiency of current antibiotics. Estevez et al. found that Se NPs were able to inhibit the growth of Mtb by damaging their cell envelope integrity, which indicated a new opportunity for the use of Se NPs as antimycobacterial agents by themselves, or for the development of novel nanosystems that combine the action of these Se nanoparticles with other drugs (Estevez et al., 2020).

Up to now, the immune escape of Mtb from phagolysosomal destruction and limited drug delivery into infected cells remain biggest challenges for TB and drug-resistant TB treatments. To synchronously solve the above issues, we combined our decade-long nanotechnology and TB immunology expertise to innovate the macrophage-targeted Se NPs for synergistic antimicrobial and bactericidal destruction of Mtb in host cells (Pi et al., 2020). The mannosylated Se NPs could not only kill Mtb directly, but could also serve as excellent carriers delivering isoniazide specifically into macrophages for enhanced intracellular Mtb killing. More importantly, Se NPs were proved to inhibit Mtb-lysosome escape, dramatically promote the fusion of Mtb into lysosomes to initiate lysosomal clearance of intracellular Mtb. Additionally, more host cell antimicrobial immunity against Mtb, including autophagy, apoptosis and M1 anti-bacterial polarization, were activated for enhanced intracellular Mtb killing. This work demonstrates the potential of Se NPs to establish macrophage-targeted synergetic bactericidal strategy with wide-range innate immunity functions and considerable low cytotoxicity. And also suggest that Se NPs may potentially serve as more effective therapeutics against TB and multidrug-resistant TB.

During the evolution together with human beings in thousands of years, Mtb has become one of the most outstanding and clever bacterial pathogens by its multiple ways to escape from immunological clearance. How to countercharge the immune escape of Mtb remains a substantial challenge for TB or drug-resistant TB treatment. Our works firstly reported that Se NPs, a kind of novel anti-bacteria agent, possessed the ability to regulate host cell immunity for intercepting Mtb immune escape (Pi et al., 2020). These results strongly suggest that Se NPs could not only serve as direct bacteria killing agent or drug delivery system, but could also be used to regulate host immunity for enhanced intracellular bacteria clearance.

Anti-Viral Infections by Selenium Nanoparticles

Virus, the most dangerous pathogens that causing millions of deaths every year, are now inducing more and more deaths due to the epidemic of COVID-19 virus. How to develop more effective treatments for virus infection becomes the most urgent demand for human health. Selenium has long been found to be directly involved in fighting against viruse infections, such as influenza virus (Yu et al., 2011), Hepatitis virus (Himoto et al., 2011), coxsackie virus (Beck et al., 1995), West Nile virus (Verma et al., 2008), and human immunodeficiency virus (HIV) (Stone et al., 2010). These anti-viral effects are not only associated with the direct virus killing, but also related to its roles in regulating the function of selenoproteins, which introduces Se NPs as ideal antiviral candidates with wide-spectrum antiviral activity (He et al., 2021).

Selenium Nanoparticles Induced Influenza Virus Inhibition

Different kinds of influenza virus are responsible for the seasonal flu epidemics each year, therefore severely threatening human health. Due to the low toxicity and excellent activity, the

antiviral capabilities of Se NPs have attracted increasing attention in recent years. Li et al. introduced oseltamivir decorated Se NPs for H1N1 virus treatment, which significantly interfered the binding of H1N1 influenza virus to host cells through inhibiting the activity of hemagglutinin and neuraminidase (Li et al., 2017). Se NPs could prevent H1N1 from infecting MDCK cells and causing cell apoptosis by blocking chromatin condensation and DNA fragmentation, along with the inhibition of ROS generation and activation of p53 phosphorylation and Akt (Li et al., 2018a). Additionally, they further demonstrated that Se NPs could protect cells and lung tissues from H1N1 virus induced damages by restraining apoptotic signal events (Lin et al., 2018). Moreover, Wang et al. also demonstrated that Se NPs could inhibit H1N1 influenza virus-induced apoptosis by inhibiting ROS-mediated AKT and p53 signaling pathways (Wang C. et al., 2020).

These works collectively suggest that Se NPs could inhibit H1N1 influenza virus-induced apoptosis as novel anti-influenza agents, which might contribute to the control of H1N1 influenza both *in vitro* and *in vivo*. However, it's worth to note that Amir et al. investigated the efficacy of hexanic extracts of Ficus carica and olive fruit and Se NPs on the immunogenicity of the inactivated avian influenza virus subtype H9N2 in broiler chickens, which indicated that the prepared Se NPs emulsions could elicit a little degree of immunity, but they could not inhibit the anamnestic response and infection (Asl Najjari et al., 2015). In spite of no significant inhibition on H9N2 infection by Se NPs was confirmed by this work, the positive effect on the immunogenicity were found by Se NPs treatment, which as worth for further investigation.

Enterovirus Inhibition by Selenium Nanoparticles

As the most common pathogens leading to severe cases of hand, foot, and mouth disease (HFMD), Enterovirus 71 (EV71) can induce different clinical symptoms and even death among infants and children under 6 years old. Unfortunately, at present, no effective treatment for EV71 is available, which requires the development of effective treatment strategies. Zhong et al. developed Se NPs as the carrier of oseltamivir to assess the anti-EV71 activity, which apparently enhanced the antiviral effect of oseltamivir to suppress EV71 proliferation and impede cell apoptosis by reducing the caspase-3 activity and ROS generation (Zhong et al., 2019). Lin et al. developed a Se NPs system with siRNA targeting EV71 VP1 gene, which indicated a remarkable interference efficiency in the nerve cell line SK-N-SH and prevented the cells to be infected and restrained host cell apoptosis induced by EV71 (Lin et al., 2020). Taken together, these works demonstrated that Se NPs could serve as a promising drug candidate and drug delivery system against EV71 virus infection, providing the possibilities for the control of EV71 infection.

Hepatitis Virus Suppression by Selenium Nanoparticles

Hepatitis virus (HV) infection is one of the most serious and prevalent health problems worldwide. The emergence of current anti-HV medications contributes to the slow down of HV induced epidemic, however, some drawbacks including adverse effects and drug resistance are requiring novel agents for more effective and safe treatments. Sodium selenite was found to suppress Hepatitis B virus (HBV) protein expression, transcription, and genome replication in hepatoma cell models in a dose- and time-dependent manner (Cheng et al., 2016), which proved that there is a close relationship between selenium and virus susceptibility. This work firstly confirmed the suppression effects of Selenium on HBV replication and indicated the potentials of Se NPs for HBV treatment.

The prevalence of HBV infection has been significantly reduced by the approved HBV vaccine, which induces strong Th2 responses against that could protect HBV infection. However, there is a vital need to stimulate Th1 prophylactic immune response for more effective control of HBV infection. Mehdi et al. introduced a novel strategy by administration of Se NPs and the HBs antigen vaccine, which could affect lymphocyte proliferation and total antibody responses, and more importantly, could increase IFN-γ level and induce Th1 response (Mahdavi et al., 2017). These immunologic results clearly showed that Se NPs possessed the ability to polarize immune system toward a Th1 pattern and thereby increase the efficacy of vaccines against viral pathogens specifically controlled by cellular immune responses.

Potential Application of Selenium Nanoparticles in COVID-19 Epidemic

COVID-19 is a widespread, highly contagious and extremely dangerous disease that has caused millions of deaths in the past year. The point-of-care tests for COVID-19 detection is of vital importance for its epidemic prevention and epidemiological investigation. Wang et al. presented a lateral flow immunoassay kit based on Se NPs-modified SARS-CoV-2 nucleoprotein, which detected anti-SARS-CoV-2 IgM and anti-SARS-CoV-2 IgG in human serum by the naked eye within 10 min (Wang Z. et al., 2020). This work demonstrated that the Se NPs based lateral flow kit could conveniently, rapidly, and sensitively detect anti-SARS-CoV-2 IgM and IgG in human serum and blood, highlighting the use of Se NPs for COVID-19 diagnosis and epidemiological investigation.

