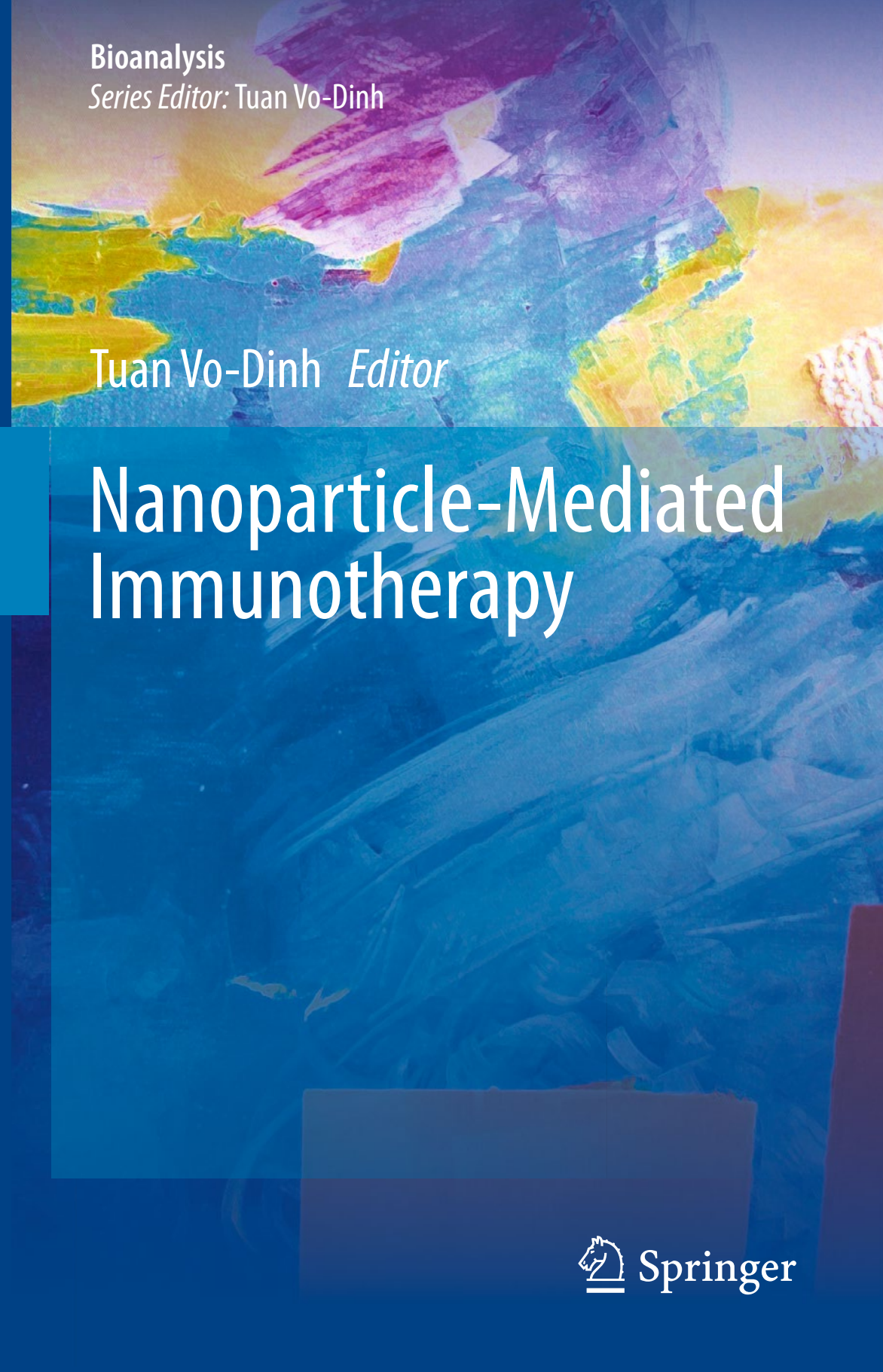
Bioanalysis

Series Editor: Tuan Vo-Dinh

Tuan Vo-Dinh *Editor*

Nanoparticle-Mediated Immunotherapy



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Nanoparticle-Mediated Immunotherapy

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Preface

The book ***Nanoparticle-Mediated Immunotherapy*** is intended to present recent scientific and technological advances in disease treatment at the intersection of nanotechnology and immunology. The book includes chapters grouped in two parts.

Part I contains chapters on basic principles and methods, including fundamental optical properties of nanoparticles and their effects on the immune system, strategies for immunotherapy from discovery to bedside, basic analysis, and imaging methods such as intravital microscopy to monitor anti-tumor immunological response, and theoretical studies of nanoparticle-mediated photothermal treatment for photoimmunotherapy.

Part II contains chapters describing various applications of nanoparticle-mediated immunotherapy. For instance, cancer immunotherapy uses the host's inherent immune system to treat cancer and imparts a memory effect on the immune system that can inhibit cancer relapse. However, tumor cells often form their own immune mechanisms to interact with tumor microenvironments in order to escape from cancer immunotherapy. To enhance the therapeutic efficacy of cancer immunotherapy, various nanostructured materials have been developed to modulate such immune suppressive factors in the tumor microenvironments. Engineered nanoparticles have also been developed and used as immune-stimulating adjuvants to strengthen the immunogenicity of antigens. Immunotherapies could synergistically benefit from targeted thermal nanotherapies, especially when hyperthermia around the tumor bed is combined with precise thermal ablation of cancer cells. Photothermal therapy combined with adjuvant immunotherapy has been developed to produce synergistic outcomes. Copper sulfide nanoparticles as well as bacterium-mimicking liposomes have been designed as adjuvant delivery systems. Various inorganic nano-agents such as gold nanostructures, carbon nanotubes, graphene oxide, CuS nanoparticles, and MoS2 nanosheets with strong near-infrared (NIR) absorbance have been explored for in situ photoimmunotherapy. Other types of nanomaterials, including liposomes, polymeric nanoparticles, quantum dots, magnetic nanoparticles, mesoporous silica nanoparticles, and carbon-based nanomaterials have been utilized to deliver photosensitizers, aiming at enhancing their tumor accumulation and therapeutic efficacy. Upconversion

nanoparticles, which can emit visible light under NIR light excitation, have been developed to allow NIR-mediated photodynamic therapy with improved tissue penetration. Plasmonic nanoparticles such as gold nanostars (GNS) have unique properties that allow them to amplify the optical properties of the excitation light and thus increase the effectiveness of light-based photothermal tumor ablation. The combination of GNS-mediated photothermal therapy with checkpoint blockade immunotherapy has shown great promise to address one of the most challenging problems in the treatment of metastatic cancer, i.e., achieve complete eradication of primary treated tumors as well as distant untreated tumors in murine models. Furthermore, GNS-mediated photoimmunotherapy has shown to elicit a long-term immunologic memory, ultimately inducing an anticancer vaccine effect.

