REVIEW

Innate and adaptive immune responses toward nanomedicines

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Abstract Since the commercialization of the first liposomes used for drug delivery, Doxil/Caelyx® and Myocet®, tremendous progress has been made in understanding interactions between nanomedicines and biological systems. Fundamental work at the interface of engineering and medicine has allowed nanomedicines to deliver therapeutic small molecules and nucleic acids more efficiently. While nanomedicines are used in oncology for immunotherapy or to deliver combinations of cytotoxics, the clinical successes of gene silencing approaches like patisiran lipid complexes (Onpattro®) have paved the way for a variety of therapies beyond cancer. In parallel, the global severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic has highlighted the potential of mRNA vaccines to develop immunization strategies at unprecedented speed. To rationally design therapeutic and vaccines, chemists, materials scientists, and drug delivery experts need to better understand how nanotechnologies interact with the immune system. This review presents a comprehensive overview of the innate and adaptative immune systems and emphasizes the intricate mechanisms through which nanomedicines interact with these biological functions.

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1. Introduction

Nanomedicines share physicochemical characteristics with pathogens: dimensions which are a fraction of the cell diameter, significant liquid−solid interfaces, and patterned surfaces[1]. Some mechanisms involved in the protection against microbes are therefore also implicated in the recognition of nanomedicines. However, all biological processes protecting against microbial colonization might not equally affect the fate of nanomedicines. While bacteria and viruses have the biological machinery necessary for proliferation, therapeutics are administered at a finite dose; some processes inhibiting the replication of microbes might not affect the clearance of nanomedicines.

The implications of individual biological responses might also be different: while immunological memory against pathogens help protect against infections, immune reactions toward therapeutic nanomedicines can impede their therapeutic effect or trigger adverse reactions. In many countries struggling with the pandemic of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), mRNA vaccines have drawn of a lot of attention. The shape, size, and chemical compositions of these vaccines strongly resemble delivery systems destined to treat cancer or genetic diseases. In light of the physicochemical similarities between vaccines and therapeutic nanomedicines, it seems timely to revise current understanding on the interactions between nano-sized materials and the immune system.

This work offers a perspective of the components of the immune systems and recapitulates how they interact with nanomedicines. The following sections will address innate immunity, the links between the innate and adaptive responses, and the adaptive immune system. Highlighting the intricate interactions between nanomedicines and each of these components will help scientists design more efficient and better tolerated nanotechnologies to treat and prevent human diseases.

2. The immune system

The innate and adaptive immune systems work in orchestrated ways to achieve two distinct purposes: clearing senescent cells and protecting against invading pathogens[2]. The former involves efficient removal of the millions of cells which undergo apoptosis every day. This physiological process must therefore be carefully regulated to avoid disproportionate inflammatory responses[3]. The second function aims at maintaining homeostasis against a variety of microorganisms which have evolved to infect mammals: viruses, bacteria, fungi, and parasites. To overcome very quick replication and possible resistance mechanisms, the control of pathogens involves amplification of biological cues, crosstalk between cells and redundant defense functions. Untamed, this response can sustain inflammation and have deleterious effects on the host.

The innate immune response is a series of biological processes involving proteins and phagocytic cells that occur without much specificity, when a naïve organism is exposed to a pathogen for the first time. Components of the innate immune systems have been conserved throughout evolution or are the result of early interactions of mammals with commensal flora[4]. The innate immune system can rapidly recognize certain molecular patterns shared by pathogens and eliminate them through sequestration in phagocytes. The production of cytokines, i.e., biological mediators that can trigger the death of infected cells and prime for a more sustained response, can further prevent the installation of infection. Examples of cytokines produced by the innate immune system are interferons, interleukins, and some fragments of the complement cascade[5].

The adaptive immune system allows a second wave of defense and prepares for future contacts with the pathogens. This delayed reaction results in both the cellular and the humoral responses, that is the development of mature immune cells and antibodies able to specifically recognize certain antigens on the pathogens[6]. In time, these specialized cells and proteins will allow a quicker and more specific reaction. Importantly, a fraction of the clones of lymphocytes produced during the adaptive response may remain after an infection, creating an immunological memory. This memory, directed against proteins, glycoproteins, and polysaccharides on the surface of the infecting pathogen, can protect the host against other microorganisms sharing structural similarities.

2.1. Cell components of the immune system

The cells of the immune system are called leukocytes or white blood cells. These cells can be separated based on their origin, their histological appearance, and their surface markers (Fig. 1). Like all blood cells, leukocytes originate from hematopoietic stem cells in the bone marrow and are derived from two progenitor lines: myeloid and lymphoid cells. Myeloid cells mature mostly into innate immune cells: monocytes, dendritic cells, mast cells, and granulocytes. The latter include neutrophils, eosinophils, and basophils. In contrast, lymphoid cells are the progenitors of natural killer cells (part of the innate immune system) and the B and T lymphocytes, which constitute the adaptive immune system. After a first stage of maturation in the bone marrow, leukocytes migrate to the blood, the lymphatic system, and tissues, where they further differentiate[6].

Histologically, the different families of blood cells can also be separated based on the structure of their nuclei. Mature red blood cells and platelets are anuclear. Monocytes, macrophages, mast cells, and lymphocytes have spherical nuclei (i.e., mononuclear) and are distinct from polymorphonuclear cells, also known as granulocytes (i.e., neutrophils, eosinophils, and basophils). The lower density of mononuclear cells allows isolation from the blood by centrifugation, and peripheral blood mononucleated cells (PBMCs) are easily accessible to study the interactions of nanomedicines with immune cells[7].

Leukocytes all express a common surface marker, the CD45 surface protein. CD45 is a large protein (180−220 kDa) which plays a key role in regulating immune functions via its phosphatase activity, notably the activation of the T- and B-cell receptors. Natural ligands of CD45 include placental protein 14, lectins (CD22, galectin-1 and -3) and pUL11, a protein found on the cytomegalovirus (CMV)[8]. Leukocytes are distributed differently among organs and tissues, which contributes to their particular immune functions (Table 1).

3. The innate immunity

3.1. Cellular functions in the innate immunity

To clear microbes, cells of the innate immune system share general functions which can be exerted alone or in collaboration with opsonins, i.e., soluble proteins acting as biological 'flags' driving cellular responses (see below).
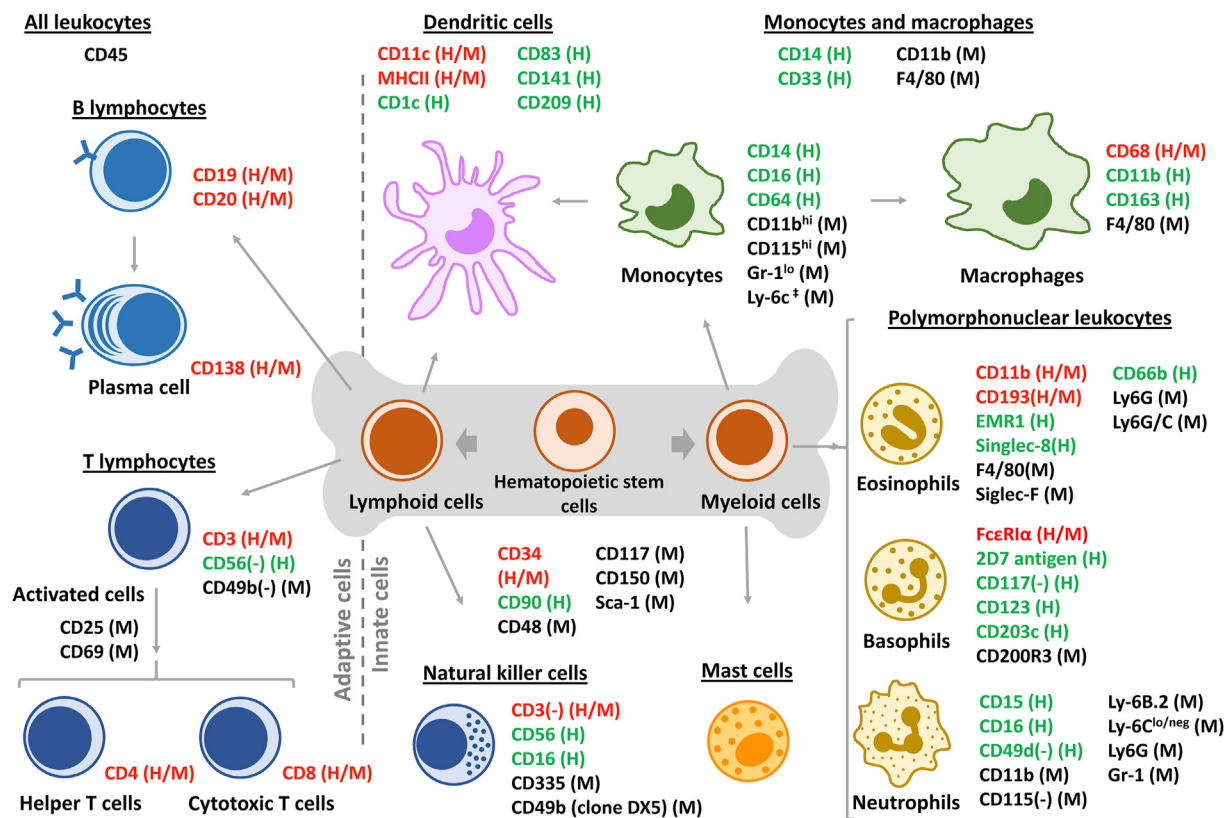
Figure 1 Leukocytes originate from hematopoietic stem cells in the bone marrow. Stem cells give rise to lymphoid and myeloid cells, which are common progenitors of leukocytes and other blood cells. Monocytes (the precursor of macrophages), mast cells and granulocytes (basophils, neutrophils, and eosinophils) originate from myeloid cells. Lymphoid cells produce B- and T-lymphocytes and natural killer cells. Dendritic cells can originate from monocytes or lymphoid precursors. All leukocytes exhibit the CD45 protein on their surface. Common receptors for each cell type in mice (M) and humans (H) are presented in red (both M/H), green (only H) or black (only M). According to their main function in the immune system, leukocytes can be subdivided in adaptive cells (left) or innate cells (right).