It's very interesting that the cure rates in some places are found to be significantly correlated with Selenium intake levels (Moghaddam et al., 2020), which suggests the potential of Selenium for COVID-19 treatment. More importantly, organic Selenium species have been proved to inhibit COVID-19 by covalently binding to the COVID-19 virion Mpro through cell membranes, which results in effective inhibition of COVID-19 infected Vero cells (Jin et al., 2020). Additionally, the ability of Se NPs to inhibit different virus and boost innate and acquired immunity provide more possibilities to use Se NPs as novel anti-viral agents for COVID-19 treatment. However, there are still few works concerning the anti-viral effects of Se NPs against COVID-19, more studies are needed to confirm the role of Se NPs in COVID-19 treatments (He et al., 2021).

Anti-Fungi Activity of Selenium Nanoparticles

Fungi are ubiquitous and form their own kingdom to threat human health as infections would affect various parts of different species and bodies. Causing a spectrum of diverse diseases, *Candida albicans* is a major opportunistic fungus that very difficult to be controlled. Its biofilms coated by an exopolymeric substance or extracellular polymeric substance matrix can protect the pathogen from adverse environmental conditions, fungicides and hosts' immunity (Mathé and Van Dijck, 2013). Guisbiers et al. investigated the effects and potential mechanism of Se NPs for inhibiting *Candida albicans* biofilms (Guisbiers et al., 2017). After adhesion on the biofilm, Se NPs could penetrate into the pathogen and then disrupt the cell structure by substituting with sulfur, which induced 50% suppression of *Candida albicans* biofilm at low Se NPs dosage. Aiming at the treatment of Candida and Aspergillus infections, Shakibaie et al. produced biogenic Se NPs to show the strong inhibitory effect on *Candida albicans* and *Aspergillus fumigatus* (Shakibaie et al., 2015). Additionally, Joshi et al. reported that mycogenic Se NPs displayed antifungal activity against *Colletotrichum capsici* and *Alternaria solani*, which highlighted the practical application of Se NPs to manage plant diseases in an ecofriendly manner (Joshi et al., 2019). The broad spectrum antifungal activity of Se NPs against different fungi provides new weapons for the arsenal against fungi induced infectious diseases.

Anti-Parasite Effects of Selenium Nanoparticles

In addition to possessing antimicrobial effect towards bacteria, virus and fungi, Se NPs also present antiparasitic properties. Biogenic Se NPs were found to display powerful cytotoxicity in killing promastigote and amastigote forms of Leishmania. Major, which suggested that Se NPs could be emerged as a promising therapeutic agent for curing cutaneous leishmaniasis (Beheshti et al., 2013). Se NPs have also been proved to show potent scolicidal effects against Echinococcus granulosus, therefore may be used in Cystic echinococcosis surgery (Mahmoudvand et al., 2014). However, the *in vivo* efficacy of Se NPs remains to be further explored.

Se NPs are found to be more effective than sodium selenite with regard to their anti-coccidial, anti-oxidant, anti-apoptotic and anti-inflammatory role against Eimeria parasite in the jejunum of mice (Alkhudhayri et al., 2018; Alkhudhayri et al., 2020). Furthermore, Dkhil et al. demonstrated the protective roles of Se NPs in mice infected with *Schistosoma mansoni*, indicating that Se NPs could possess therapeutic anti-schistosomal activity in the treatment of intestinal schistosomiasis (Dkhil et al., 2019). Additionally, biogenic Se NPs were also found to show anti-Toxoplasma effects against Toxoplasma gondii in mice with no considerable toxicity, demonstrating the therapeutic effects of Se NPs for toxoplasmosis *in vivo* (Keyhani et al., 2020a; Keyhani et al., 2020b; Shakibaie et al., 2020). These *in vivo* findings along with the previously mentioned *in vitro* results collectively demonstrated that Se NPs could be served as nutritional supplements with powerful anti-parasite effects.

CONCLUSION, PERSPECTIVE AND OUTLOOKS

Selenium toxicity can occur with acute or chronic ingestion of excess selenium in humans, and similarly, the excess selenium contents would result in inevitable toxicity against bacteria, virus, fungi and parasite. The strong toxicity of selenium thus makes Se NPs a kind of anti-infection agents with direct killing or inhibition effects on different pathogens. However, it would be very tricky to control the dosages of Se NPs for anti-infection therapy if only the direct killing effects of Se NPs are applied, as the excessive selenium contents might also induce systemic toxicity of normal cells or tissues. Thus, other functions of Se NPs must be involved for more effective anti-infection treatment with low cytotoxicity.

In infectious disease, there are many ways that the host metabolism and immune status can be affected, leading to a dysregulation of redox homeostasis and immunosuppression. Selenium has long been found to be closely associated with different pathogen infections by regulating the antioxidant defense system through selenoprotein functions. As part of antioxidant defense, selenoproteins, such as GPXs and TXNRDs, play an important role in controlling oxidative stress, which therefore results that reduced selenoprotein expression would induce weaken of the defense against infectious diseases (Guillin et al., 2019). Dietary supplementation to provide adequate or supranutritional selenium supply has been proposed to confer health benefits for patients in some important infectious diseases, which indicates the possibility to apply Se NPs as a kind of nutritional supplements for infectious disease defense.

The effective drug delivery and controlled drug release ability of nanosystem are providing more effective tools to enhance the targeting effects of drugs against the specific disease sites. Our previous works have proved that drug delivery system could significantly enhance the intracellular pathogen clearance efficacy of antibiotic by targeting host cells (Pi et al., 2019). Furthermore, we also introduced Se NPs as antibiotic delivery system to promote the intracellular pathogen clearance, which strongly indicated that Se NPs could be also be used as effective drug delivery system to inhibit or kill pathogens in host cells (Pi et al., 2020). These *in vitro* works have confirmed the drug delivery capacity of Se NPs for infectious diseases treatment, however, more *in vivo* works are needed for their future applications.

Up to now, most therapeutics against infectious diseases are focusing on the antibiotic treatments, however, the increasing emergence of drug-resistant mutants or multiple drug-resistant mutants requires novel treatments rather than more antibiotics that might worsen the drug-resistance conditions. Immune therapy is now serving as one of the most effective treatments in some important disease, such as tumor. It's very attractive that Selenium status may affect the function of cells both in adaptive and innate immunity. We have recently demonstrated the ability of Se NPs to regulate host immunity against intracellular pathogens, which dramatically countercharge the immune escape of intracellular pathogens, such as lysosomal escape, host cell apoptosis/autophagy and macrophage polarization (Pi

et al., 2020). This means that Se NPs could be served as effective immunomodulation agents for innate immunity regulations against infectious diseases.

Supranutritional selenium intake was shown to regulate adaptive immunity by favoring proliferation and differentiation of activated CD4-positive T cells toward Th1 cells, which play important roles in infectious disease defense (Steinbrenner et al., 2015). Although there are few works to elaborate the effects of Se NPs against infectious diseases by regulating adaptive immunity, lots works have also demonstrated the potentials of Se NPs to manipulate adaptive immunity against other diseases (Hu et al., 2019). It would be an attractive topic to explore the effects of Se NPs on adaptive immunity, which would further extend the application of Se NPs for more effective infectious disease treatments.

Taking the advantages of direct inhibition/killing effects on pathogens, targeted drug delivery against host cells or disease sites, chemosensitization effects on the current drugs, and the innate and adaptive immunity regulation effects, Se NPs are expected to be served as effective anti-infectious agents simultaneously manipulating the above activities. Considering the low toxicity, antioxidant and immunity regulation capabilities and other merits of Se NPs, it is desirable and reasonable to further explore the effects and mechanisms of Se NPs against different pathogens. With the development of nanotechniques, we hope that Se NPs can play more and more important roles in fighting against infectious pathogens, which would finally benefit the diagnosis, prevention and treatment of infectious diseases.