The goal of this book is to provide a forum that integrates interdisciplinary research and development of interest to scientists, engineers, manufacturers, teachers, students, and clinical providers. As nanotechnology is rapidly becoming an important tool and a powerful weapon in the armory of the modern physician, it is our hope that this book will stimulate a greater appreciation of the usefulness, efficiency, and potential of the synergistic combination of nanotechnology and immunotherapy in modern medicine.

Durham, NC, USA Tuan Vo-Dinh

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About the Editor

Tuan Vo-Dinh is *R. Eugene and Susie E. Goodson* Distinguished Professor of Biomedical Engineering, Professor of Chemistry, and Director of the Fitzpatrick Institute for Photonics at Duke University. Dr. Vo-Dinh completed high school education in Saigon (now Ho Chi Minh City) and pursued studies in Europe where he received a B.S. in physics in 1970 from EPFL (École Polytechnique Fédérale de Lausanne), Lausanne and a Ph.D. in physical chemistry in 1975 from ETH (Swiss Federal Institute of Technology), Zurich, Switzerland. Before joining Duke University in 2006, Dr. Vo-Dinh was Director of the Center for Advanced Biomedical Photonics, Group Leader of Advanced Biomedical Science and Technology Group, and a Corporate Fellow, one of the highest honors for distinguished scientists at Oak Ridge National Laboratory (ORNL). His research has focused on the development of advanced technologies for the protection of the environment and the improvement of human health. His research activities involve nano-biophotonics, nanosensors, laser spectroscopy, molecular imaging, medical theranostics, and photoimmunotherapy.

Dr. Vo-Dinh has authored over 500 publications in peer-reviewed scientific journals. He is the author of a textbook on spectroscopy and editor of 8 books. Elected Fellow of the National Academy of Inventors (NAI), he holds over 59 US and international patents. Dr. Vo-Dinh has received seven *R&D 100 Awards* for Most Technologically Significant Advance in Research and Development for his pioneering research and inventions of inno-

vative technologies. He has received the *Gold Medal Award,* Society for Applied Spectroscopy (1988); the *Languedoc-Roussillon Award* (France) (1989); the *Scientist of the Year Award*, ORNL (1992); the *Thomas Jefferson Award*, Martin Marietta Corporation (1992); two *Awards for Excellence in Technology Transfer*, Federal Laboratory Consortium (1995, 1986); the *Inventor of the Year Award*, Tennessee Inventors Association (1996); the Lockheed Martin *Technology Commercialization Award* (1998); the *Distinguished Inventors Award,* UT-Battelle (2003); and the *Distinguished Scientist of the Year Award*, ORNL (2003). In 1997, Dr. Vo-Dinh was presented the *Exceptional Services Award* for distinguished contribution to a Healthy Citizenry from the U.S. Department of Energy. Dr. Vo-Dinh received the 2017 *Award for Spectrochemical Analysis* from the American Chemical Society (ACS), and the 2019 *Sir George Stokes Award* from the Royal Society of Chemistry (United Kingdom).

Part I
Basic Principles and Methods

The New Frontier in Medicine at the Convergence of Nanotechnology and Immunotherapy

Tuan Vo-Dinh

1 Nanoparticles and the Immune System

1.1 Stepping into the Nanoworld

Thousands of years ago, the Greek philosophers Leucippus and Democritus have suggested that all matter was made from tiny particles like atoms. The advent of nanotechnology in the modern era has triggered the development of a new generation of imaging instruments capable of revealing the structure of these tiny particles conceived since the Hellenic Ages. For scale appreciation, let us look up in the dimension scale, from the cell up to the outer edges of our "local universe", the Milky Way, a galaxy of 100–400 billion stars. This universe revealed to us has the dimension of 50,000 light-years from the outer edges to its center. A light-year is the distance that light travels in 1 year at the speed of approximately 300 million (300,000,000) meters per second, which corresponds to approximately 10,000,000,000,000,000 (sixteen zeros) or 10^{16} m. The meter, a dimension unit closest to every-day human experience, is often considered as the basic dimension of reference for human beings. Therefore, the distance from the center to the outer edge of the Milky Way is 500,000,000,000,000,000,000 or 5×10^{20} m. Let's now look down in the other direction of the dimensional scale, down to a nanometer, which is a billion (1,000,000,000) times smaller than a meter (i.e., 10^{-9} m). The word *nano* is derived from the Greek word meaning "dwarf." In dimensional scaling

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nano refers to 10^{-9}—i.e., one billionth of a unit. Diameters of atoms are on the order of tenths of (10^{-1}) nanometers whereas the diameter of a DNA strand is about a few nanometers. Thus, *nanotechnology* allows us to enter, explore and interact with the innerworld of a cell within the dimensional scale at the level of atoms and molecules.

It is now generally accepted that nanotechnology involves research and development on materials and species at length scales from 1 to 100 nm. The average animal cell size is approximately 10–20 microns, which is 10–20 thousand nanometers. Due to their small sizes, nanoparticles (NPs) can enter and/or be absorbed deep inside cells, where they can interact with and affect many biological species contained in the cell, which have molecular structures at the nanoscale levels. These species comprise a wide variety of basic structures such as proteins (e.g., antibodies), polymers, carbohydrates (e.g., sugars), and lipids, which have a great variety of chemical, physical, and functional properties affecting the immune system. By evolutionary modification over trillions of generations, the immune system of living organisms have perfected an armory of molecular machines, structures, and processes to defend against invaders and eradicate illnesses. The immune system of the living cell, with its myriad of biological components, may be considered the ultimate "nano machinery". It is conceivable that nanoparticle systems could interact with affect and actually "manipulate" individual atoms and molecules in the immune system in very specific ways, thus inducing new properties and triggering new functions in the immune system. Upon entering the body, NPs can be taken up by phagocytes, which are an important component of both immunosuppression and immunostimulation. The ability to produce NPs can be designed to be in the range of chemical ligands, bioreceptors, proteins and equipped with appropriate biochemical entities in order to achieve new therapeutic possibilities for a variety of illnesses. For instance, NPs could be designed to carry payloads such as antigens and/or immunomodulatory agents including cytokines, ligands for immunostimulatory receptors or antagonists for immunosuppressive receptor to have improved therapeutic effectiveness. Nanotechnology is of great importance to molecular biology and medicine because immunological processes are maintained by the action of a series of biological molecular nanomachines in the cell machinery.