3.1.1. Phagocytosis

Phagocytosis is responsible for the sequestration of pathogens as well as the removal of senescent cells from tissues. It consists of the actin-dependent engulfment of microbes or debris inside a phagocyte, usually without the involvement of clathrin[9]. Neutrophils, macrophages, and dendritic cells are called "professional phagocytes", but other cell types, like fibroblasts and endothelial cells, can also participate in the clearance of apoptotic bodies[10].

Phagocytosis involves 1) recognition of the microbe/particle, 2) internalization, and 3) maturation of the phagosome. Multiple successive events initiate phagocytosis: the engagement of extracellular receptors triggers their clustering on the cell membrane and intracellular phosphorylation events which induce actin polymerization and the remodeling of the cell cytoskeleton[9,10]. These events culminate in the wrapping of the phagocyte membrane around the target and its internalization in an intracellular vesicle. The maturation of the phagosome which follows serves two distinct functions: the degradation of the internalized pathogen and the sensing of its composition to drive additional responses, if needed.

Phagocytes have a variety of cellular receptors that allow the detection and engagement of particles in their environment[10]. In mammals, these receptors can be separated in three different classes: pattern-recognition receptors, opsonic receptors, and apoptotic corpse receptors (Fig. 2). Pattern-recognition receptors are a group of receptors able to recognize common chemical characteristics conserved by microbes. Examples of ligands include various components of the wall of bacteria and fungi like lipopolysaccharides, lipoteichoic acid, and various β-glucans[11]. In contrast, opsonic receptors engage pathogens following conformational changes in endogenous fluid-phase proteins. For example, Fc receptors (FcR) recognize the Fc fragments of immunoglobulins when they are patterned on immune complexes. The FcRs expressed on phagocytes have distinct affinity for the different isotypes of immunoglobulins. Other important ligands of opsonic receptors are proteins of the complement system which will be described in more details below. Finally, apoptotic corpse receptors detect conformational changes in the phospholipid membrane of apoptotic cells. During apoptosis, phosphatidylserine relocates from the cytoplasmic leaflet to face the extracellular environment, increasing the concentration of this phospholipid by ~300-fold in the outer monolayer of the cell membrane[12]. Macrophages can bind phosphatidylserine directly or via the involvement of soluble proteins (e.g., MFG-E1, Gas6, or protein S)[10].

Recruitment of V-ATPases from the cytosol to the phagosome membrane drives gradual acidification of the lumen via the pumping of H^+ and Cl^- ions[10]. Acidification to a pH of 4.5−5.0 restricts bacterial growth, facilitates hydrolysis, and regulates the functions of proteolytic proteins. The NOX2 enzyme consumes protons from the lumen to form reactive oxygen species (ROS) and superoxide anions able to further degrade pathogens.

Table 1 Approximative distribution of leukocytes in mouse organs and human blood.

Cell type		Lung	Heart	Liver	Spleen	Small intestine	Kidney	Eye	Skin	Human blood
	T cells	31%	9%	17%	38%−42%	34%	20%	8%	40%	15%−34%
	B cells	23%	10%	11%	38%−42%	30%	5%	4%	−	2%−10%
	NK cells	6%	2%	2%	−	−	1%	2%	−	2%
	Neutrophils	12%	7%	10%	15%	1%	3%	5%	0.6%	45%−75%
	Eosinophils	2%	0.7%	0.7%	0.7%	2%	0.7%	−	4%	1%−7%
	Basophils	−	−	−	−	−	−	−	−	1%
	Mast cells	−	−	−	−	−	−	−	13%	−
	Dendritic cells	5%	1.5%	3%	2%	3%	4%	5%	20%[a]	−
	Monocytes	2%	2%	2%	−	1%	3%	4%	0.6%	4%−10%
	Macrophages	19%	62%	54%[b]	1%	28%	63%	71%[c]	22%	−
	Blast cells	−	−	−	4%	−	−	−	−	−

[a]Includes Langerhans cells.
[b]also known as Kupffer cells.
[c]Includes Microglia.

Myeloperoxidase also uses hydrogen peroxide (H_2O_2) and chloride ions to form the strong oxidizer hypochlorous acid (HOCl)[13]. Finally, cytosolic vesicles fuse with the phagosome to deliver antimicrobial peptides and proteins. These molecules interfere with functions of the pathogen by restricting access to essential metal cofactors: for example, lactoferrin binds ferric ions (Fe^{3+}) and the natural resistance-associated macrophage protein 1 (NRAMP-1) binds Zn^{2+} and Mn^{2+}. The maturation of the phagosome into the phagolysosome also implicates proteins with direct hydrolase activities: lysozyme can degrade β_{1-4} glycosidic bonds, while different pH-dependent cathepsins cleave peptide bonds, at various stages of phagosome maturation[10].

Strictly speaking, phagocytosis describes the internalization of particles with diameters above 0.5 μm[9,10]. In rat alveolar macrophages, cultured *in vitro* in the presence of polystyrene beads (diameters between 1 and 9 μm), phagocytosis was found to be maximal for particles with a diameter of 2−3 μm, irrespective of opsonization[14]. Another *in vitro* study suggests that murine bone marrow macrophages can eventually ingest IgG-opsonized particles with diameters *ca.* 20 μm, over a period of 60 min[15]. Beyond this size, or if particles present an elongated aspect-ratio and inadequate orientation[16], the spreading of the membrane on the particle can occur and drive frustrated phagocytosis[15,16].

3.1.2. Molecular sensing

Pattern-recognition receptors on the cell surface, like C-type lectin receptors (CLRs) and Toll-like receptors (TLRs), participate in the internalization of pathogens, but other pattern-recognition receptors, like RIG1-like receptors and NOD-like receptors, are also distributed intracellularly[2]. Together these receptors bind molecular patterns associated with pathogens or tissue damage, respectively named pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs)[2]. Activation of the receptors induces as series of signaling events and the production of cytokines that affect the phagocyte and surrounding cells.

In innate immune cells, TLRs are particularly important because they participate both in extracellular and intracellular sensing of pathogens. TLRs are a family of transmembrane proteins highly conserved between species. Until now, 10 human and 12 murine TLRs have been identified[17]. TLR1, 2, 4, 5, 6, and 11 are found on the plasma membrane. In collaboration with coreceptors, they bind lipopolysaccharides (LPS), lipopeptides, peptidoglycans, and bacterial flagella by hydrophobic and electrostatic interactions. The binding of LPS to TLR4 and its coreceptor MD2 induces internalization[17]. Intracellular TLRs (3, 7, 8, and 9) are expressed in endosomes, lysosomes, and the endoplasmic reticulum, and can be recruited to the phagosome to sense its content. Examples of molecules that can be sensed by intracellular TLRs are single and double-stranded RNA, as well as CpG DNA motifs. These molecules are ligands for TLR7/8 (sRNA), TLR3 (dsRNA), and TLR9 (DNA). Activation of TLRs by their ligand induces cascades of signaling events which can translate into a type 1 interferon response or the production of inflammatory cytokines [interleukin-1β (IL-1β), IL-6, IL-12, IL-18, and tumor necrosis factor-alpha (TNFα)][17]. These cytokines will impact the polarization of surrounding cells (see below).
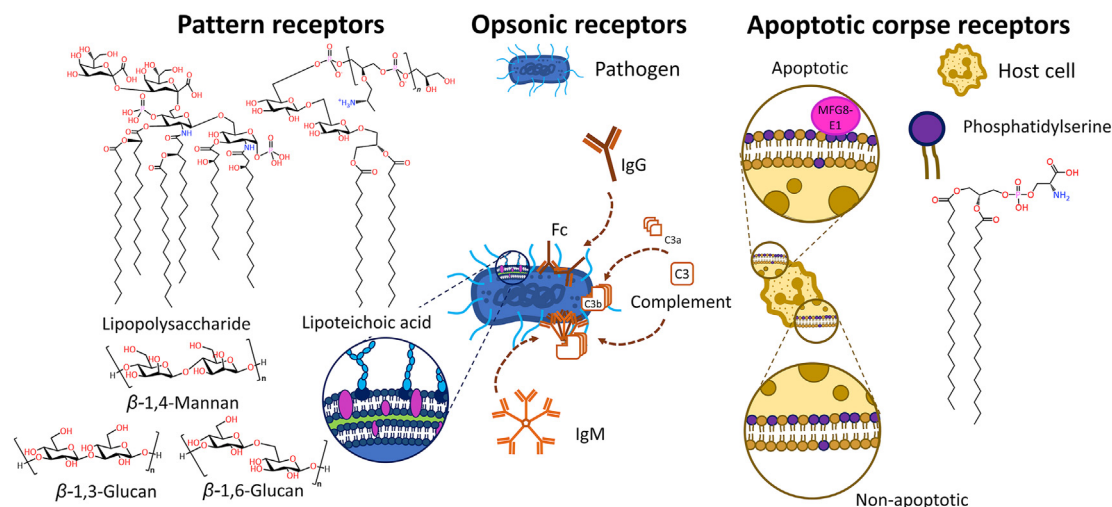
Figure 2 Phagocytes have different types of receptors enabling phagocytosis. Pattern receptors directly recognize molecules on the surface of pathogens. Opsonic receptors recognize changes in conformation of soluble proteins when the latter bind to a pathogen. Apoptotic corpse receptors recognize extracellular exposure of phosphatidylserine on the surface of dying cells.

3.1.3. Extracellular degranulation

A third function shared by some innate immune cells is the ability to secrete, in the extracellular fluid, cytotoxic and antibacterial molecules similar to those found in the phagosome[18]. This additional protection mechanism can prevent the replication of pathogens by damaging them in situ and interfering with some of their metabolic functions. Due to the presence of intracellular granules, mast cells, and granulocytes (neutrophils, eosinophils, and basophils) are well equipped for these secretory functions[10]. Frustrated phagocytosis or direct activation of cellular receptors by ligands coming from the pathogen can trigger extracellular degranulation. Upon release, the content of these granules, which also include cytokines, acts as biological cues for surrounding cells. For example, the granules of eosinophils contain both pro- and anti-inflammatory cytokines, as well as IL-5, chemokines, and growth factors which will influence chemotaxis and immune responses[19,20].