FUTURE DIRECTIONS

As a kind of novel nanomaterials, Se NPs have drawn increasing attentions to address the dilemma of antibiotic resistance, thus showing attractive potentials for future clinical infectious diseases treatment. However, there are still some inevitable challenges that need to be addressed before their clinical transformations. During these challenges for Se NPs, the most urgent issue is the biocompability, which is the ability of a material being compatible with living tissue. Ideal biocompatible nanomaterials would not produce unexpected toxicity or immunological response when exposed to the body or bodily fluids. However, the toxicity of excess selenium is a dangerous assumption that Se NPs may introduce. The toxicity of Se NPs have been widely reported for anticancer or anti-infection treatment, however, the toxicity of Se NPs against normal cells or tissues remains to be further investigated. Thus, it would be important to understand the bridge between the Selenium and Se NPs, especially for their molecular events that are responsible for the therapeutic differences and toxicity effects that are critical for their biocompability. Otherwise, the degradation of Se NPs in body is still unclear, which might introduce unknown toxicity after long-term administration. Thus, more concerns about the degradation of Se NPs after long-term administration should be paid to verify the safety of Se NPs for clinical uses. Therefore, how to develop functional Se NPs with good biocompability and degradation property would be the most critical issues for the further clinical application of Se NPs against different infectious diseases.

Another critical issue for the further studies of Se NPs would be focused on the exploration of their anti-infectious mechanisms, especially their effects and mechanism on immune regulations. The direct killing effects of Se NPs against different pathogens are the mostly investigated parts for their potential anti-infection applications. However, as widely known, one of the most important issues in infectious diseases would be the immune responses for infection controls. The immunity regulation functions of Selenium are thought to be closely associated with selenoproteins, which play critical roles in both metabolism and immune system. However, how selenium from Se NPs affect the immune responses by regulating selenoprotein activity remains to be further investigated. And in our opinion, Se NPs may also strongly influence the phagocyte functions to further regulate the immune responses. Thus, it would be important to explore the effects of Se NPs on phagocyte functions when they are used for infectious disease treatment, which may also activate anti-infection immunity for infections control.

AUTHOR CONTRIBUTIONS

WL drafted the manuscript, JZ helped to revise the manuscript, J-FX and JP was responsible for leading this work and revising the manuscript.

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Berberine-Loaded Biomimetic Nanoparticles Attenuate Inflammation of Experimental Allergic Asthma *via* Enhancing IL-12 Expression

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Asthma is one of the most common chronic pulmonary disorders, affecting more than 330 million people worldwide. Unfortunately, there are still no specific treatments for asthma so far. Therefore, it is very important to develop effective therapeutics and medicines to deal with this intractable disease. Berberine (Ber) has fabulous anti-inflammatory and antibacterial effects, while its low water solubility and bioavailability greatly limit its curative efficiency. To improve the nasal mucosa absorption of poorly water-soluble drugs, such as Ber, we developed a platelet membrane- (PM-) coated nanoparticle (NP) system (PM@Ber-NPs) for targeted delivery of berberine to the inflammatory lungs. *In vivo*, PM@Ber-NPs exhibited enhanced targeting retention in the inflammatory lungs compared with free Ber. In a mouse model of house dust mite- (HDM-) induced asthma, PM@Ber-NPs markedly inhibited lung inflammation, as evident by reduced inflammatory cells and inflammatory cytokines in the lung compared with free Ber. Collectively, our study demonstrated the inhibitory actions of nasally delivered nanomedicines on HDM-induced asthma, primarily through regulating Th1/Th2 balance by enhancing IL-12 expression which could potentially reduce lung inflammation and allergic asthma.

Keywords: berberine, biomimetic nanoparticles, platelet membrane, asthma, IL-12

INTRODUCTION

As one kind of chronic inflammatory disease of the airway, asthma is still regarded as a great medical and socioeconomic burden worldwide (Barberand et al., 2021; Klimek et al., 2021). In the past decades, asthma treatment is mainly through the inhalation or systemic administration of bronchodilators and anti-inflammatory agents, such as glucocorticoids (Wu et al., 2021). However, it presents major challenges in drug delivery and therapeutic efficacy due to the mucus obstruction in airway inflammation diseases; thus, the pathological symptoms cannot be controlled even with high doses of the recommended drugs (Chakraborty et al., 2021). Besides, chemotherapeutic agents and glucocorticoids always show serious side effects in the body (Cangemi et al., 2021). To address the problems in clinical asthma treatment, we synthesized platelet-derived extracellular vesicles- (PEVs-) cloaked biomimetic nanoparticle (NP) carrier for targeted delivery of

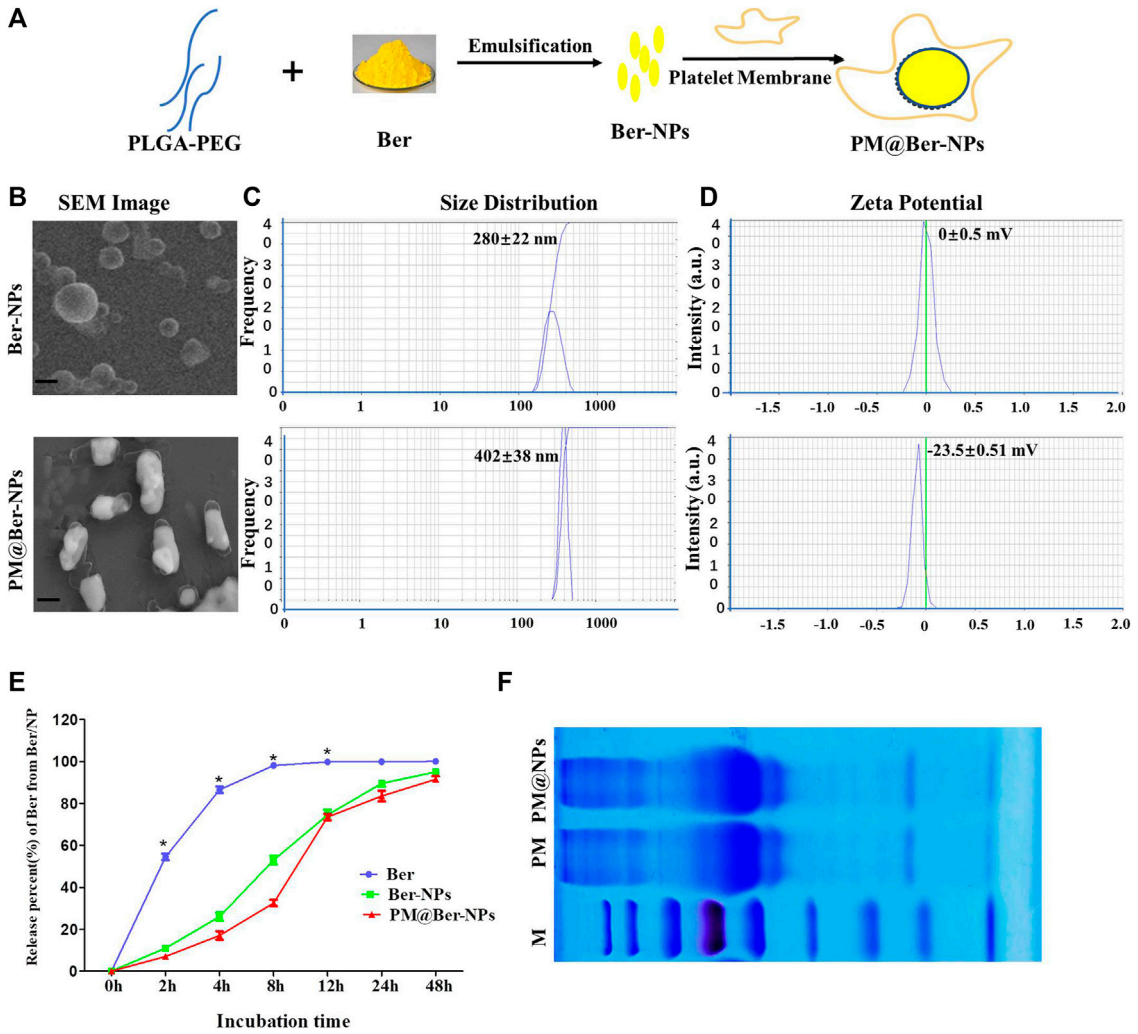

FIGURE 1 | Characterization of Ber-loaded NPs. **(A)** Scheme of preparation of biomimetic-based platelet-cell membrane-coated nanoparticles (PM@Ber-NPs). **(B)** Scanning electron microscope (SEM) image of Ber-NPs and PM@Ber-NPs. Scale bar = 100 nm. **(C)** Mean size and **(D)** zeta potentials of Ber-NPs and PM@Ber-NPs. **(E)** *In vitro* release kinetics of berberine from nanoparticles (NPs) in PBS (0.01 M, PH = 7.4). **(F)** SDS-PAGE protein analysis of platelet membrane (PM) and PM@NPs. *$p < 0.05$.