1.2 The Immune System

Figure 1 shows a schematic diagram of the possible effects of nanoparticles on the body's immune system. Upon entering the body, NPs interact with cells and proteins and could stimulate or suppress the innate immune response, and similarly activate or avoid the complement system. Their physicochemical properties of NPs, including size, shape, hydrophobicity and surface modification, are the factors that could influence the interactions between NPs and the innate immune system

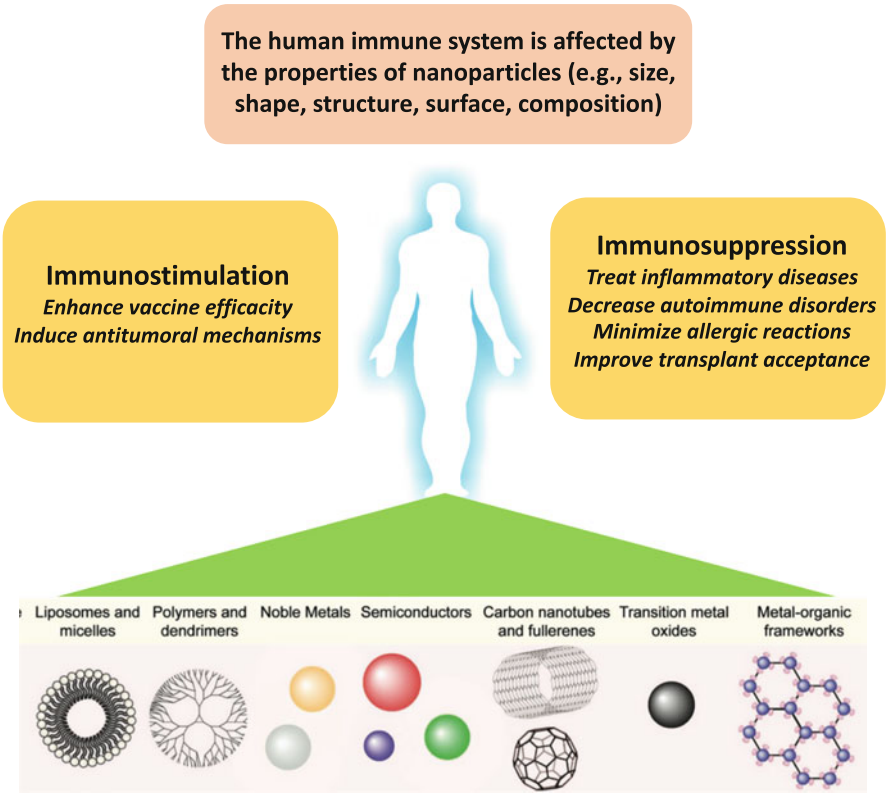

Fig. 1 Effect of Nanoparticles on the Immune System. Inside the body, nanoparticles could stimulate or suppress the innate immune response. The properties of nanoparticles, such as chemical composition, structure, size, shape, hydrophobicity and surface modification, are the factors that could influence the interactions between them and the innate immune system

[1].The immune system is designed to react to foreign threats and entities entering the body in order to protect the host and maintain homeostasis [2]. Two basic subsystems control the immune response: the innate immunity and the adaptive immunity. The innate immunity is the first line of defense, producing a non-specific inflammatory response upon the detection of conserved biological motifs; in general these motifs are often associated with bacteria and viruses. On the other hand, the adaptive immune system responds with a more specific defense mechanism by which antibodies that are highly-specific to detected antigens are produced; the initial antigen production is often followed by creation of memory cells for future immunological protection [3]. The acquired immune system is activated by mechanisms whereby antigen presenting cells (APCs) present acquired antigens to T cells.

As they enter the body, NPs usually interact with the innate immune system first; this interaction could generate an immunomodulatory response based on their

physicochemical properties [4]. Therefore, knowledge of the interactions NPs with the innate immune system could provide useful insight into developing immune-compatible, immune-modulating, or immune-stimulating NP platforms for specific medical applications of interest. The innate immune system is a broad-based, non-specific defense mechanism, which involves molecular (complement system, cytokines) and cellular (phagocytes and leukocytes) components that recognize classes of molecules such as frequently encountered pathogens. The innate immune system recognizes pathogens mainly via pattern-recognition receptors (PRPs). Also there is a highly organized complement system, which involves a set of serum proteins that are usually in an inactive state; however, these proteins can be converted into an active state to damage and clear pathogenic organisms. Activation of the complement system induces the formation of potent proteins that elicit physiological responses such as chemoattraction (attract phagocytes to sites of injury or inflammation) and enhanced vascular permeability [5]. Several circulating and tissue-specific cell types, such as natural killer (NK) cells, granulocytes (neutrophils, basophils, eosinophils, mast cells) and antigen-presenting cells (macrophage and dendritic cells (DC)) are part of the innate immune system.

In the innate immune response, APCs and neutrophils can recognize pathogens via pattern recognition receptors (PRRs) PRRs, which identify pathogen-associated molecular patterns (PAMPs), which can identify two classes of molecules: pathogen-associated molecular patterns (PAMPs), which are associated with microbial pathogens, and damage-associated molecular patterns (DAMPs), which are associated with components of host's cells that are released during cell damage or death. Upon identification, the cells uptake and digest the pathogens and induce an inflammatory response [6]. Activation of the innate immune system induce changes that can cause cytokine secretion (e.g. interleukins (ILs), tumor necrosis factor (TNF-α) [6]. It is noteworthy that activation of PRRs is process of the inflammatory immune response that allows the host cell to distinguish "self" from "non-self". PRRs are expressed on either the cell membrane (such as Toll-like receptors (TLRs) and C-type lectin receptors (CLRs)) or in the cytosol (such as NOD-like receptors (NLRs) and RIG-I-like receptors (RLRs) [7]. Physicochemical properties of NPs are important factors affecting the contact of NPs with the innate immune system and the resulting immune response.