Degranulation can originate from the total lysis of the cell (i.e., cytolytic degranulation) or from "piecemeal" degranulation, a process that maintains viability and ulterior functions[20]. One type of cytolytic degranulation is the production of extracellular traps by neutrophils[21] and eosinophils[19]. These structures consist of entanglements of DNA and bactericidal proteins which are expelled from the cell by the disruption of the plasma membrane. These structures physically entangle pathogens and act as strong DAMPs that can be sensed by surrounding cells, they appear to have protective functions in sepsis[22], but might also be implicated in disease[23].

3.1.4. Cytotoxic activity

Finally, a subset of innate cells from lymphoid origin, natural killer lymphocytes (NK cells), also help to maintain homeostasis[24]. NK lymphocytes can trigger apoptosis by directly discharging bactericidal molecules to the cytoplasm of cells expressing distress signals. This process shares similarities with the activity of cytotoxic CD8+ lymphocytes discussed below, notably the need for a cell−cell synapse and the involvement of adapter proteins. An important distinction between NK cells and cytotoxic lymphocytes is that the formers do not require maturation to engage pathogens. This allows rapid control of

proliferation in infected cells without the need for clonal selection and expansion. NK cells also have a role in the immunosurveillance against the spontaneous development of cancer[25].

Receptors on the surface of NK cells stem from germline encoded genes, in contrast to receptors from B and T cells which originate from somatic recombination (see below). Another distinction is that, although clones of B and T lymphocytes each express one single antigen-specific receptor, NK cells possess random amounts of multiple receptors[26]. This ensures phenotypic diversity despite a limited repertoire[24].

The effector functions of NK cells are closely regulated by adaptor proteins and activating or inhibitory receptors. An important activating receptor is FcRγRIIIA (CD16) which binds to the Fc portion of IgG immune complexes to trigger antibody-dependent cellular cytotoxicity (ADCC)[25]. This receptor, which bridges innate immunity and the presence of antigen-specific antibodies, is implicated in the efficacy of some therapeutic monoclonal antibodies[27]. NK cells can also be activated in the absence of immunoglobulins, via natural cytotoxicity receptors[28]. These receptors bind diverse ligands with activating or suppressing functions, including components of the extracellular matrix, proteins upregulated in cancer and viral infections, proteins of the complement, lectins (e.g., galectin-3) and growth factors[28].

Finally, like other immune cells, NK cells are impacted by surrounding cytokines. The presence of IL-15, IL-12, and IL-18, secreted by proximal macrophages or dendritic cells, appears necessary to fully prime NK cell functions[25]. Accordingly, NK cells also affect their environment via the production of pro-inflammatory interferon-γ (IFN-γ) and TNFα, as well as anti-inflammatory IL-10[29]. Importantly, the lysis of target cells by NK cells promotes the release of free antigens which can be captured and presented by dendritic cells, eventually playing a role in the adaptive immune response[25].

3.2. Interactions of nanomedicines with innate immune cells

Even if the diameter of nanomaterials is below the range where phagocytosis is the most efficient, interactions of nanomedicines with monocytes and macrophages remains an important factor governing their biological fate[29]. Seminal studies in the 1990s

showed that steric protection on the surface of liposomes[30,31] and polymer nanoparticles[32] decreased unwanted distribution to the liver and the spleen, and prompted enhanced blood circulation times in rodents. This spurred interest in studying the *in vitro* interactions of nanoparticles with monocytes[33,34] and macrophages[35], and eventually their distribution in the organs of the mononuclear phagocyte system (MPS) *in vivo*.

Walkey et al.[35] evaluated the uptake of gold nanoparticles with different poly(ethylene glycol) (PEG) densities in cultures of reticulum cell sarcoma (J774A.1 cells, a cancerous cell line resembling macrophages). After 4 h of incubation *in vitro*, they reached three conclusions: 1) the cellular uptake of nanoparticles with no/low PEGylation (<32 PEG chains per 100 nm^2) was higher than that of nanoparticles with denser steric protection, 2) the uptake of larger particles (60 and 90 nm in diameter) was more efficient than that of smaller colloids (15 and 30 nm), and 3) cellular uptake became independent of the presence of surface proteins as the density of PEG on nanoparticles increased. Looking at human neutrophils isolated from peripheral blood, Bisso and colleagues[36] similarly confirmed that *in vitro* internalization increased with larger diameters until *ca.* 200 nm, and that protein adsorption could increase or decrease uptake based on the nanoparticle characteristics. Interestingly, in other *in vitro* settings, incubation of neutrophils with gold nanorods[37], cationic silica nanostructures[38], inorganic calcium phosphate nanoparticles[39], or cationic lipid nanoparticles[40] triggered the rapid degranulation of extracellular traps. The relevance of extracellular traps on the *in vivo* fate of nanoparticles remains unclear.

In vivo, the involvement of the MPS on the clearance of nanomedicines was evidenced notably by studying the impact of the dose on circulation times[41,42]. For nanoparticles with no steric protection (*i.e.*, high intrinsic clearance), independent studies showed that augmenting the injected dose resulted in non-linear increase in blood exposure[41−44]. This phenomenon was attributed to the limited quantity of opsonins available in the bloodstream[41] or to the saturation of liver Kupffer cells and other phagocytes[42−44]. Interestingly, for nanoparticles which have lower affinity for the MPS due to their steric protection (*i.e.*, PEGylated), increasing the dose within a 100-fold range did not prolong circulation times[42,43]. Recently, our group confirmed that a threshold of approximately 20 PEG chains per 100 nm^2 might be necessary to prevent early clearance of polymer nanoparticles by the MPS, but that higher PEGylation densities did not necessarily translate into higher blood exposure[45].

Various groups have highlighted the importance of circumventing distribution to the MPS to increase the efficacy of therapeutic nanomedicines. For example, the injection of large liposomes containing clodronate can efficiently deplete Kupffer cells in the liver[46]. This model was used to increase the circulation times and tumor distribution of PEGylated doxorubicin liposomes and other types of nanoparticles[47,48]. Although this strategy can result in increased therapeutic efficacy[47], its clinical relevance remains questionable as it might also make animals (and potentially humans) more susceptible to bacterial infections[47]. Interestingly, in both aforementioned studies, the depletion of Kupffer cells resulted in increased splenic distribution[47,48]. This supports the role of the spleen to sieve colloids that are not efficiently retained by the liver[49].

Decoy colloids can also be used to partially bypass the MPS. For example, the tumor distribution and efficacy of small PEGylated nanoparticles was significantly increased when mice were pre-dosed with high quantities of non-PEGylated liposomes (375 mg/kg), 1.5 h before treatment[50]. Likewise, in a thorough and elegant study, Nikitin and colleagues[51] proposed that pre-dosing rodents with allogeneic anti-erythrocytes IgG2a 12 h before the injection of nanomedicines could prolong the circulation times of various colloidal systems, including PEGylated liposomal doxorubicin. The antibodies induced the forced clearance of red blood cells and resulted in increased circulation times compared to those observed in animals primed with vehicle. Although particles with very high clearance benefited the most from the phenomenon, forced clearance of erythrocytes appeared to induce a 1.6-fold increase in the blood exposure of PEGylated liposomal doxorubicin[51]. Importantly, in a murine model of melanoma, PEGylated liposomes showed improved antitumor efficacy when the MPS was saturated.

Finally, in a provocative but well-designed study, Chan and his group[52] suggested recently that the capacity of the MPS might be regulated in terms of numbers of particles instead of mass. Using a variety of techniques, including intravital imaging, they showed that the clearance rates of gold nanoparticles significantly increased when a minimum threshold of 10^{12} injected particles per mouse was reached. Doses above this number resulted in decreased accumulation in macrophages and increased tumor deposition, for gold and silica nanoparticles, as well as PEGylated liposomes[52]. To further showcase the relevance of this threshold, one single administration of a fixed quantity of PEGylated doxorubicin liposomes (*ca.* 5×10^{12} particles, 2 mg/kg of doxorubicin) had significantly increased antitumor efficacy when delivered with a large quantity of empty particles (*ca.* 5×10^{13} empty particles). Time will tell if this threshold can translate to humans by allometric conversions, and whether the phenomenon, obtained with single dose administrations, holds true for the multiple dose regimens used in the clinics.

In patients receiving PEGylated liposomes encapsulating irinotecan[53] or a camptothecin analog[54], Zamboni and his group[53,54] observed that individuals with higher plasma clearance also had a more significant decrease in blood monocytes. In these single intravenous administration studies, blood exposure to the drug was measured by the plasma concentration *vs.* time curve (AUC), and monocyte counts were measured at nadir, (*i.e.*, 9 or 11 days after the dosing of the camptothecin analog and irinotecan, respectively). For both drugs, younger patients (<60 years old) experienced a larger decrease in monocytes than patients >60 years of age[53,54]. The authors propose that uptake in monocytes partly explains the clearance of the liposomes and that the delayed monocytopenia is a consequence of the encapsulated cytotoxic payload[53,54]. Interestingly, both liposomal drugs appeared to be 1.5- to 2.5-fold more toxic for monocytes than for neutrophils. In comparison, the non-encapsulated camptothecin analog was more toxic and affected monocytes and neutrophils equally[54]. It remains unclear if these interactions between the liposomes and the monocytes occur in the bloodstream or in the bone marrow, and whether differences in initial counts of monocytes existed between young and older patients before the administration of liposomes.

Multiple-dose pharmacokinetic studies in patients also support the role of the MPS in the clearance of nanomedicines. In an open label study in 15 patients, Gabizon et al.[55] have observed that repeated administration of PEGylated liposomal doxorubicin resulted in gradually increasing plasma exposure to the drug. Over three cycles of intravenous treatments given every 4 weeks, the AUC of the drug increased by $>40\%$. The authors ascribed this increase in blood exposure to damage caused by the drug to the mononuclear phagocyte system[55]. A follow-up analysis suggested

that patients who had important decreases in monocytes also experienced higher gradual increase in blood exposure, over the 3 cycles[56].