natural herbal ingredients berberine (Ber) to the asthma airway, which is expected to alleviate the symptoms and progression of pulmonary inflammatory disease (as shown in **Figure 1A**).

Berberine is a quaternary ammonium isoquinoline alkaloid, which is extracted from Chinese medicine *Berberis pruinosa*. *Berberis pruinosa* has been used in Chinese traditional medicine to treat infection, diabetes, arrhythmia, tumors, osteoporosis, etc. (Mujtaba et al., 2021; Ye et al., 2021) for thousands of years, which indicates the good biological safety of Ber. However, the therapeutic effects of Ber are greatly limited owing to its poor bioavailability and off-targeting properties.

NP-based delivery systems have been extensively developed to load and deliver Ber to disease sites for enhanced biocompatibility purposes (Zhao et al., 2021). Cell membrane-cloaked biomimetic drug delivery system has recently attracted increasing attention due to endowing NPs with enhanced biocompatibility, low immunogenicity, and active targeting abilities (Vijayan et al., 2018). Platelets are small blood cells (1~3um) that have superior inflammation targeting properties. However, it might evoke inflammation in the body through directly adopting platelets as drug carriers. To solve this problem, researchers developed a PEV platform. PEVs are nanosized membrane vesicles (100–150 nm) derived from platelets. PEVs-cloaked carriers could avoid phagocytic uptake by macrophages and could target and adhere to injured parts of the vasculature, which can enhance the binding between platelet-adhering pathogens and inflammatory tissues (Ma et al., 2020).

In this work, to address the off-targeting properties of Ber *in vivo*, the biomimetic NPs were synthesized to load and deliver Ber to the inflammatory lungs. The biomimetic NPs could be targeted and retained in the inflammation sites due to the inflammatory targeting characteristics of platelet membrane (PM) which is

coated onto the surface of NPs. In this study, we found that Ber-loaded biomimetic NPs could successfully attain inflammatory lungs at 2 h after NPs administration, while the Ber was released from NPs from 2 to 48 h. It implied that NPs could deliver and release the Ber into lungs that play anti-inflammation effects and subsequently attenuate allergic asthma.

MATERIALS AND METHODS

Mice

Eight-week-old female C57BL/6 mice were obtained from Laboratory Animal Center, Southern Medical University (Guangzhou, China). The experimental protocols were conducted according to the National Institutes of Health guidelines on the use of laboratory animals. The animal care and study protocols were approved by the Institutional Animal Care and Use Committee of Guangdong Medical University (GDY2002094).

Antibodies

Anti-mouse CD11c (N418), MHC-II (M5/114.15.2), CD11b (M1/70), Ly6G (RB6-8C5), SiglecF (E50-2440), CD4 (RM4-5), TCRβ (H57-597), IL-4 (11B11), IL-13 (eBio13A), and IL-12p40 (C17.8) antibodies were obtained from eBioscience. Anti-mouse IL-5 (TRFK5) was obtained from BD Biosciences.

Cell Culture and NP Uptake

A549 cells were grown in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin-streptomycin (Gibco) at 37°C in a 5% CO_2 incubator. In experiments to determine the involvement of NPs uptake, the cells were incubated with 50 μg/mL house dust mite (HDM, *Dermatophagoides farinae,* Greer Laboratories) for 24 h. Then, A549 cells were treated with naked Cou6 NPs or PM@Cou6 NPs for 2 h. After the cells were washed two times, A549 cells treated with or without HDM were imaged by fluorescence microscopy (Olympus IX70 Inverted Microscope).

Preparation Berberine-Loaded Nanoparticles (Ber-NPs)

Ber-NPs were prepared using emulsification and evaporation methods, as previously described with some modifications (Liu et al., 2010). Briefly, 20 mg of Ber (Sigma-Aldrich) and 80 mg of PEG/PLGA (PEG5000-PLGA28,000, Sigma-Aldrich) were dissolved in 5 mL of dichloromethane as the oil (O) phase, while 20 mL of PVA (1%, w/w, Sigma-Aldrich) was used as external the water (W) phase. The oil phase was sonicated for 40 s at 100 W on ice to form the first emulsification. Then, it was dropped into the W phase and sonicated for another 40 s to form the second emulsification. 250 μg of Coumarin 6 (Cou6, Sigma-Aldrich, United States) was added to the oil phase as the fluorescence marker of NPs. Finally, the NPs were harvested by centrifuging at 12,000 rpm for 20 min and washed three times with water.

Preparation of PM-Coated Ber-Loaded NPs

Fresh whole blood samples were drawn from healthy mice. Platelets from whole blood were isolated through gradient centrifugation. Briefly, 2.5 mL whole blood (from five mice) was centrifuged at 200 g for 10 min and the supernatant was isolated as platelet-rich plasma (PRP). Then, the PRP was centrifuged at 1800 g for 20 min at 4°C, followed by discarding the supernatant. The platelets were accumulated in the precipitates and were washed twice with PBS before use.

Platelets were suspended in deionized water with protease inhibitor and frozen at −80°C, followed by thawing at room temperature. After three repeated freeze-thaw cycles and sonication, the platelets were mixed with Ber-NPs and sonicated for 30 s.

Characterization of PM@Ber-NPs

The binding capacity of PM coating on Ber-NPs was calculated by measuring the concentrations of unbound PM in the supernatant and in the wash solution. The entrapment efficiency of Ber by the different types of NPs was assessed by high-performance liquid chromatography (HPLC). The size distribution and zeta potential of the NPs were determined using a Zetasizer Nano ZS (Malvern Instruments, United Kingdom). The morphology of the as-prepared Ber-NPs was captured using the ZEISS scanning electron microscope (SEM) operated at a 15.0 kV accelerating voltage.

To measure the Ber loading rate of Ber-NPs or PM-Ber-NPs, 10 mg lyophilized NPs were dissolved in 1 mL of methanol, and then the amount of Ber in the solution was determined by HPLC. HPLC detection was performed using a C18 column (5 μm, 250 mm × 4.6 mm), whereas the mobile phase, consisting of acetonitrile and phosphate buffer [0.05 mol/L potassium dihydrogen phosphate and 0.05 mol/L sodium heptane sulfonate (1:1)] (40:60), was maintained at a flow rate of 1.0 mL/min. The ultraviolet detector wavelength was 263 nm and the injection volume was 20 μL.

HDM-Induced Asthma

Eight-week-old female C57BL/6 mice were sensitized intranasally with HDM (200 μg in 50 μL saline per mouse; *Dermatophagoides farina*, Greer Laboratories) on day 0 and day 1. These mice were subsequently challenged (intranasally) with HDM (30 μg in 50 μL saline per mouse) on day 14 for five consecutive days. The HDM + (Ber-NPs) group and the HDM + (PM@Ber-NPs) group of mice were administered intranasally with Ber (2 mg/kg body weight) 1 h before HDM challenge from day 14 to day 21. Bronchoalveolar lavage fluid (BALF), sera, and lung samples were collected 48 h after the last HDM challenge.