1.3 Nanoparticle Size Effect

An important factor that has a significant impact on the uptake of these materials by cells, the initiation of innate immune response, and their overall bio-distribution in vivo if the size of NPs [4, 8]. The interactions with the innate immune system is affected by the surface to volume ratio of NPs. Endocytosis, the general process by which cells engulf external substances, gathering them into special membrane-bound vesicles contained within the cell. The endocytotic uptake of NPs involve several endocytic uptake mechanisms: pinocytosis, macropinocytosis, phagocytosis

and clathrin/caveolar-mediated endocytosis. Pinocytosis and micropinocytosis, are non-specific processes by which NPs in liquid droplets are ingested by living cells. Phagocytosis is a process by which particles, microbes or fragments of dead cells are engulfed and internalized, usually by specific membrane receptors. Clathrin-mediated endocytosis, also called receptor-mediated endocytosis (RME) is a process that absorb particles, metabolites, proteins, etc. by the inward budding of the plasma membrane (invagination) and forms vesicles containing the absorbed substances; RME is strictly mediated by receptors on the surface of the cell as only the receptor-specific substances can enter the cell through this process.

Various studies have shown that uptake of NPs into cells could involve a combination of several processes. For example, 600-nm polystyrene NPs were engulfed by phagocytosis/macropinocytosis, while 40-nm NPs were internalized by both clathrin-mediated endocytosis as well as phagocytosis or macropinocytosis by macrophages [9]. Smaller 10-nm gold NPs per cell were uptaken in greater amounts than larger 50-nm gold NPs into cells via a clathrin/dynamin-dependent mechanism by DCs [10]. Nanoparticle size, surface charge and composition have been demonstrated to be important for imunostimulatory activity. For example, NPs with small sizes ranging from <40 to 50 nm have been shown to be very effective in stimulating both humoral immunity and MHC-I restricted CD8+ T cell immunity [11–13]. The effect of NPs of different sizes on the immune systems were investigated. Most NPs accumulate in the liver (Kupffer cells), the amount increasing with the size of NPs and smaller NPs exhibiting higher retention than larger NPs [14, 15]. Smaller NPs (<200 nm) are rapidly drained to the lymph nodes, where they were taken up by resident DC, whereas larger NPs (>500 nm) depended on cellular transport by DC, immigrating from the injection site to the lymph nodes [16]. These results indicated that larger NPs prefer interacting with tissue-resident APCs, while smaller NPs (<200 nm) could circulate through vein and lymphatic drainage, providing better antigen presentation.

Nanoparticle sizes can be controlled so that they passively accumulate in tumors due to the enhanced permeability and retention (EPR) effect of tumor vasculature. The EPR effect is a result of the inherent leakiness of the tumor vasculature, which is underdeveloped and allows nanoparticles to escape the circulation and accumulate passively in tumors. In addition, retention of nanoparticles in the tumor is enhanced by the lack of an efficient lymphatic system which would normally carry extravasated fluid back to the circulation. To take advantage of the EPR effect nanoparticles can be engineered to have a narrow size range between approximately 10 and 100 nm. For example, gold nanostars can take advantage of the EPR effect because they can be synthesized to have hydrodynamic sizes that fit well in the 10–100 nm size ranges. Figure 2 shows results of a study of gold nanostars' biodistribution and intratumoral uptake using radiolabeling, as well as X-ray computed tomography (CT) and optical imaging in a sarcomas mouse model. The data show that the 30-nm GNSs exhibit the most optimal tumor uptake, compared to the smaller 12-nm and the larger 60-nm GNSs [17].

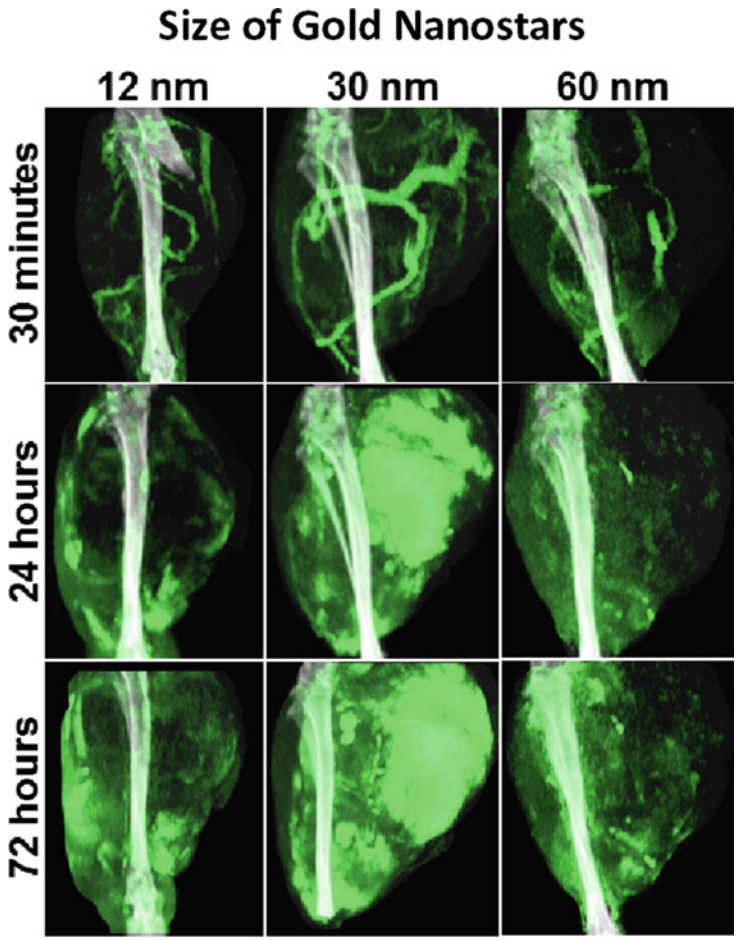

Fig. 2 Effect of the Size of Gold Nanostars on Accumulation into Tumors. Depending on their sizes, nanoparticles have a natural propensity to accumulate in and around cancer cells due to the enhanced permeability and retention effect. Computed tomography images of hind leg primary sarcomas following injection gold nanostars with different sizes (12-nm, 30-nm and 60-nm diameter) at 30 minutes, 24 hours, and 72 hours. The 30-nm GNSs exhibit optimal tumor uptake, compared to the smaller 12-nm and the larger 60-nm GNS (Adapted from Ref [17]

1.4 Nanoparticle Shape, Structure, and Surface Effect

The shape of NPs has also been demonstrated to affect interactions with the innate immune system [6, 18]. Figure 3 shows some examples of metal (gold, silver) nanoparticles that can be engineered to have different shapes (prism, cube, rod, pyramid, dumbbell, star). Gold nanoparticles (GNPs) have received great interest for biomedical applications because they offer several advantages, including