In the clinics, both PEGylated and non-PEGylated nanomedicines are used (Table 2). Liposomal doxorubicin (Myocet®), liposomal vincristine (Marqibo®), and liposomes containing a synergistic combination of daunorubicin and cytarabine (Vyxeos®) are examples of nanomedicines formulated without PEG coating. In contrast, PEGylated liposomal doxorubicin (Doxil®/Caelyx®), PEGylated liposomal irinotecan (Onivyde®), and patisiran lipid complexes (Onpattro®) have steric protection. At least for liposomes, the absence of PEG does not compromise clinical efficacy; encapsulation of doxorubicin in PEGylated and non-PEGylated liposomes significantly increases the blood exposure compared to the free drug, 260-fold with PEGylated liposomes and 20-fold with their non-PEGylated counterpart[57].

3.3.   Proteins of the innate immunity

3.3.1.   The complement system
The complement cascade includes approximately 50 proteins[58], which circulate in the fluid-phase (plasma, lymph, and interstitial fluids)[59] or are attached to the extracellular membrane of leukocytes, platelets, erythrocytes, epithelial cells, endothelial cells, skin keratinocytes, and kidney podocytes[60]. Upon contact with the surface of pathogens, these proteins are cleaved in sequence and their enzymatic functions are activated, triggering a self-amplifying cascade. The deposition of complement proteins on pathogens decorates them with signals that facilitate phagocytosis and send messages to cells of the adaptive immune system. These proteins are called opsonins. Fragments of the complement cascade which do not bind to the pathogen can also act as chemokines and trigger effects more systemically.

The two key components of the complement cascade are the C3 and C5 proteins which are responsible for most of the biological effects of the cascade. These proteins are cleaved into smaller fragments (C3a/C3b and C5a/C5b) by three well-known activation mechanisms: the alternative, classical, and lectin-mediated pathways. Each activation route leads to the formation of distinct C3 and C5 convertases, molecular complexes responsible for the conversion of C3 and C5 into their activated fragments (Fig. 3A).

The alternative pathway is initiated by the spontaneous hydrolysis of C3 protein into C3a and C3b. The C3b fragment can covalently bind to carbohydrates and amines on the surface of the pathogen, *via* a thioester moiety which is exposed upon activation. The C3a protein does not directly interact with the pathogen but is involved in recruiting neutrophils, monocytes, and macrophages to the site of infection. The classical pathway involves the binding of C1q protein to the Fc portion of immunoglobulins found on the surface of pathogens (IgM or IgG). C1q also bind to lipid A, β-sheet amyloid fibrils, pentraxins, and apoptotic cells[60]. The lectin pathway differs from the classical pathway only in the initial stimuli responsible for the conversion of C4 into its bioactive fragments. Instead of relying on the presence of surface antibodies, the lectin pathway is initiated by lectin binding to mannose residues of glycoproteins and glycolipids, or by ficolins binding to *N*-acetylated surfaces. Each mannose binding lectin (MBL) or ficolin associates with serine proteases, forming the MBL-associated serine proteases (MASPs) complexes.

All activation pathways result in the production of different C3 convertases protein complexes (Fig. 3). These complexes amplify the cascade by cleaving additional C3 protein and releasing more C3a and C3b. At this point, additional C3b molecules can join the C3 convertase complex and form the C5 convertase. Cleavage of the protein C5 by the C5 convertase leads to the release of the anaphylatoxin C5a, while the larger fragment, C5b protein, remains bound to the C5 convertase. The enzymatic cascade ends on the formation of C5b-9, terminal complement complex (TCC), or membrane attack complex, resulting from the association of C5b with the proteins C6, C7, C8, and C9. The TCC creates a pore in the microorganism's membrane, leading to its lysis (Fig. 3B).

In general, the complement cascade produces cytokines, opsonins, and terminal lytic complex. Cytokines C3a, C4a, and C5a recruit macrophages and mast cells, which eliminate the antigens marked by C1q, C4b, C3b, or other fragments of C3 (iC3b and C3c). Besides these immediate actions, complement proteins can also participate in the adaptive humoral response[58].

3.3.2.   Nanomedicines and the complement cascade
For almost 30 years, evaluating how nanomedicines activate the complement cascade *ex vivo* has been a common and practical assay to study interactions with biological systems[61−65]. These interactions are regulated by dynamic interfacial forces and physicochemical properties of the material, such as charge, size, shape, hydrophobicity, hydrophilicity, chemical composition, and coverage by functional groups[61,62,64,65]. As such, they offer a convenient way to discriminate between materials, and the challenges and opportunity associated with studying complement−nanomedicine interactions have been detailed elsewhere recently[66].

However, the impact of complement activation on the fate of nanomedicines *in vivo* remains poorly understood. Despite the necessary role of complement proteins to fight some pathogen infections[67,68], *bona fide* demonstrations of the impact of complement on the blood clearance of nanoparticles remain scarce. As early as the 1990s, some reports showed that *in vitro* complement activation by liposomes was not always predictive of circulation times *in vivo*[69]. Szoka's group[70] showed that inhibiting the complement system in mice did not significantly alter the pattern of gene expression, in the lungs, liver, and spleen, observed after intravenous injection of cationic transfecting liposomes.

More recently, our group compared the circulation profiles of PEGylated nanoparticles in C57bl/6 wildtype controls and transgenic animals unable to activate the cascade of the complement, that is C3 knockout (KO) mice[45]. Nanoparticles with long and short circulation times were tested (*i.e.*, 7-fold variation in $AUC_{0-6\ h}$ between the fastest and slowest clearance). For all nanoparticles, intravenous injection resulted in similar pharmacokinetics in $C3^{-/-}$ animals and control animals. Similar results were reproduced by depleting the complement activity in BALB/c mice, *via* the injection of intraperitoneal cobra venom factor (CVF)[71]. This toxin acts as a soluble C3 convertase, and its injection to animals temporarily depletes circulating levels of C3 and their ability to activate the complement system. In this follow-up study, the circulation profiles of a first dose of PEGylated nanoparticles were superimposable, irrespective of the animal's ability to activate complement[71]. We also looked at the circulation profiles of PEGylated and non-PEGylated liposomes in BALB/c mice and Sprague Dawley rats treated with vehicle or CVF[72]. Again, comparable circulation profiles were observed in animals with or without the ability to activate the complement, when 20 mg/kg of PEGylated and non-PEGylated liposomes were injected. However, when non-PEGylated liposomes were

Table 2 Name and indications of various nanomedicines used clinically.

Disease	Formulation	Tradename	Active ingredient	Lipid-based	PEG	Approved indication
Oncology	PEGylated liposomal doxorubicin	Doxil/Caelyx®	Doxorubicin	Yes	Yes	Kaposi's sarcoma, multiple myeloma (USA), metastatic breast cancer (Canada), ovarian cancer (2nd line)
	PEGylated liposomal irinotecan	Onivyde®	Irinotecan	Yes	Yes	Pancreatic cancer (2nd line, with fluorouracil and folinic acid)
	Liposomal doxorubicin	Myocet liposomal®	Doxorubicin	Yes	No	Metastatic breast cancer (Europe, Canada)
	Liposomal daunorubicin/cytarabine	Vyxeos®	Daunorubicin/ cytarabine (1:5 molar ratio)	Yes	No	Newly-diagnosed therapy-related acute myeloid leukemia (in adults), AML with myelodysplasia-related changes (in adults)
	Liposomal vincristine	Marqibo®	Vincristine	Yes	No	Philadelphia chromosome negative acute lymphoblastic leukemia (2nd line, in adults)
	Hafnium oxide nanoparticles	Hensify®	Hafnium oxide nanoparticles (radioenhancer)	No	No	Locally advanced tissue sarcoma (Europe)
	Albumin-bound paclitaxel	Abraxane®	Paclitaxel	No	No	Pancreatic cancer, non-small cell lung cancer (with carboplatin), metastatic breast cancer (2nd line)
Infectious disease	Liposomal amphotericin B	Ambisome®	Amphotericin B (antifungal)	Yes	No	Cryptococcal meningitis, leishmaniasis, and fungal infections (immunocompromised and neutropenic patients or renal sensitivity)
	Liposomal amikacin (suspension for oral inhalation)	Arikayce®	Amikacin (antibiotic)	Yes	No	Refractive *Mycobacterium avium* complex infections
Orphan disease	Patisiran lipid complex	Onpattro®	Patisiran (siRNA)	Yes	Yes	Hereditary transthyretin-mediated amyloidosis
Vaccines	Tozinameran	Comirnaty®	mRNA encoding SARS-CoV-2 spike protein	Yes	Yes	Vaccination against COVID-19 (USA: emergency use; Europe: conditional marketing approval)
	mRNA-1273	Not available	mRNA encoding SARS-CoV-2 spike protein	Yes	Yes	Vaccination against COVID-19 (USA: emergency use; Europe: conditional marketing approval)
Others	Liposomal verteporfin	Visudyne®	Verteporfin (photodynamic therapy)	Yes	No	Phototherapy in subfoveal choroidal neovascularization in age-related macular degeneration
	Ferumoxytol	Feraheme®	Iron oxide nanoparticles	No	No	Iron-deficiency in adults with chronic kidney disease
	Loteprednol etabonate (ophthalmic suspension)	Eysuvis®/Inveltys®	Loteprednol nanocrystals	No	Yes	Short-term treatment of dry eye disease (<2 weeks), post-operative inflammation and pain following ocular surgery

A) The different pathways of complement activation

Alternative pathway
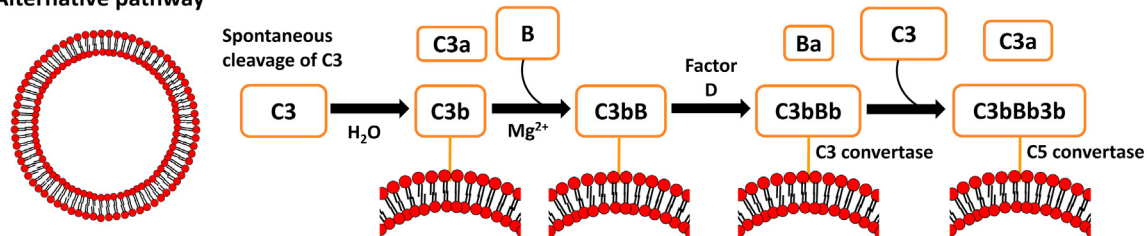
Classical pathway
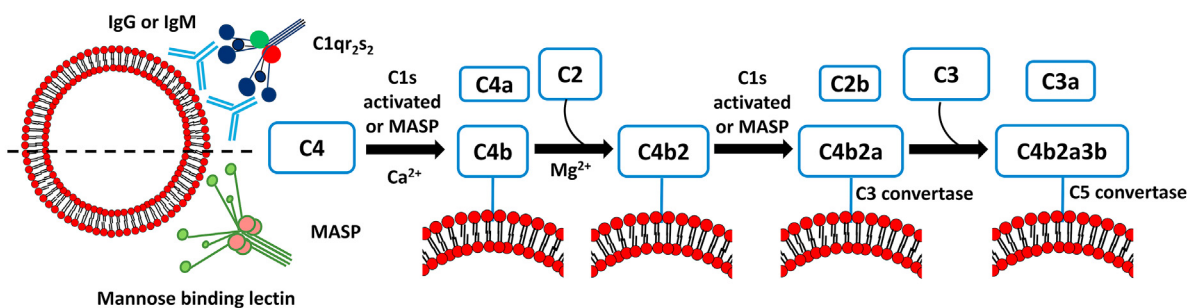
Lectin pathway