IVIS Imaging

HDM-induced C57BL/6 mouse asthmatic models were intranasally administered with 50 μL of naked PLGA NPs and PM-coated biomimetic NPs (Cou6 was loaded into the NPs as fluorescence marker). For semiquantitative analysis, the image and fluorescence intensities of mice were collected 2 h after

intranasal administration of NPs and determined using the Kodak Multi Mode Imaging System.

Histology

Lung tissues were fixed and processed for hematoxylin and eosin (H&E) staining. Briefly, lung tissues were fixed by 5 min instillation of 10% PBS-buffered formalin through trachea catheterization at a transpulmonary pressure of 15 cm H_2O and then kept overnight at 4°C with agitation. After paraffin processing, the tissues were cut into semithin 5 μm thickness and stained with H&E for histological analysis.

Bronchoalveolar Lavage Fluid Collection and Lung Mononuclear Cell Isolation and Flow Cytometry

For BALF collection, lung tissues were lavaged with 1 mL cold PBS for three times, and the supernatant was collected. Lung mononuclear cells were prepared as previously described (Han et al., 2018). Briefly, lung tissues were removed, minced, and digested with 1 mg/mL collagenase IV (Life Technologies) in RPMI-1640 (Hyclone) with 5% FCS (Hyclone) for 45 min at 37°C. Cells were enriched by using a 38% Percoll gradient (GE Healthcare Life Sciences). Red blood cells were lysed with ACK lysis buffer (R&D Systems). Cells were harvested for analyses. For surface staining, cells were stained with antibodies in PBS containing 1% FCS (Hyclone) on ice for 30 min. For intracellular staining, cells were stimulated with PMA (Sigma-Aldrich) and ionomycin (Sigma-Aldrich) for 5 h in the presence of Golgistop (BD Biosciences) before being stained according to the manufacturer's instructions (eBioscience). The samples were acquired on a FACSCantoII (BD) or LSRFortessaTM X-20 (BD) and analyzed with FlowJo software (Treestar).

Blood Collection and ELISA

Blood was collected from the mouse heart and centrifuged at 5000 g at 4°C for 10 min. After being aspirated to blood supernatant, the sera were obtained for ELISA and stored at −20°C. The concentrations of IL-4, IL-5, IL-13, and IL-12 in blood sera were determined by ELISA kits (Invitrogen), according to the manufacturer's protocol. The linear range of the detection was 0–25,000 pg/mL.

Real-Time RT-PCR Analysis

To determine the expression of mRNA in lungs, lungs from asthmatic mice were lysed with a tissue homogenizer in TRIzol (Invitrogen). Total RNA was extracted by TRIzol according to the manufacturer's instructions. Real-time PCR analysis was performed with primers using Power SYBR Green Master Mix from Life Technologies. All gene expression results (mRNA abundances) were expressed as arbitrary units relative to the abundance of GADPH mRNA. The fold change was calculated through the $2^{-\Delta\Delta Ct}$ method. The primers used were as follows: IL-4, forward primer: 5′-GGTCTCAACCCCCAGCTAGT, reverse primer: 5′-GCC GATGATCTCTCTCAAGTGAT; IL-13, forward primer: 5′-CCTGGCTCTTGCTG CCTT, reverse primer: 5′-GGT CTTGTGTGATGTTGCTCA; IL-5, forward primer: 5′-CTCTGT TGACAAGCAATGAGACG, reverse primer: 5′-TCTTCAGTATGTCT AGCCCCTG; IL-12, forward primer: 5′-CAACCATCAGCAGATCATTCTA, reverse primer: 5′-GAGTCCA GTCCACCTCTACAAC.

Statistical Analysis

Results are expressed as mean ± SD. Statistical significance was determined by one-way ANOVA with a Games-Howell post hoc analysis for multiple-group comparisons. Two-group comparisons were analyzed by the two-tailed unpaired Student *t*-test.

RESULTS

Preparation and Characterization of Ber-NPs and PM@Ber-NPs

To improve the water solubility and bioavailability of Ber, biodegradable polymer PLGA was employed to encapsulate Ber to form soluble NP carriers, and for further increasing the targeting and biocompatibility of NPs, PM-derived vesicles were coated onto the surface of NPs (**Figure 1A**). The morphology (**Figure 1B**) and size distribution (**Figure 1C**) of the obtained Ber-NPs was approximately 280 nm which increased to 400 nm after coating with the cell membrane. According to the SEM results, we assumed that the increased size of NPs might be due to the PM-derived vesicles coating on the surface of NPs. Moreover, the polydispersity of PM@Ber-NPs was <0.3, indicating the uniform dispersion NPs in water.

There are two sides of the cell membrane: one side is with a positive charge and the other side is with a negative charge; the vesicles from the platelet-cell membrane could be coated onto the surface through electrostatic interactions under strong mechanical force. The surface charge (zeta potential) of Ber-NPs was ~0 mV (**Figure 1D**), while it increased to −23 mV when coated with PM, which indicates that the PM-derived vesicles have been successfully coated on the surface of NPs. The loading rate of Ber into the NPs was 8.1%, and the encapsulation rate was 81.02%, respectively, as determined by the HPLC method.

In Vitro Release Kinetics of Berberine From NP-Based Delivery System

The *in vitro* release of Ber in its free form was almost completely released within 4 h (98%), with no further increase after 6 h (99%, **Figure 1E**). Release of Ber from all two types of NPs at 6 h was only 50–60%, with subsequent progressive increases in release at 12 h (65–75%), 24 h (75–85%), and 48 h (90–98%). These data suggested that loading of Ber into NP carriers could delay the release of Ber compared with free berberine and facilitated steady drug release for approximately 24 h.

In addition, SDS-PAGE was performed on PM and PM@NPs (**Figure 1F**), which indicated that PM@NPs contained

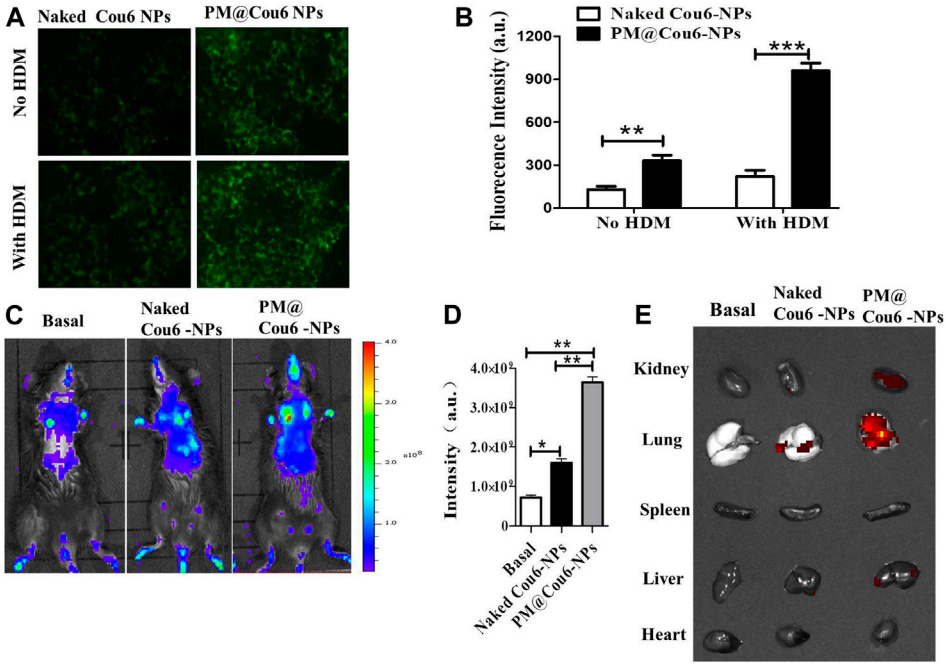

FIGURE 2 | Enhanced retention of drugs in murine inflammatory lungs *via* biomimetic modification of NPs with PM. Cou6 was encapsulated into the NPs as a fluorescence marker. **(A)** The intracellular uptake of Cou6-NPs and PM@Cou6-NPs in A549 cells with or without HDM treatment. **(B)** The fluorescence intensity in A549 cells cocultured with Cou6-NPs and PM@Cou6-NPs. **(C)** Biodistribution of naked Cou6-NPs without modification and PM@Cou6-NPs, determined by the IVIS imaging system. **(D)** The mean fluorescence intensity (MFI) of NPs in the lungs, determined by the IVIS imaging system ($n = 3$). **(E)** Biodistribution and retention of PM@blank-NPs, naked Cou6-NPs without modification, and PM@Cou6-NPs in organs, determined by IVIS imaging system at 2 h after NP intranasal administration. $^{*}p < 0.05$; $^{**}p < 0.01$.