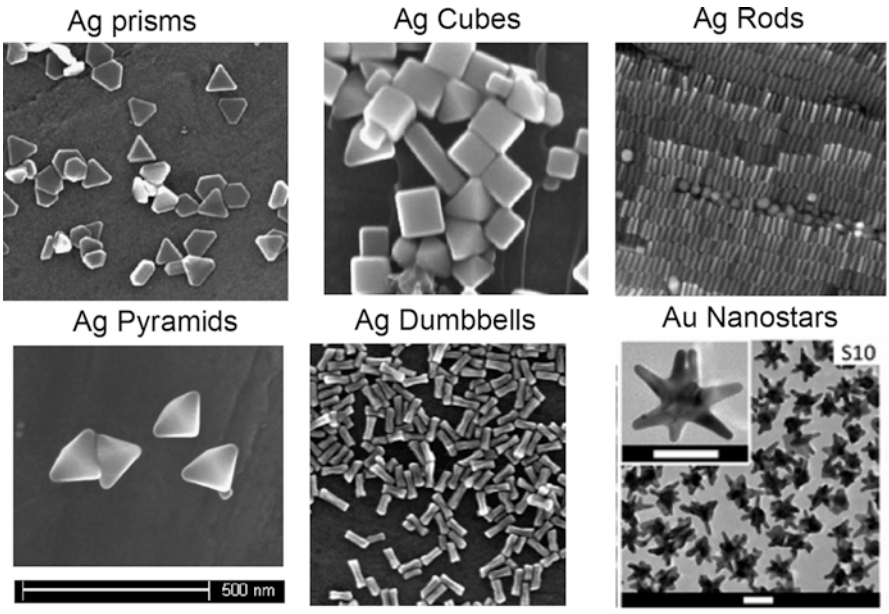

Fig. 3 Examples of Different Shapes of Engineered Metallic Nanoparticles

biocompatibility, the ease of adding functional ligands, efficiency in penetrating cells, and the possibility to heat them using near-infrared light. The effect of size and shape on the biodistribution of a set of gold nanoparticles (GNPs) after intravenous administration in mice has been investigated [17]. The efficiency of cellular uptake of the gold nanoparticles with different shapes was found to rank in the following order from lowest to highest: stars, rods, and triangles [19]. Different shapes tended to use the various endocytosis pathways in different proportions. The results indicated that (a) the size and the shape greatly influence the kinetics of accumulation and excretion of GNPs in filter organs; (b) spherical and star-like GNPs showed the same percentage of accumulation, but a different localization in liver; (c) only star-like GNPs are able to accumulate in lung; (d) changes in the geometry did not improve the passage of the blood brain barrier [20]. Gold nanostars have shown to be an effective theranostic nanoprobes for brain tumor delivery, and spatially controlled blood-brain-Barrier permeation in a mouse study [21]. Systemically administered 1^{24}-labeled GNSs via iv tail vein injection were shown to cross the blood-brain barrier in glioblastoma mouse model and permeate the neoplastic vasculature and accumulated in brain tumor intracellular vesicles [22] The uptake of gold nanorods by macrophages was found to be more efficient than that of nanospheres [23]. Blocking experiments and electron microscopic studies indicated macropinocytosis as the major uptake mechanism.

The effect of surface modifications on cellular uptake of gold nanorods in human primary cells and established cell lines was investigated [24]. The results indicated

that the surface properties of gold nanorods affected their cellular uptake, and the cationic surface tended was advantageous for uptake, but it depended on the cell types. The size and shape of various nanostructures can also affect the immunological response in biological systems. GNPs of varied shapes coated with West Nile virus envelope (E) protein can induce different cytokine secretion behaviors in dendritic cells: rod-shaped GNPs induced the secretion of the inflammasome-related cytokines, interleukin 1beta and interleukin 18, while or cubic AuNPs induce the secretion of the pro-inflammatory cytokines 18 at high levels [25]. A study of the shape effect of glyco-nanoparticles on macrophage cellular uptake and immune response showed that spherical GNPs were internalized to a much greater extent than cylindrical GNPs [26]. This phenomenon was attributed to different endocytosis pathways, spherical GNPs being internalized based on clathrin- and caveolin-mediated endocytosis while cylindrical GNPs mainly involving on clathrin-mediated endocytosis.

Grafting of poly(ethylene oxide) (PEO) onto the nanorods was found to significantly delay their internalization for several hours. It was also observed that neutrophil granulocytes did not fully internalize the particles but trapped them in their extracellular structures. Human cytokine induced killers cell cells loaded with gold nanoprisms have been used as theranostic platform for targeted photoacoustic imaging and enhanced immuno-photothermal combined therapy [27]. Titanium dioxide NPs of different shapes could raise levels of proinflammatory cytokines, increase maturation, and increase expression of costimulatory molecules on DCs [28]. A study of various sizes and shapes of polystyrene NPs showed that particles possessing the longest dimension exhibited maximum tendency to attach to macrophages as these size particles exhibit the strongest binding to the membrane ruffles of the macrophage surface [29].

Chitosan-coated hollow CuS nanoparticles that assemble the immunoadjuvants oligodeoxynucleotides containing the cytosine guanine (CpG) motifs have been developed for combined NIR laser-induced photothermal ablation and immunotherapy [30]. In this method, photothermal ablation-induced tumor cell death reduces tumor growth and releases tumor antigens into the surrounding milieu, while the immunoadjuvants potentiate host antitumor immunity. These hollow CuS nanoparticles are biodegradable and can be eliminated from the body after laser excitation.