B) Cleavage of C5 and formation of terminal complement complex
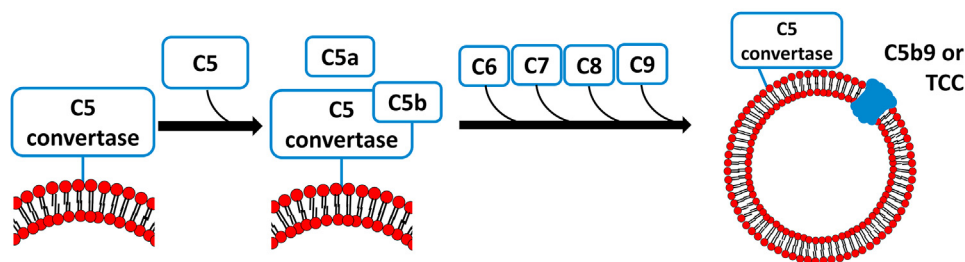
Figure 3   The complement cascade can be initiated by the alternative, classical, or lectin pathways (A). Independently of the pathway, the binding of C6, C7, C8, and C9 proteins to the C5b protein leads to formation of C5b9, also known as terminal complement complex (B).

administered at a lower dose of 2 mg/kg (which resulted in a very fast clearance), a 1.3- and 1.5-fold increase in $AUC_{0-24\ h}$ was observed when complement was depleted in mice and rats, respectively. This increase in blood exposure was mostly attributable to the blood concentrations measured between 6 and 24 h after the injection, when residual circulating levels were low[72].

Other groups have also observed that complement minimally impacted the clearance rates of PEGylated emulsions[73], iron oxide nanoparticles[51], but also alphaviruses[74] and adenoviruses[75]. Some evidence exist that complement proteins pre-adsorbed on the surface of nanomaterials could be dynamically exchanged in vivo[76]. To reach this conclusion, the groups of Moghimi and Simberg[76] coated paramagnetic iron-oxide nanoworms with complement proteins and injected them to C3 KO animals. Five minutes after injection, they recovered the nanomaterial from the blood and observed no trace of the initial complement proteins. If dynamic exchanges also occur with other materials, the phenomenon could explain the difficulty of observing the impact of complement in vivo.

Finally, when investigating the tissue distribution of fluorescently-labeled, non-PEGylated liposomes in the presence

and absence of complement, we observed that depletion of the cascade resulted in decreased distribution of liposomes to splenic B cells[72]. Twenty-four hours after injection, animals injected with CVF had a 4-fold decrease in the proportion of splenic B cells containing liposomes, compared to mice treated with vehicle. Others have also observed that paramagnetic iron-oxide nanoworms had different distribution in circulating leukocytes, in wildtype and C3 KO mice[77]. It is therefore possible that complement could qualitatively affect the distribution of nanomedicines to organs of the MPS, while not always significantly affecting the levels found in circulation.

3.3.3.   The importance of other proteins on the clearance of nanomedicines

Recently, Schöttler et al.[78] studied the uptake of 100-nm polystyrene nanoparticles with relatively sparse steric protection (8−10 chains per 100 nm^2) in cultures of leukemic macrophages (RAW264.7 cells). Like in other reports[36], they observed that cellular uptake could be prevented by pre-incubation of the nanoparticles with plasma proteins. Interestingly, they showed that the adsorption of the protein clusterin (apolipoprotein J) on the

surface of nanoparticles could inhibit the *in vitro* uptake in macrophages by >70%[78]. In our own hands, pre-incubation of PEGylated nanoparticles with clusterin before intravenous injection to healthy mice resulted in increased blood circulation times only for particles with very low PEG densities (<20 PEG chains per 100 nm^2)[45]. In the same work, we also showed that nanoparticles with low steric protection were cleared much faster in transgenic mice which did not express apolipoprotein E (ApoE), a protein responsible for lipid trafficking in the blood. It is therefore possible that clusterin and other apolipoproteins, which interact physiologically with hydrophobic biological constituents, bind to the surface of nanoparticles with low PEG densities to somehow stabilize them. This effect would be less perceptible with systems which have inherently higher steric protection[45].

Further evidences also support a possible role for apolipoproteins on the *in vivo* fate of nanomedicines. The 60-nm hepatitis B virus is known to highjack apolipoproteins to evade immune mechanisms and enter hepatocytes[79]. Likewise, lipid nanoparticles encapsulating siRNA lose their gene silencing properties *in vitro* in the absence of ApoE, and *in vivo* in $Apoe^{-/-}$ mice[80]. Finally, we showed that, irrespective of PEG coverage, the circulation times of polymer nanoparticles in animals that did not express the receptor for low density lipoproteins ($Ldlr^{-/-}$) were longer than those observed in wildtype animals[45]. Further investigation might therefore consolidate a more definitive role for apolipoproteins in the biological fate of nanomedicines.

Finally, two recent reports highlighted the possible importance of "natural" antibodies on the fate of nanomedicines[81,82]. These antibodies are non-specific immunoglobulins which circulate in the blood in the absence of infection. *In vitro* incubation of PEGylated liposomal doxorubicin (LipoDox®), PEGylated liposomal irinotecan (Onivyde®), and carbohydrate coated iron oxide nanoparticles (FeraHeme®), resulted in decreased complement activation, when the plasma of healthy donors and breast cancer patients was depleted of antibodies by exposure to protein A[81]. Complement activation could be restored with the reintroduction of polyclonal IgGs, but not monoclonal (non-specific) trastuzumab. In a parallel study, the quantity of natural IgM adsorbed on the surface of peptide-targeted PEGylated liposomes were correlated with decreases in circulation times in mice and rats[82].

4. The links between innate and adaptive immunity

4.1. Type 1 and type 2 immune responses

Tissue-resident macrophages and infiltrating neutrophils, as first responders to tissue aggression, recruit additional monocytes, and macrophages during the initiation phase of inflammation. The cytokines released in the tissue will influence how recruited cells will respond. For example, most of the neutrophils that accumulate in tissues do not return to the circulation. Neutrophils that phagocytose particles enter phagocytosis-induced cell death which prompts their clearance by macrophages[3]. The sensing of pathogens or infected cells, notably *via* TLR signaling, promotes the production of interferon and inflammatory cytokines. This induces a pro-inflammatory phenotype in newly arrived and tissue-resident cells, that is the type 1 response[83]. Type 1 response translates into increased phagocytic and cytotoxic activities. In opposition, when immune cells sense anti-inflammatory cytokines, for example the minimal danger cues associated with phagocytosis of apoptotic corpses, they will adopt a type 2 response. This phenotype initiates

the resolution phase. The balance between type 1 and 2 responses is closely regulated and threads a fine line between fighting a pathogen and maintaining tissue functions[84].

Type 1/2 polarization was first described for T helper lymphocytes (CD4$^+$, T$_H$ cells)[83], but the observation that macrophages responded to similar biological cues prompted a model where pro- and anti-inflammatory phenotypes were coined as M1 and M2, respectively. Although the dichotomous M1/M2 paradigm is practical, it does not fully appreciate the plethora of macrophage with mixed M1/M2 phenotypes that exist in health and disease[85]. It is increasingly clear that the phenotype of macrophages in tissues initiates the polarization of T$_H$ cells, and not the other way around[84]. Likewise, while macrophages can maintain their phenotype without the involvement of lymphocytes, implication of polarized T$_H$ cells potentiates their effect. Type 1 and 2 responses therefore represent cascades of biological responses implicating a variety of cells.

At the onset, Type 1 response is prompted by IL-12, but the cascade is sustained mostly by the production of IFN-γ, IL-2, IL-6, and TNFα[83]. Pro-inflammatory macrophages are effector cells and produce bactericidal nitric oxide (NO) and ROS, but also high quantities of IL-1β, TNFα, and IL-6 which act as potent positive amplification signals[84]. The pro-inflammatory immune response inhibits and kills pathogens; it is necessary to fight leishmania, bacterial, mycobacterial, and fungal infections[83]. However, untamed type 1 response can cause tissue damage, predispose toward neoplastic transformation or promote insulin resistance[84].

In contrast, type 2 response is driven by IL-4, but also IL-10 and IL-13. Physiologically, most tissue-resident macrophages are polarized toward an anti-inflammatory phenotype which drives growth and healing[84]. In this state, macrophages produce IL-10 and tissue growth factor-β (TGF-β) which sustain the type 2 phenotype. Anti-inflammatory macrophages have high levels of scavenger, mannose, and galactose-type receptors. Disproportionate type 2 response is associated with tissue fibrosis and allergy[83,84].