endogenous membrane proteins preserved by PM. Our results indicated that the vesicles from platelets were successfully coated or conjugated on the surface of the NPs.

Enhanced Uptake of PM@Ber-NPs in Inflamed Epithelial Cells Induced by HDM

To determine whether the biomimetic NPs could target the pulmonary epithelial cells at inflammatory stimulation, we treated A549 cells with HDM for 24 h. As shown in **Figures 2A,B**, we coincubated the different NP systems with rested or inflammatory (HDM-stimulated) A549 cells for 1 h. In rested A549 cells, NPs uptake represented no significant difference between PLGA NPs and PM@NPs; however, it showed significantly increased cell uptake of PM@NPs in HDM-induced inflammatory A549 cells comparing with PLGA NPs without PM modification. These results demonstrated that PM@NPs could target the inflammatory pulmonary epithelial cells, thus showing higher biocompatibility *in vitro*.

In vivo Biodistribution of NPs in Asthma Mouse Model

In vivo and *ex vivo* fluorescence imaging was used to evaluate the pulmonary inflammation targeting capability of Cou6-labeled PLGA NPs (Cou6-NPs) and Cou6-labeled PM@ PLGA NPs (PM@Cou6-NPs). Strong fluorescence signals were observed at lung tissues 2 h after administration with NPs (**Figure 2C**), demonstrating the enhanced targeting ability of the biomimetic NPs. The statistical data by quantitative region-of-interest (ROI) analysis (**Figure 2D**) also showed that the fluorescence intensity of lungs of PM@Cou6-NPs was much higher than those of the Cou6-NPs group, indicating that PM@Cou6-NPs had good inflammation targeting properties. In order to investigate which organ PM@Cou6-NPs accumulate in or whether PM@Cou6-NPs pass blood-brain barrier, organs of mice with Cou6-NPs and PM@Cou6-NPs treatments were separated. The results showed that PM@Cou6-NPs mainly accumulate in the lung (**Figure 2E**; **Supplementary Figure S1**).

Ber-Loaded Biomimetic NPs Reduce Cell Infiltration of Airway in HDM-Induced Asthma Mouse

To investigate the efficacy and specificity of Ber and its NPs *in vivo*, we performed experiments in HDM-mediated eosinophilic airway inflammation. In this HDM-induced asthma model, we treated the mice with Ber and its NPs, during the sensitization and challenge stage of this chronic model (**Figure 3A**). As shown in **Figure 3B**, Ber and its NPs reduced total infiltrated cells numbers in BALF of HDM-induced asthmatic mice. Among them, PM@Ber-NPs were the least in reducing cell infiltration into mouse

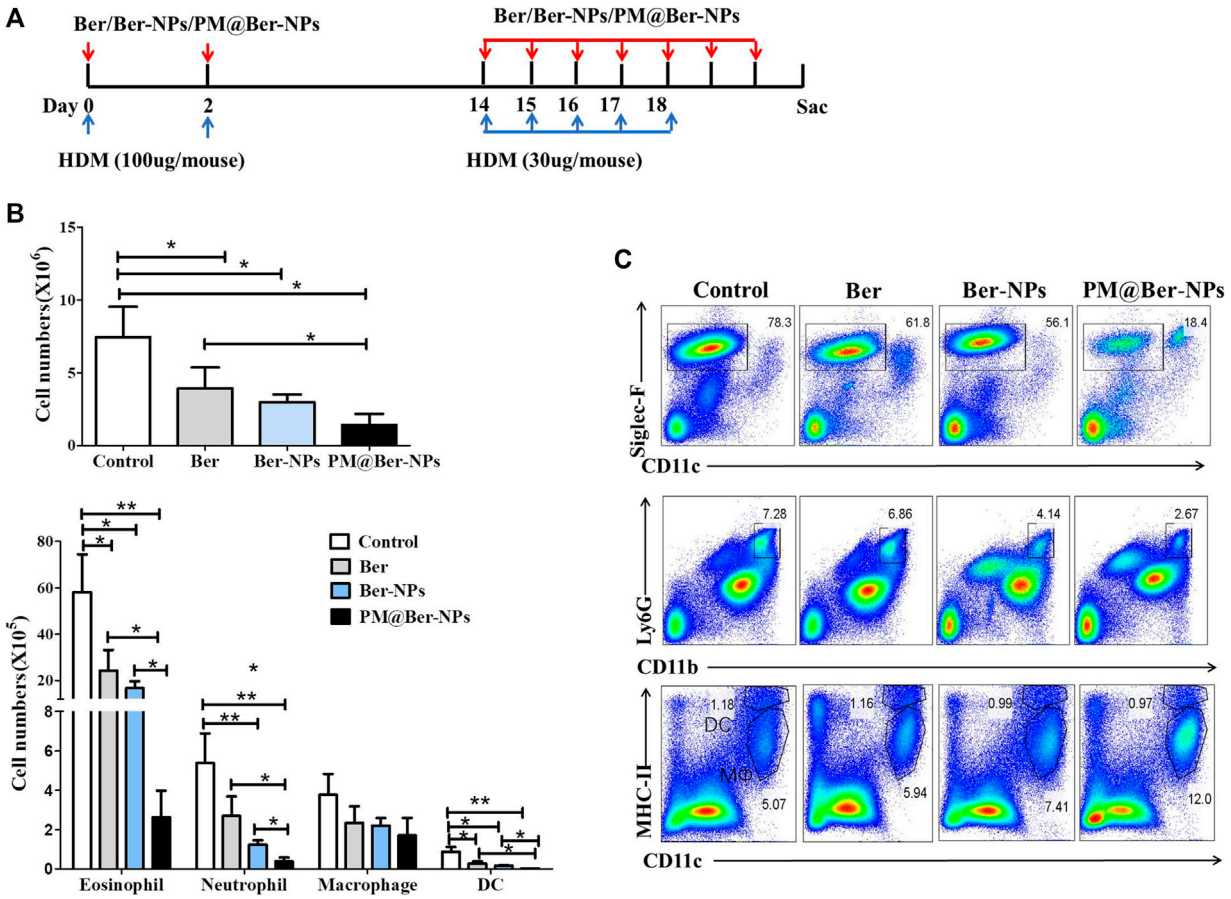

FIGURE 3 | Ber-loaded biomimetic NPs reduce airway cells infiltration in HDM-induced mouse asthma. **(A)** Eight-week-old C57BL/6 female mice (n = 5 mice per group) were subjected to the HDM-induced asthma model. PBS (Control), Ber, Ber-NPs, and PM@Ber-NPs were administered to the mice (as described in Methods). Two days after the last challenge, cells in the BALF sera and lungs were collected to further analysis. **(B)** Total cell counts, eosinophils, neutrophils, macrophages, and dendritic cells in the BALF were quantified. **(C)** Infiltrated cells in the BALF with HDM-induced asthma (n = 5) were isolated and followed by flow cytometry analysis. $^{*}p < 0.05$; $^{**}p < 0.01$.

airway. Moreover, flow cytometry showed that Ber and its NPs reduced not only total eosinophil numbers but also total neutrophil numbers and dendritic cells numbers in BALF. But Ber did not affect total macrophage (MΦ) numbers in BALF (**Figures 3B,C**). Notably, PM@Ber-NPs could reduce much more infiltrated cells in the airway than that of Ber and Ber-NPs. These data indicated that Ber and its NPs inhibited airway inflammation in the HDM-induced asthma model. Additionally, PM@Ber-NPs exhibited the best efficacy to reduce airway inflammation.