Before interacting with the immune system, NPs must first enter the cell. Therefore, it is important to engineer NPs for effective delivery systems that could offer various advantages: (1) effective accumulation into cellular systems of interest; (2) site-specific delivery of drugs, peptides, and genes for specific applications; (3) improved in vitro and in vivo stability; and (4) minimum side effect profile. Two-dimensional Raman imaging were used to investigate nanoprobe uptake in single cells by monitoring spatial and temporal tracking via Raman-labeling and modulation of surface charge [31]. To study the efficiency of cellular uptake, silver nanoparticles functionalized with three different positive, negative-, and neutrally-charged Raman labels [4-mercaptobenzoic acid (4-MBA), 4-aminothiophenol (4-ATP), and 4-thiocresol (4-TC)] were co-incubated with cell cultures, and allowed to be taken up via normal cellular processes. The results showed that the 4-ATP

particles are taken up into the cells more efficiently than particles functionalized with 4-MBA (Fig. 4a). The surface charge on the nanoparticles was observed to modulate their uptake efficiency during co-incubation, demonstrating a dual function of the surface modifications as tracking labels and as modulators of cell uptake. Uptake of nanoparticles in the nucleus of a cell was demonstrated with a

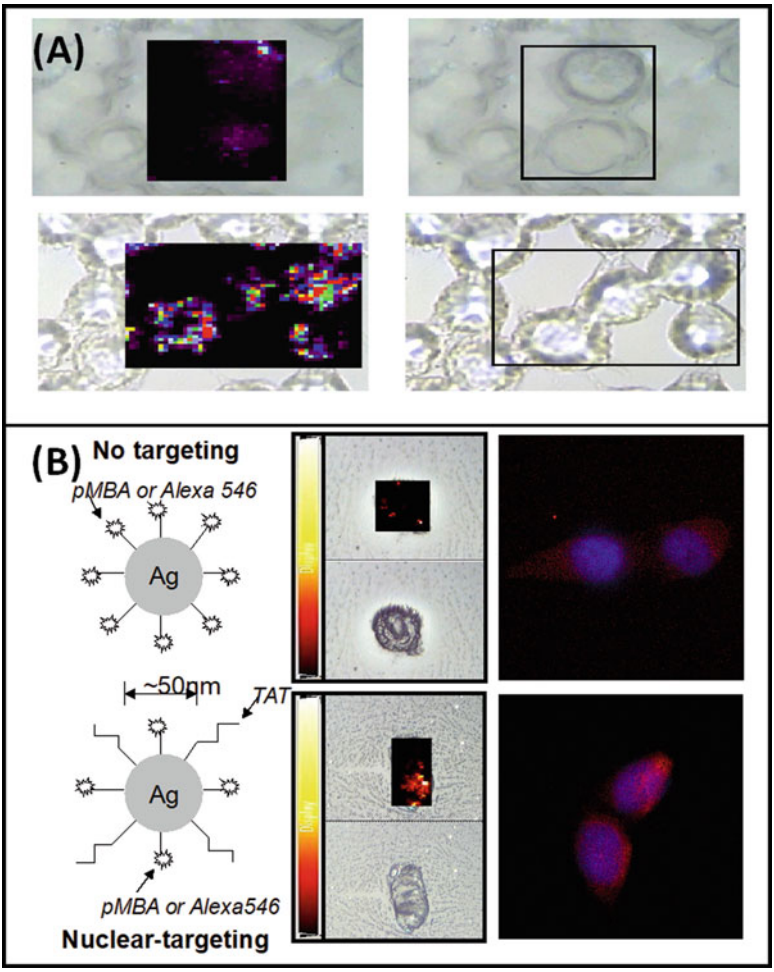

Fig. 4 Effect of Chemical Ligands on Cellular Uptake. (**a**) Two-dimensional surface-enhanced Raman scattering (SERS) map showing cellular distribution of with 4-ATP-labeled silver nanoparticle labeled with 4-MBA dye (top) and 4-AT dye (bottom) in J774 cells; the 4-ATP particles are taken up into the cells more efficiently than particles functionalized with 4-MBA (Adapted from Ref. [31]); (**b**) Two-dimensional SERS mapping was used to track the spatial and temporal progress of cell uptake and localization of nanoparticles. Silver nanoparticles co-functionalized with the TAT peptide (bottom) showed greatly enhanced uptake into the nucleus over the nanoparticles without the targeting moiety (top). (Adapted from Ref. [32])

co-functionalized nanosensing/biodelivery platform combining a nuclear targeting peptide (NTP) for improved cellular uptake and intracellular targeting, and p-mercaptobenzoic acid (pMBA) as a surface-enhanced Raman scattering (SERS) reporter (Fig. 4b) [32]. The nuclear targeting peptide, an HIV-1 protein-derived TAT sequence, has been previously shown to aid entry of cargo through the cell membrane via normal cellular processes and, furthermore, to localize small cargo to the nucleus of the cell. Silver nanoparticles co-functionalized with the TAT peptide showed greatly enhanced cellular uptake over the control nanoparticles lacking the targeting moiety.

The size, chemical structure and composition of NPs could play an important role in their interaction with the immune system. For example, some NPs could trigger immunostimulatory responses mediated by the production of inflammatory cytokines. Secretion of inflammatory cytokines has been shown for a variety of nanomaterials themselves including gold colloids, dendrimers, polymers, and lipid NPs [12, 33–37]. Cationic liposomes facilitate secretion of inflammatory cytokines such as TNF-α, IL-12, and IFN-γ, and increase the expression of CD80/CD86 activation markers on the surface of DCs than anionic or neutral NPs [38, 39]. Therefore, great care should be taken with therapies that use NPs and their safety should be well evaluated before clinical use.

2 Nanoparticles, Antitumor Immunity and Cancer Immunotherapy

2.1 Nanoparticle and Cancer Immunosurveillance and Tumor Microenvironment

Nanoparticles have great potential to produce novel therapeutic strategies that target malignant cells through the ability of nanoparticles to get access to and be ingested preferentially by tumors due to the EPR effect. The accumulation of NPs in tumors and specific effects on the immune system are topics of extensive research. Nanoparticles have been reported to exhibit properties capable of stimulating antitumor immunity [40]. In cancer research, it has been observed that while the immune system can recognize and potentially attack tumors, the tumor could develop immunosuppressive systems that affect the immune system and protect it against anti-tumor immunity [41, 42]. Extensive research effort have been devoted to cancer immunosurveillance and to the so-called concept of ‘cancer immunoediting’. Cancer immunoediting consists of three phases: elimination (i.e., cancer immunosurveillance), equilibrium, and escape. Cytotoxic lymphocytes, which are major protagonists of immunosurveillance and immunotherapy of cancer, are capable of eradicating malignant cells. The selective killing of transformed cells requires a precise molecular recognition of “malignant self” [42]. Better understanding of the

immunobiology of cancer immunosurveillance and immunoediting could lead to the development of more effective immunotherapeutic approaches to eradicate human cancers.