4.2. Nanomedicines and immune polarization

Due to imbalances in the production of IL-4 and IL-12, the responses of certain inbred strains of mice are skewed toward type 1 or type 2 reactions[83]. While C57BL/6 mice exhibit a general susceptibility to type 1 polarization, the response of BALB/c mice is biased toward type 2. In a very interesting study, the teams of Bear and DeSimone[86] have compared the clearance of nanoparticles in type 1- and type 2-biased mouse strains. During the 2 h that followed the intravenous injection of cylindrical, negatively charged, 300-nm PEG-hydrogels, type 1-biased mice (C57BL/6 and B10D2) had at least a 4-fold higher blood exposure than their type 2 counterparts (BALB/c and DBA2)[86]. These differences were also noticeable for 30-nm quantum dots, but not for 6-μm microparticles. The authors ascribe these distinctions to a higher expression of mannan receptors on the surface of phagocytes from BALB/c mice, compared to C57BL/6 animals. It is unclear how this phenomenon translates to other materials. In this experiment, the clearances of the studied materials were relatively fast, as all had <25% of the initial signal remaining in the blood 2 h after injection[86]. Independently, we also compared the pharmacokinetics of 90-nm polymer nanoparticles with different PEG densities in BALB/c and C57BL/6 mice[45]. Over 6 h after intravenous injection, the blood exposure observed with nanoparticles with low steric protection (*i.e.*, 15 PEG chains per 100 nm^2) was

approximately 1.8-fold higher in C57BL/6, compared to BALB/c mice. Interestingly, these differences disappeared for nanoparticles with slower clearances[45]. Altogether, this suggests that differences between type 1/2-biased strains might be more important for nanoparticles which are quickly removed from the circulation than for longer-circulating systems.

In cancer, systemically injected nanoparticles can preferentially distribute to solid tumors[87] where they can target cancer cells and macrophages alike[88]. The ability of nanomedicines to polarize tumor-associated macrophages toward an anticancer phenotype has therefore raised significant interest. The general concept relies on using the sensing machinery in the macrophage (*i.e.*, TLRs and other pattern receptors) to locally prompt the phagocyte towards a type 1 immune response, for example by encapsulating TLR agonists[89], IL-12, or CpG motifs[90].

In a recent report, Chen's group[91] has designed hybrid nanovesicles decorated with signal regulatory protein alpha (SIRPα) and combining the tumor homing properties of platelet-derived exosomes and the pro-inflammatory macrophage-derived extracellular microvesicles. Three intravenous injections of these sophisticated nanovesicles to C57BL/6 mice bearing subcutaneous B16F10 melanoma, resulted in increased tumor levels of IFN-γ, TNFα, and IL-12, and reduced amounts of IL-10, strongly supporting a shift toward a type 1 response[91]. In this metastatic-prone cancer model, treatment with nanovesicles was able to prevent cancer recurrences after surgical tumor ablation and significantly prolong survival. Interestingly, the nanovesicles were much less potent in a triple-negative breast cancer model (4T1 cells) implanted in BALB/c mice. To achieve comparable efficacy in these type 2-biased animals, encapsulation in nanovesicles of cyclic GMP-AMP, a strong ligand of the intracytoplasmic pattern receptor STING, was necessary[91].

4.3. Antigen presentation and the major histocompatibility complex

Dendritic cells, macrophages and B lymphocytes are professional antigen presenting cells (APCs) which contribute to the adaptive immune response *via* their ability to present antigens to T lymphocytes[92]. T cells can only recognize peptides with a length of 10−30 amino acids[93]. Therefore, APCs specialize in intracellularly processing danger signals and presenting representative segments on their surface *via* dedicated transmembrane proteins, the major histocompatibility complex (MHC) molecules.

In mammals, MHC molecules are highly polymorphic (*i.e.*, large differences exist between individuals) and represent a unique and necessary footprint used by lymphocytes to discriminate between self and non-self. Two categories of MHC molecules exist: MHC class I molecules present what is inside the cytoplasm, and MHC class II what has been phagocytosed. In other words, class I provides surveillance against cellular distresses that are replicating in the cytoplasm (including pathogens), while class II molecules are used by phagocytes to display what they have internalized[94]. Because all nucleated cells can be infected, MHC class I molecules are ubiquitous. In contrast, mostly professional APCs present MHC class II molecules. For reasons inherent to their effector functions detailed below, CD8$^+$ cytotoxic T cells recognize antigenic peptides presented on MHC class I molecules, while CD4$^+$ helper lymphocytes bind only peptides on MCH class II complexes (*i.e.*, interact with APCs).

Antigen presentation is implicated in three important immune functions: 1) the differentiation of naïve T cells toward an antigen-specific response (for this function, APCs are mostly dendritic cells), 2) the T-dependent production of antibodies (APCs are B cells, in lymphoid organs), and 3) the response of effector T lymphocytes (APCs are mostly peripheral macrophages)[84]. The first two functions exploit the mobility of antigens and immune cells through the lymphatic circulation to facilitate contacts between dendritic cells and lymphocytes (Fig. 4). Peripheral dendritic cells that have internalized a pathogen and sensed inflammatory signals, notably the presence of IFN-γ, can migrate toward lymph nodes to present antigens to naïve lymphocytes. The presence of chemokines, notably ligands of the receptor CCR7 expressed on dendritic cells and lymphocytes, coordinates the movement of both populations towards the lymph nodes[6]. During their journey, if dendritic cells sense sufficient danger signals from the internalized pathogen (*e.g.*, activation of TLR by DAMPs), it will upregulate the costimulatory protein B7 involved in the activation of T cells. Free antigens from the extracellular fluid can also passively drain to lymphoid organs where they can be internalized and presented by residing dendritic cells or B cells[95]. Some evidence also exists that monocytes can enter tissues without differentiation, up-regulate expression of MHC class II molecules, take up antigens and deliver them to lymph nodes[96]. Finally, the last function of APCs, which relates to their interactions with effector cells, relies on the patrolling of differentiated T cells in the bloodstream and their chemotaxis to infected peripheral tissues.

4.4. Nanomedicines in the lymphatic circulation

In healthy organs, the interstitial fluid is passively drained to lymph nodes *via* afferent lymphatic vessels (Fig. 4)[6]. Lymphatic vessels have a diameter between 10 μm and 2 mm and contain the lymph, a mixture of extracellular fluid, leukocytes and free antigens[97,98]. In the absence of hydrodynamic pressure, the progression of the lymph is slow (superficial velocities range from 3 to 10 μm/s), and is sustained by the contraction of smooth muscles and the presence of unidirectional valves[98]. The thoracic duct returns the fluid back to the venous circulation *via* the brachiocephalic vein (Fig. 4).

Lymph nodes are capsular tissues consisting of multiple side-by side lobules surrounded by sinuses[97]. Their basic structure is made of a fibrovascular tissue filled with lymphocytes, macrophages, dendritic cells, and erythrocytes[97]. Afferent lymphatic vessels empty in the subcapsular space, while the efferent vessels, the vein, and the artery stem from the medulla. Antibody-producing B cells also reside in the medulla. Each lobule can be subdivided in the superficial cortex, which contains follicles of naïve B cells, and the paracortex which contains T cells[97].

The size of a free antigen can affect how fast it leaves the extracellular fluid to reach the lymph, but also its diffusion through the lymph nodes. Protein antigens with small sizes (molecular weight <70 kDa) can distribute to the follicular region of afferent lymph nodes within minutes of intradermal injection[95,99]. In contrast, lymph-borne vesicular stomatitis viruses (70 nm × 180 nm cylinders) are captured by macrophages in the subcapsular sinus[100]. These distinctions might be of importance to the biological fate of nanomedicines and exploited to design functional materials.

The diffusion of fluorescent colloids through the dermal tissue can offer an elegant way to monitor lymphatic drainage in health and disease[101]. Fluorescent PEG conjugates and liposomes were
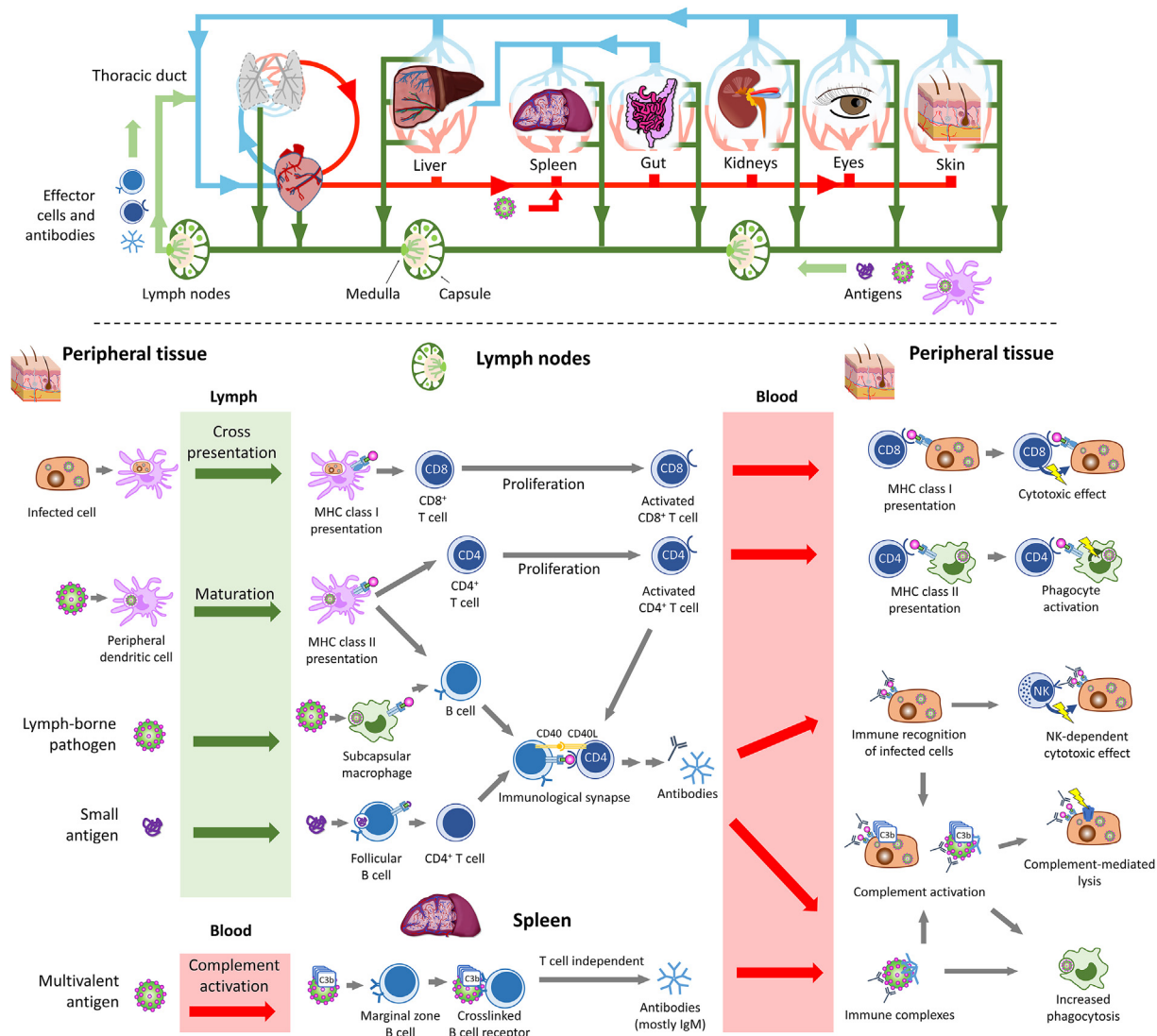
Figure 4 Leukocytes and antigens travel through the blood and the lymph. The lymph is a mix of extracellular fluids and leukocytes drained from tissues of the entire body. The lymph flows from tissues to lymph nodes, reentering the venous circulation through the thoracic duct. Adaptative immune response results from the encounters between antigens and leukocytes.