Ber-Loaded Biomimetic NPs Alleviate Lung Inflammation of Asthmatic Mouse

To further examine the effect of Ber and its NPs on the HDM-induced asthma model, we isolated infiltrated cells in lungs from asthmatic mice. Data by flow cytometry showed that the cell numbers and percentage of eosinophil and neutrophil were decreased by Ber and its NPs (**Figures 4A,B**). Moreover, the cell numbers and percentages of infiltrated cells in the lung by PM@Ber-NPs have significant differences compared to other groups. H&E staining also showed that Ber and its NPs reduced leukocytes infiltration in mouse airway of the lung (**Figure 4C**). These results are consistent with the infiltrated cell numbers in BALF. Combined with all data together, it suggests that Ber and its NPs could alleviate lung inflammation. Among them, PM@Ber-NPs exhibited the best beneficial effects to reduce airway inflammation in HDM-induced asthma.

Ber-Loaded Biomimetic NPs Reduce Airway Inflammation of Mice *via* Enhancing IL-12 Expression

To understand the mechanisms involved in inhibiting airway inflammation by Ber, we examined the expressions of cytokines by flow cytometry in asthmatic mice. The data showed that Ber and its NPs inhibited Th2 type cytokine expression (**Figures 5A,B**) but increased the IL-12 expression (**Figures 5A,B**). We also examined the Th2 type cytokines and IL-12 secretion in asthmatic mice's sera. The results showed that Ber and its NPs

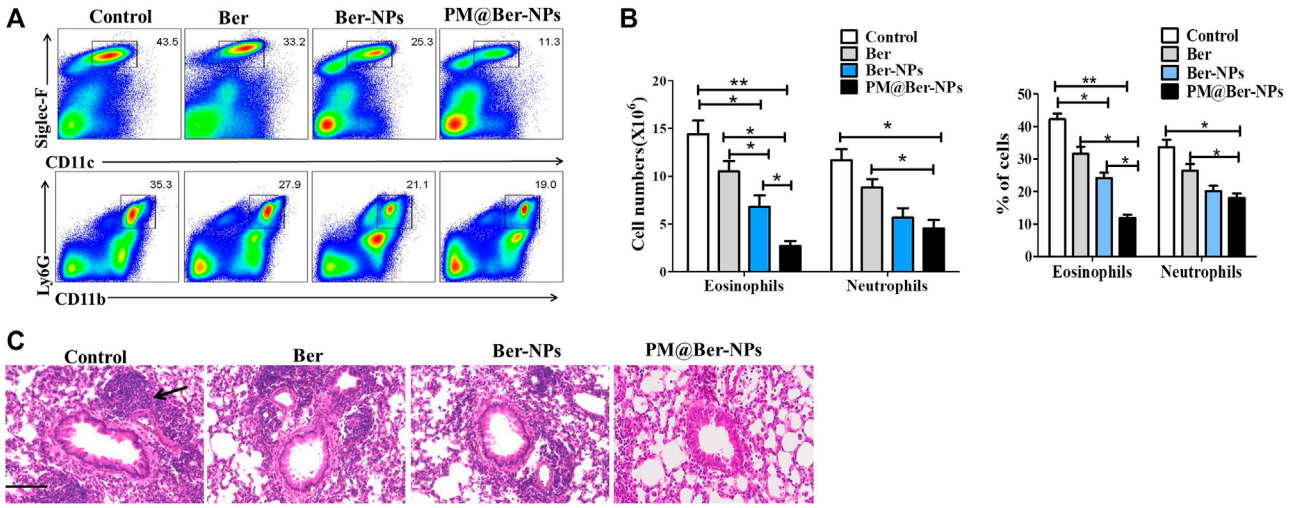

FIGURE 4 | Ber-loaded biomimetic NPs alleviate lung inflammation of asthmatic mice. **(A)** Infiltrated cells in the lung with HDM-induced asthma ($n = 5$) were isolated and followed by flow cytometry analysis. **(B)** The cell numbers and percent of different infiltrated cells were calculated for each mouse. **(C)** Hematoxylin and eosin (H&E) staining of a lung section from mice with HDM-induced asthma. Arrow shows infiltrated cells from blood vessels accumulated around mice's lung airway. Scale bars, 100 μm. $^{*}p < 0.05$; $^{**}p < 0.01$.

reduced Th2 type cytokine secretion but increased IL-12 secretion (**Figure 5C**). qPCR also showed that Ber and its NPs reduced IL-13 mRNA expression (**Figure 5D**). PM@Ber-NPs significantly suppressed IL-4, IL-5, and IL-13 expression and exhibited higher inhibitory effects. In addition, Ber and its NPs increased IL-12 expression (**Figures 5A–D**). These results indicated that Ber modulates Th1/Th2 balance. Thereby, Ber and its NPs suppressed airway inflammation through enhancing IL-12 expression.

DISCUSSION

Chinese herbal medicine treatment of chronic asthma has a long history in China and around the world (Ouyang et al., 2020; Wong et al., 2021). It has been reported that Ber has various activities including anti-inflammatory effects and has been used in treating many diseases including asthma (Yang et al., 2014; Li et al., 2016; Tew et al., 2020). However, the low bioavailability of Ber limits its clinical application as an anti-inflammatory agent. Moreover, Ber also presents other drawbacks such as poor oral absorption and first-pass effect in the intestine and liver (Liu et al., 2010). It remains in the tissue for a short time after 24 h because of drug metabolization and clearance. To overcome these issues, the development of targeted drug delivery based on nanotechnology presents a new approach for the loading and delivery of Ber to improve its bioavailability (Wu et al., 2014; Mirhadi Rezaee, et al., 2018; Li et al., 2019; Lu et al., 2019; Niu et al., 2020). But Ber based on nanotechnology in asthma treatment has not been reported until now. Thereby, in this study, we tried to develop a new Ber based on nanotechnology to treat asthma.

Biomimetic cell membrane-coated NPs have been broadly applied because of their superior biochemical properties. Cell membrane-coated NPs have been demonstrated to create a platform for a variety of applications, including detoxification (Hu et al., 2013a; Pang et al., 2015), drug delivery (Hu et al., 2011), and vaccination (Hu et al., 2013b; Fang et al., 2014). Functionalization with PM enables biomimetic targeting by taking advantage of the natural interactions between platelet surface markers and different targets, including damaged airway and pathogens (Hu et al., 2015a; Hu et al., 2015b). Given the wide range of biological interactions that PM participates in, the potential of PM-coated NPs extends far beyond the traditional nanodelivery applications. The reason might be that the biodetoxification of PM coated with NPs serves as an ideal substrate for interaction with biological toxins, enabling their neutralization and subsequent clearance. Recently reported papers have shown that PM coated with NPs has a good target for treatment of lung diseases (Bahmani et al., 2021; Zhou et al., 2021). However, PM coated with NPs for targeted treatment of asthma still was little known.