The therapeutic efficacy of cancer immunotherapy for many patients is mainly limited by the immunosuppressive tumor microenvironment (TME). The key to current immunotherapy strategies is modifying the tumor microenvironment such that the tumor-mediated immunosuppression is reduced, immune recognition of the tumor is supported and the immune system effectively attacks the tumor. There is increasing interest in developing strategies based on NPs to change and modulate the tumor microenvironment (TMI) in order to stimulate innate and adaptive immune systems and achieve effective anti-tumor immune responses. The ultimate goal is to trigger, modulate and expand the capabilities of the patient's immune system to attack and eradicate tumors. Immunomodulation of immunosuppressive factors and therapeutic immune cells (e.g., T cells and antigen-presenting cells) could be designed to reprogram the immunosuppressive TME. Nano-biomaterials can be rationally designed to modulate the immunosuppressive TME in a spatiotemporal manner for enhanced cancer immunotherapy [43]. A syringeable immunomodulatory multidomain nanogel (iGel) that overcomes the limitation by reprogramming of the pro-tumoral TME to antitumoral immune niches [44]. Local and extended release of immunomodulatory drugs from iGel deplete immunosuppressive cells, while inducing immunogenic cell death and increased immunogenicity. The iGel approach has the potential to offer an immunotherapeutic platform that can reshape immunosuppressive TMEs and synergize cancer immunotherapy with checkpoint therapies, with minimized systemic toxicity.

Figure 5 depicts a schematic diagram of molecular processes in anti-tumor immunity**,** depicting the three different phases of immunization, T-cell activation, and immunosuppression in the tumor microenvironment [45]. In the immune system, tumor-associated antigens are captured by dendritic cells (DCs). The DCs process the captured antigens and expose them onto the major histocompatibility complexes (MHC) I or II and migrate to draining lymph nodes. In the lymph node, antigen presentation to T cells will elicit responses depending on the type of DC maturation stimulus received and on the interaction of T-cell costimulatory molecules with their surface receptors on DCs. For instance, interaction of CTLA4 with CD80/86 or PD-1 with PD-L1/PD-L2 will suppress T cell responses. Antigen-educated T cells (along with B cells and NK cells) will exit the lymph node and enter the tumor bed, where a host of immunosuppressive defense mechanisms can be produced by tumors [45].

2.2 Nanosystems with Tumor Antigens

Tumors often produce antigenic species that could induce immunogenic responses from the patient's body; however, these immune responses are usually not sufficiently strong or are suppressed by various mechanisms. Processes that suppress

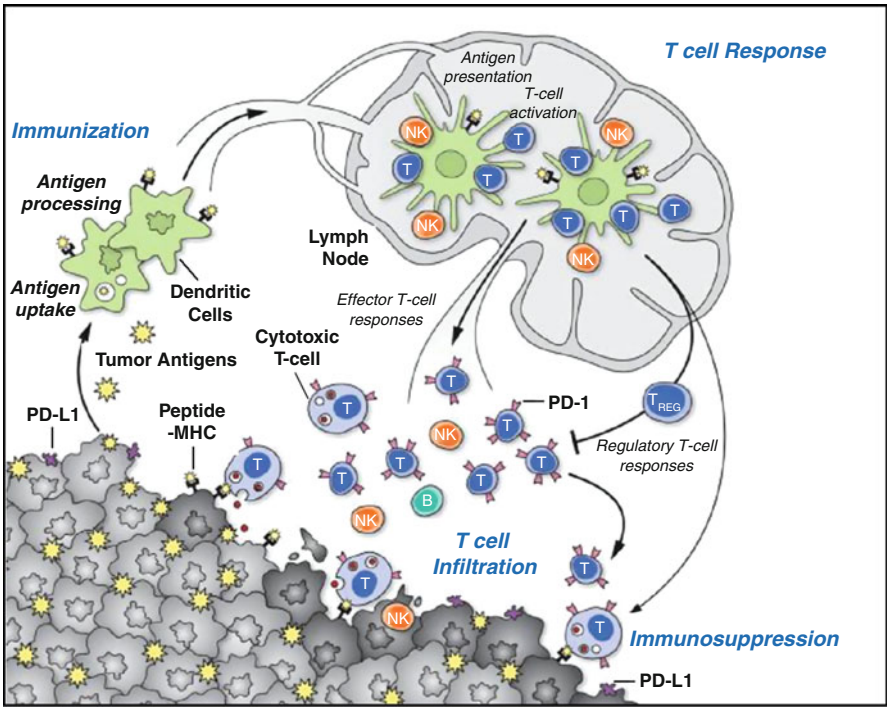

Fig. 5 Schematic Diagram of Molecular Processes and Biological Species involved in Anti-Tumor Immunity. The three different phases of immunization, T-cell activation, and immunosuppression in the tumor microenvironment are depicted (Adapted from Ref [45])

anti-tumor responses of the immune system could involve recruitment of immunosuppressive leukocytes. A possible strategy for effective delivery system tumor antigens is to use NPs that are loaded with tumor antigens; phagocytes will then take up these NPs, become stimulated and present antigens to T cell, the main cell type in anti-tumor immune responses. Also tumor-associated immunosuppressive phagocytes, which are present in the tumor microenvironment of solid tumors, and cells that are critical for tumor progression, are constantly recruited to the tumor, and therefore could carry NPs to tumors. In this case the antigen-loaded NPs stimulate phagocytic cells to become activated immunostimulatory antigen-presenting cells (APC), resulting in a more effective adaptive immune response. In line of this strategy, various nanoplatforms loaded with tumor antigens have been developed to enhance the response of the immune system against tumors [46–48]. Cancer vaccines loaded with tumor-associated antigens are able to induce antigen-specific immunities against tumors, rather than non-specific immunological responses triggered by other methods such as the checkpoint-blockade therapy. Also, cancer vaccines may offer a long-term immune-memory effect that could be helpful to prevent cancer recurrence.

2.3 Nanoparticles Having Adjuvant Activity

As discussed previously, the process of tumor antigen presentation is important in activating and enhancing the adaptive immune system. However, tumor antigen presentation by APCs is often an ineffective process. A method to achieve this enhancement effect involves addition of an adjuvant to NP payloads in order to modulate immunosuppressive APCs, such as dendritic cells (DCs), into immunostimulatory phenotypes in the tumor microenvironment. DCs are important regulators of the immune system, with the ability to induce and maintain primary immune responses as well as tolerance. It has been shown that phagocytic APCs tend to ingest NPs preferentially as compared to other cells [49]. Toll-like receptors (TLRs), an important type of receptors that recognize pathogen-associated constituents, are expressed in APCs [50]. Activation of these receptors is an effective strategy to activate APCs and stimulate an effective adaptive immune response [51]. It is noteworthy that some vaccines used adjuvant substances that activate TLRs or other "danger" signal receptors in order to stimulate the innate immune response, leading to a more effective adaptive immune response.