allowed to evidence how metastases in sentinel lymph nodes could impact lymphatic flow[101]. In a parallel study, intradermal injection of non-PEGylated polymer nanoparticles with diameters of 25 and 100 nm showed very different patterns of diffusion to the lymphatics[102]. Twenty-four hours after injection, significant quantities of ultra-small nanoparticles were detected in the subcapsular sinus of the afferent lymph nodes, internalized by CD11c$^+$ dendritic cells and macrophages. In comparison, the uptake in dendritic cells was 8-fold lower for nanoparticles with a diameter of 100 nm. Beyond size, this work also evidenced that activation of the complement by the nanoparticles prompted maturation of the dendritic cells and more potent immune response[102].

5. The adaptive immunity

Adaptive immunity regroups T and B lymphocytes which have very distinct functions: T cells are mostly effector cells which can adapt to fight pathogens, while B cells are responsible for the production of antibodies. Both T and B lymphocytes are produced

in the bone marrow in the form of individual clones with exceptional ability to recognize antigens.

Through a gene rearrangement process unique to B and T cells called V(D)J recombination, each clone expresses a single and randomly generated surface receptor. Although the biological mechanisms responsible for clonal diversity are beyond the scope of this review, the outcome is that each B- or T-cell receptor on immature lymphocytes recognizes a unique peptide. At this stage, the receptors that bind antigens are generated randomly and without consideration to self or non-self. Cells which are not useful or possibly harmful are eradicated from the repertoire by the selection of clones. Cells that bind self MHC class molecules too weakly or too strongly and those that recognize peptides belonging to the host (*i.e.*, possibly self-reactive) will be eliminated by apoptosis. By the end of this process, each mature lymphocyte can recognize one out of 10^6 different possible foreign peptides.

From the thymus and the spleen where T and B cells respectively mature, they return to the lymph and blood to wander

between secondary lymphoid organs (*e.g.*, lymph nodes and mucosae). Clonal expansion occurs when a T cell with the correct specificity binds an APC with the right antigen−MHC complex[24]. The APCs responsible for priming T cells are mostly dendritic cells. While phagocytosed antigens can be presented on MHC class II molecules (to activate CD4$^+$ cells), the MHC class I presentation (necessary for CD8$^+$ activation) is usually restrained to antigens which have invaded the cytoplasm. Dendritic cells possess a unique cross presentation capacity, that is the ability to phagocytose infected or diseased cells in the periphery, release phagocytosed antigens in their cytoplasm and present them on MHC class I molecules[103].

Clonal expansion requires a triad of signals: 1) antigen-specific activation of the T cell receptor complex, 2) costimulatory signals between the lymphocyte and the APC, and 3) the presence of cytokines[24]. Costimulatory signals originate from the binding of lymphocyte receptors to proteins on primed APCs, notably the involvement of the receptors in the CD28 superfamily which act as stimulatory (CD28, ICOS) and inhibitory (PD-1, CTLA-4) immune checkpoints. Cytokines can originate from the lymphocyte itself (*e.g.*, in an IL-2-dependent autocrine loop) or from surrounding cells (*e.g.*, CD4$^+$ cells help the expansion of CD8$^+$ cells). Under these conditions, antigen-specific CD4$^+$ clones expand 1000 to 10,000-fold, and CD8$^+$ clones up to 50,000 times, significantly increasing the numbers of lymphocytes that can recognize the antigen. Differentiated CD4$^+$ cells also start expressing CD40L which is key to their effector functions.

After clonal expansion, differentiated T cells return to peripheral tissues where they can exert their function, but some CD4$^+$ cells also migrate within the lymphoid organ to the B cell-rich follicle, to help drive the humoral response.

5.1. Cellular immune responses

Via MHC class I molecules, differentiated CD8$^+$ cytotoxic lymphocytes recognize cells that have been infected by pathogens or are expressing distress signals (*e.g.*, cancer cells). Like NK cells, CD8$^+$ lymphocytes can trigger apoptosis by discharging cytotoxic proteins (perforin and granzymes) to the cytoplasm of infected cells. The immunological synapse involves the binding of the specific T cell receptor to its antigen, the binding of the coreceptor CD8 to the MHC class I molecules, as well as the involvement of activating costimulatory signals. Costimulatory signals involve immune checkpoints like those discussed for clonal expansion, but also LFA-1 on the T cell and integrin ICAM-1 on the distressed cell. CD8$^+$ lymphocytes can also induce apoptosis by directly binding the FAS death receptor on the distressed cells by the expression of the protein FAS ligand.

In parallel, CD4$^+$ helper lymphocytes recognize antigens presented on MHC class II molecules through interactions between 1) the presented antigen and T cell receptor, 2) CD4 and the MHC class II molecules, and 3) CD40L and CD40, a protein constitutively expressed on APCs. The immunological synapse sustains a positive amplification loop skewed toward a type 1 or type 2 response, based on the cell polarization and surrounding milieu. T_H1 cells produce cytokines that recruit and activate additional phagocytes and directly stimulate the production of bactericidal molecules by the macrophage involved in the synapse. This enhances the phagocyte's ability to kill its intracellular content by increasing the production of digestive enzymes, and in turn augments the number of digested antigens presented on its surface. Immunological synapses involving T_H2 cells suppress the inflammatory phenotype by secretion of IL-4 and IL-10 and contribute to the activation of eosinophils by secretion of IL-5. When T_H2 cells form immunological synapses with B cells, the humoral response can be skewed toward the production of IgE.

5.2. The humoral immune response

The humoral response corresponds to the production of neutralizing antibodies that bind pathogens and enhance their clearance. Through recombination and maturation, naïve B lymphocytes exhibit a diversity of random receptors comparable to that of T cells. However, while receptors of T cells bind only peptides, those of B cells can recognize peptides, proteins, polysaccharides, lipids, and small chemicals. Furthermore, B cells are distinct from T cells in that they act both as APCs and effector cells[104]. This allows B cells to bind and internalize free antigens in the form of digested peptides, full proteins, or complex structures with repeated surface patterns.

The first step of the humoral response involves the binding of an antigen to a B cell receptor exhibiting the correct specificity[104]. This happens when free antigens diffuse to the follicle of the lymph nodes or when blood-borne pathogens reach the spleen. The mechanisms of B cell activation differ based on whether the antigen is a globular protein or a larger polyvalent pattern.

After binding their specific B cell receptor, antigenic proteins are internalized by naïve B cells and traditionally presented on MHC class II molecules. While these events are not enough to stimulate the production of antibodies, they induce phenotypic changes in the B cell's chemokine receptors and initiate its migration toward the T cell zone of the lymphoid tissue[104]. At this location, the activated B cell presents its surface antigen to a differentiated CD4$^+$ T follicular lymphocytes sharing specificity for the same antigen. Prompted by the secreted cytokines and the activation of its own CD40 by the T cell's CD40L, the antigen-specific B cell proliferates, forming a germinal center in the follicle. There, under the influence of T helper lymphocytes and follicular dendritic cells, B cells undergo a series of hypermutation and selection events which translate into the development of antibodies with very high affinity toward the antigen. Under continuous stimuli from CD40 and cytokines (notably IL-2, IL-4, and IL-6), B cells eventually adopt a plasma cell phenotype able to secrete antibodies.

Contrary to antigenic proteins, large polyvalent patterns like polysaccharides and nucleic acids can trigger antibody production in the absence of T cell involvement. This is due to their large repeating structures which can crosslink multiple B cell receptors simultaneously. In that context, complement proteins deposited on the antigen also bind to coreceptors CD21 (complement receptor 2), fully engaging the response[105]. B cells stimulated in the absence of T helper lymphocytes adopt a short-lived plasma cell phenotype, and rarely induce memory responses.

5.3. Nanomedicines and adaptive immune functions

PEG was initially chosen for its inert character, but it is now appreciated that its patterning on the surface of a nanomedicine can increase interactions with the immune system[71]. Dams and colleagues[106] showed that the clearance of PEGylated liposomes was much increased when rats and Rhesus monkeys had received a first 'sensitizing' dose 5−7 days prior. They showed that the phenotype could be transferred from sensitized to naïve animals by plasma transfusions.

Significant efforts subsequently devoted to study the phenomenon confirmed that increased clearance was due to anti-PEG IgM[107], which were not observed in splenectomized animals[108]. The T-independent nature of the immune response was established by the observation that mice without T cells (nude BALB/c) still developed this phenotype, but not animals without T and B cells (SCID)[109]. Our group and others evidenced that, in animals with anti-PEG IgMs, activation of the complement cascade was in part responsible for the accelerated clearance[71,73].

Beyond liposomes, the phenomenon was observed with various types of PEGylated colloids, including polymer nanoparticles[71], lipid complexes of nucleic acids[110], proteins and viruses[111]. The physicochemical characteristics of the nanomedicines appear to play a role in the production of anti-PEG IgM[112], but also the injected dose[113] and the encapsulation of anticancer payloads[114]. In the latter condition, it is believed that cytotoxic nanomedicines would kill the B cells upon internalization and prevent their proliferation. This is consistent with the absence of accelerated clearance seen in patients who received multiple doses of anticancer nanomedicines. However, multiple reports have evidenced the presence of anti-PEG antibodies in the sera of healthy donors, without known prior exposure to PEGylated therapeutics[115−117]. In these studies, conducted in patients from the U.S., Austria, and China, the prevalence of anti-PEG IgM and IgG ranged between 20% and 70% of samples analyzed[115−117]. The impact of these antibodies on the performance of nanomedicines in patients remains unclear.