In the present study, *in vitro* results showed that PM@Ber-NPs exhibited enhanced cellular uptake in the inflammatory microenvironment compared with free Ber and Ber-NPs. Moreover, *in vivo* fluorescence imaging revealed that more PM@Ber-NPs were targeted to lung tissues than free Ber and Ber-NPs. In a mouse model of HDM-induced asthma, the PM@Ber-NPs markedly inhibited lung inflammation as evidenced by the reduced number of inflammatory cells and the expression of inflammatory cytokines in the lung compared with free Ber and Ber-NPs treatments. In addition, in this study, the administrated dose of Ber-loaded NPs was only 2 mg/kg body weight, which is lower than that of previous studies (Li et al., 2016). These results suggested that delivery of PM@Ber-NPs has improved its bioavailability than free Ber and Ber-NPs.

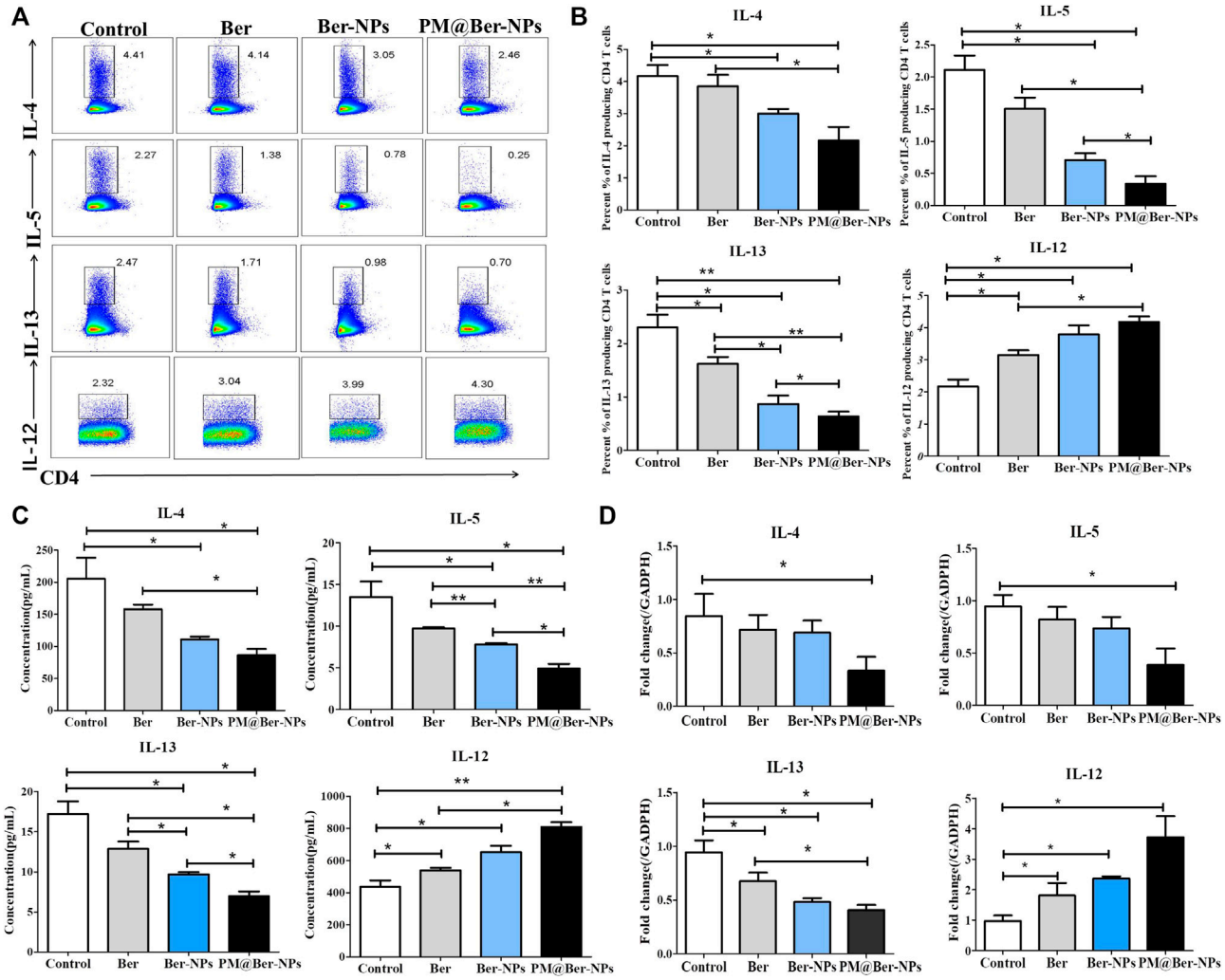

FIGURE 5 | Ber-loaded biomimetic NPs decrease Th2 type cytokines expression but enhance IL-12 expression in lungs of asthmatic mice. **(A)** Cytokines secretory in the lungs from HDM-induced asthma mice (n = 5) were isolated and followed by flow cytometry analysis. **(B)** Percentages of cytokines secretory in the lungs from HDM-induced asthma mice were counted. **(C)** Th2 type cytokines (IL-4, IL-5, and IL-13) and IL-12 serum from asthmatic mice were determined by ELISA kit. **(D)** The mRNA expression of cytokines was examined by real-time RT-PCR. *p < 0.05; **p < 0.01.

It has been reported that imbalanced Th1/Th2 cells exist in patients with allergic asthma (Shi et al., 2011). The alternation of the Th1/Th2 ratio is confirmed to be an initial factor for asthma that increases airway inflammation (Carneiro et al., 2010). Therefore, it could be a potentially beneficial remedy for asthma treatment if the imbalanced status of Th1/Th2 cells can be reversed. To further investigate the mechanism of Ber in asthma, we detected cytokines expression associated with Th1 and Th2 cells by flow cytometry and ELISA. Our findings showed that Ber markedly inhibited expressions of Th2 cytokines (including IL-4, IL-5, and IL-13) and elevated levels of Th1 cytokines (IL-12). Allergic asthma is primarily mediated by Th2 cells, which secrete the characteristic cytokines IL-4, IL-5, and IL-13. In contrast to Th2 cells, Th1 cells have an opposite effect. IL-12 is the predominant cytokine produced by Th1 cells. IL-12 is involved in the antagonism of Th2-cell responses and IgE synthesis to restrain the progress of asthma. Accordingly, examination of the levels of Th1/Th2 cytokines is an important index in the evaluation of asthma. To further confirm the effects of Ber on Th1/Th2 cytokines, we determined the mRNA expressions of IL-12, IL-4, IL-5, and IL-13 in lung tissues. As expected, the result of real-time PCR is consistent with that of flow cytometry and ELISA. This result suggests that Ber could be able to regulate the immune response of Th1/Th2 cells. Because of the increased IL-12 expression in asthma, Ber inhibits airway inflammation in asthmatic mice. These roles of Ber in other anti-inflammation diseases have similarly been shown (Kim et al., 2003).

In conclusion, Ber encapsulated into biomimetic PLGA NPs indicated an unbalanced Th1/Th2 response for effective delivery of drugs to the inflammatory tissue in the asthma model. PM@Ber-NPs were prepared and characterized,

which ameliorate HDM-induced asthma. The novel drug nanodelivery system may provide a promising platform for improving asthma treatment.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**; further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee of Guangdong Medical University.

AUTHOR CONTRIBUTIONS

SO proposed and supervised the project. HJ, JL, MZ, PL, RL, WZ, JZ, and JP performed the experiments and analyzed the results. SO and HJ wrote the paper. WZ, ZM, and XD helped to revise the manuscript. XL and GH helped to edit the manuscript. All authors have given approval to the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.724525/full#supplementary-material

Supplementary Figure S1 | The drugs accumulate in murine inflammatory lungs, but not in the brain *via* biomimetic modification of NPs with PM. Cou6 was encapsulated into the NPs as a fluorescence marker. **(A)** Biodistribution and retention of PM@blank-NPs, naked Cou6-NPs without modification, and PM@Cou6-NPs in organs, determined by IVIS imaging system at 2 h after NP intranasal administration. **(B)** The mean fluorescence intensity (MFI) of NPs in lungs, determined by the IVIS imaging system. * $p < 0.05$; ** $p < 0.01$.

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