Nanoparticle-delivered adjuvants have been used to 'precondition' or alter the vascular and immunological biology of the tumor to enhance its susceptibility to thermal therapy [52]. The 'preconditioning' process involves the use of a bioactive agent (e.g., TNF-alpha, arsenic trioxide,. and interleukins) to modify the vascular and/ or immunological components of the tumor microenvironment in order to make the tumor tissue more susceptible to a secondary treatment, such as thermal therapy. A gold nanoparticle tagged with a vascular targeting agent (i.e., TNF-alpha) demonstrated the possibility of preconditioning through reduction in tumor blood flow and induction of vascular damage, which recruits a strong and sustained inflammatory infiltrate in the tumor.

Some ligands are used to induce an immunostimulatory effect in NPs. Polyethyleneimine (PEI) is a positively charged polymer that has shown to induce anti-tumor immune effect. PEI-based nanoparticles encapsulating siRNA were preferentially and engulfed by regulatory DCs expressing CD11c and programmed cell death 1–ligand 1 (PD-L1) [53]. PEI-siRNA uptake transformed these DCs from immunosuppressive cells to efficient antigen-presenting cells that activated tumor-reactive lymphocytes and exerted direct tumoricidal activity, both in vivo and in situ. In particular siRNA-PEI nanoparticles were shown to stimulate TLR5 and TLR7 stimulation of siRNA-PEI nanoparticles and synergize with the gene-specific silencing activity of siRNA; this process transform tumor-infiltrating regulatory DCs into DCs capable of promoting therapeutic antitumor immunity. PEI-based nanoparticles have been developed for use as vaccine adjuvants. Other types of NP generating polymer were also shown capable of stimulating TLRs. For instance, polymethyl vinyl ether-co-maleic anhydride (PVMA)-coated NPs were also successfully used as an adjuvant to activate DCs by stimulating TLR2/4 stimulation. These findings indicate that NPs engineered to include adjuvant activity have considerable potential for cancer immunotherapy. Gold NPs have been shown

to be effective vaccine adjuvants and enhance the immune response via different cytokine pathways depending on their sizes and shapes [54].

2.4 Virus-like Nanoparticles and Cancer Immunotherapy

Virus-like particles (VLPs) are multiprotein structures that mimic the organization and conformation of authentic native viruses but are non-infectious by lacking the viral genome. The FDA has approved several VLP-based vaccines to prevent infection by viruses that cause cancer. Examples of these VLP vaccines include the one against human papilloma virus (HPV) that causes human cervical cancer, and a VLP vaccine that protects against hepatitis B virus (HBV) infection, which is associate to liver cancer. The main idea behind the use of VLP vaccine technology against infectious disease or tumors is based on the facts that the VLPs can carry a payload but also can be recognizable by the immune system as a pathogen and can activate APCs [54, 55]. Like other NPs, VLPs also can be engineered to incorporate exogenous molecules, such as antigens, giving them potential value for immunotherapy, and as vaccines for a range of cancers and other diseases. Virus-based cancer vaccines have receiving increasing interest in the field of cancer immunotherapy; it has been observed that most of the immune responses they elicit are against the virus and not against the tumor. On the other hand, targeting tumor associated antigens is effective, however the identification of these antigens remains challenging. To address this challenge a multi-vaccination strategy focused on an oncolytic virus artificially wrapped with tumor cancer membranes carrying tumor antigens has been developed. This nanoparticle platform can control the growth of aggressive melanoma and lung tumors in vivo both in preventive and therapeutic setting, creating a highly specific anti-cancer immune response [56].

A VLN was developed as a nanoplatform to co-deliver CRISPR/Cas9 [Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-associated protein 9 (Cas9)] system and small molecule drugs for effective malignant cancer immunotherapy [57]. VLN has a core-shell structure, in which small molecule drugs and CRISPR/Cas9 system are loaded in the mesoporous silica nanoparticle (MSN)-based core, which is further encapsulated with a lipid shell. Upon reaching tumors, VLN releases the CRISPR/Cas9 system and small molecule drugs in response to the reductive microenvironment, resulting in the synergistic regulation of multiple cancer-associated pathways.

3 Nanoparticle-Mediated Hyperthermia and the Immune System

3.1 Hyperthermia Effect on the Immune System

Another area of increasing interest for immune-mediated anti-tumor nanotechnology in hyperthermia is the use of NPs that are dormant by themselves but can be activated using external energy sources. As one of the first effective systemic cancer treatments, hyperthermia (HT) aims to increase tumor temperature above the normal value (~36 °C) to trigger local and systemic antitumor effects and/or ablate cancer cells. HT at high temperature (>55 °C) can actually induce immediate thermal death (ablation) to targeted tumors. On the other hand, HT at mild fever-range can be used to improve drug delivery to tumors, improve cancer cell sensitivity to other therapies, and trigger potent systemic anti-cancer immune responses [58–61]. Fever-range thermal stress was shown to increase tumor cell susceptibility to NK cells via increased expression of NK target molecule [62]. Upon hyperthermia treatment, cancer cells experience stress and induce heat shock proteins (HSPs) as a part of the defense mechanisms. HSPs released after heat-induced necrosis can activate APCs through TLR signaling pathways and exhibit immunostimulatory properties [63]. Increased production of inflammatory cytokines, such as TNF-α, IL-1β, and IL-12 was observed following the binding of HSP70 to TLR2 and TLR4 on DCs [64, 65]. Several studies demonstrated that HSPs can enhance the adaptive anti-tumor immune response by inducing cross-presentation of cancer antigen to prime cytotoxic CD8+ T cells [66–68].

Traditional HT modalities such as microwaves, radiofrequency and ultrasound can control macroscopic heating around the tumor region, but cannot precisely target or ablate cancer cells in a timely manner. Cancer treatment using photothermal therapy (PTT), which exploits high temperature transduced from photon energy is a promising method offering high efficiency and specificity because cancer cells are more sensitive to elevated temperature (>42 °C) than normal cells.

3.2 Nanoparticle-Mediated Hyperthermia

Nanoparticle (NP)-mediated thermal therapy has demonstrated the potential to combine the advantages of precise cancer cell ablation. NPs have a natural propensity to extravasate from the tumor vascular network and accumulate in and around cancer cells due to the enhanced permeability and retention (EPR) effect. Selective absorption of NPs into the tumors using external energy sources can induce nanoparticle-mediated local hyperthermia. The NP-mediated thermal techniques are, in general, minimally invasive and an achieve superior localized heating of the entire tumor mass while sparing surrounding normal tissues. This