The interactions of nanomedicines with the adaptive immune system can also be exploited therapeutically. For example, the physiological PD-1/PD-L1 immunoregulatory pathway is an inhibitory checkpoint which prevents CD8$^+$ from eliciting their cytotoxic activity[118]. In healthy tissues, the binding of PD-1 to PD-L1 protects against self-reactivity, but these cell−cell interactions can also be exploited by cancer cells to bypass immune surveillance. Anti-PD-1 and anti-PD-L1 antibodies, which disrupt the inhibitory effect, achieve very high success rates in patients suffering from melanoma, non-small cell lung cancer, and other types of cancers, but some tumors remain refractive[118].

To increase immune responsiveness, Lebel et al.[119] used 80-nm protein nanoparticles derived from the papaya mosaic virus to trigger a polarizing IFN-α response in tumor-bearing animals. Injected intratumorally, these nanoparticles act as TLR7 agonists and induce the production of interferon gamma inducible proteins-10 (IP-10), monocyte chemoattractant protein-1 (MCP-1), and other inflammatory cytokines. The ensuing tumor infiltration of CD8$^+$ cytotoxic lymphocytes translates into prolonged survival in a model of B16F10 melanoma[119]. Importantly, in the same model, the treatment with the nanoparticles synergized strongly with anti-PD1 antibodies. The authors ascribe this synergy to the increased capacity of their nanoparticles to prime CD8$^+$ T cells, which can freely exert their cytotoxic effect in the presence of checkpoint inhibitors.
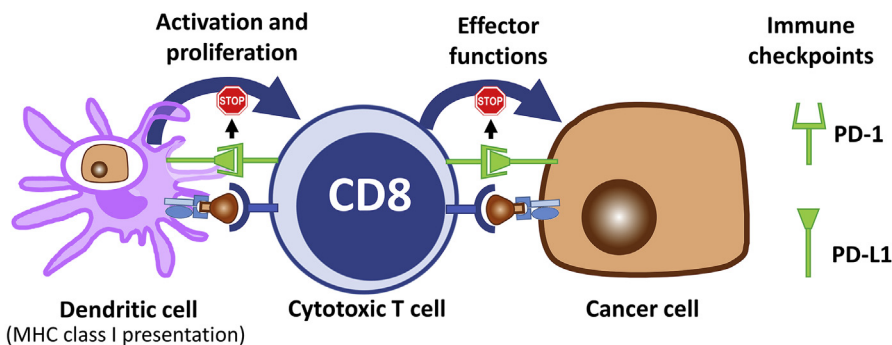
More recently, other systems have been proposed to potentiate the therapeutic inhibition of immune checkpoints and further mitigate the ability of cancer cells to stall cytotoxic lymphocytes. For example, nanomedicines exhibiting polyvalent PD-L1 surface ligands were shown to be have higher affinity and selectivity than free antibodies toward cancer cells, *in vitro* and *in vivo*[120]. In parallel, targeted nanoparticles were also used to silence PD-L1 expression and significantly decrease tumor volume in a murine model of non-small cell lung cancer[121].

Beyond implications of the PD-1/PD-L1 pathway, Oberli et al.[122] described lipid nanoparticles encapsulating mRNA that were able to efficiently transfect phagocytes to express protein antigens. Their rationale was that proteins expressed in the cytoplasm of APCs could be presented to CD8$^+$ cells and induce clonal expansion. *In vivo* screening of a small library of nanoparticles (composed of phospholipids, cholesterol, surfactants, and ionizable and PEGylated lipids) identified formulations which resulted in the prevalence of antigen-specific CD8$^+$ lymphocytes[122]. Using a mock reporter luciferase mRNA, they showed that subcutaneously injected lipid nanoparticles transfected ∼5% of the dendritic cells in the draining lymph node, ∼3% of the neutrophils and ∼1% of the macrophages. mRNA encoding the tumor antigen TRP2, transfected using a formulation which contained the TLR−agonist LPS, resulted in prolonged survival in a B16F10 melanoma model[122]. Similar strategies, combining mRNA to other TLR agonists, like the small molecule R848, have also been proposed[123].
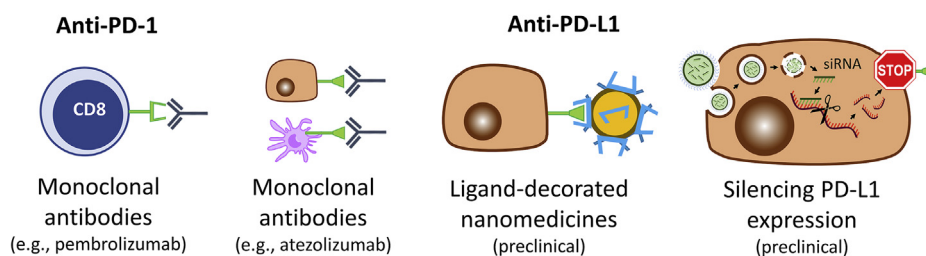
In clinical oncology, T cells can be transfected *ex vivo* with chimeric antigen receptor (CAR) to confer them specificity toward tumor cells, irrespective of their original receptor configuration[124]. Reinjection of autologous transfected CAR T cells to patient results in large numbers of tumor-specific lymphocytes that can fight the cancer, without the need for antigen presentation and clonal expansion. Tisagenlecleucel (Kymriah®, Novartis) and Axicabtagene ciloleucel (Yescarta®, Kite/Gilead) are examples of CAR-T cell technologies approved by the U.S. Food and Drug Administration (FDA) which cost upward of 300,000 $ per patient. To palliate to the complex and costly process of transfecting autologous cells *ex vivo*, CD3-targeted polymer-based nanomedicines to transfect the CAR to T cells *in vivo* have been proposed[125]. Intravenous injection of these antibody-coated nanomedicines containing mRNA resulted in efficient transcription in approximately 8% of the splenic T cells, 3% of macrophages and 2% dendritic cells. Expression was transient and lasted for approximately 7−10 days. Using the cancer specific 1928z CAR, the authors showed that weekly intravenous injections could significantly prolong survival of immunocompetent mice models of leukemia and solid tumor[125]. Importantly, the authors also showed the potential of the technology to induce cellular response against hepato-cellular carcinoma caused by hepatitis B virus. Altogether, these approaches confirm the potential of mRNA nanomedicine to vaccinate against cancer. Should these promises be confirmed in patients, they could significantly alter the practices in cancer immunotherapy, including making CAR technology more accessible for a variety of prevalent cancers. Examples of cancer immunotherapy approaches that can be enabled by nanomedicines are presented in Fig. 5.

Due to the global SARS-CoV-2 pandemic, mRNA vaccines to protect against pathogens have also attracted tremendous attention. The topic has been discussed recently in many excellent reviews[126]. Briefly, the American company Moderna (candidate mRNA-1273)[127], the European collaboration between BioNTech and Pfizer (tozinameran, BNT162b2)[128] and other groups[129] have developed comparable technologies where molecules of messenger RNA are encapsulated in PEGylated lipid nanoparticles. These systems all contain proprietary ionizable lipid allowing endosomal escape, which enables the cytoplasmic translation of mRNA coded protein. The mRNA used by Moderna and Pfizer/BioNTech encodes a stabilized version of the full transmembrane SARS-CoV-2 spike protein, while the mRNAs of the Chinese company Suzhou Abogen Biosciences (candidate ARCoV) encode solely the receptor-binding domain of the spike protein. In an unprecedented accomplishment in the field of vaccine development, these technologies entered

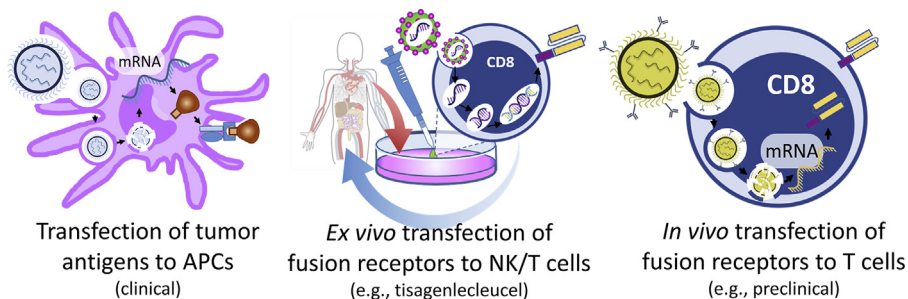## Cells involved in adaptive cytotoxic immune response

Figure 5   Nanomedicines can impact the field of cancer immunotherapy and prompt the immune system to fight tumors more efficiently.

clinical evaluation only a few months after the SARS-CoV-2 genome was made available.

A report on the preclinical development of ARCoV offers some insight on the mechanisms of mRNA vaccines against COVID-19[129]. They showed that intramuscular injection translated into rapid transfection of the injected muscle and the liver, mainly in monocytes, macrophages, and dendritic cells, but also in hepatocytes[129]. Intravenous injection of their candidate also resulted in significant plasma concentration of the encoded protein, but this administration method is not currently used in clinical vaccination regimens. A two-dose vaccination treatment (14 days apart) elicited neutralizing antibodies, a TH1-biased $CD4^+$ response and specific $CD8^+$ cells in mice and non-human primates.

High neutralizing IgG titers, T_H1 biased $CD4^+$ polarization and $CD8^+$ response against subunit S1 of the spike were also obtained in mice with the mRNA-1273 candidate from Moderna[130]. A follow up study in primates showed that two intramuscular injections of 100 µg of mRNA-1273, at a 4-week interval, could raise a neutralizing humoral response in rhesus macaques[127]. In that study, the presence of spike-specific $CD4^+$

follicular lymphocytes and T_H1 cells was confirmed, and no T_H2 polarization nor specific $CD8^+$ T cells were detected. Four weeks after the second vaccination, antibodies could protect animals against intratracheal challenges with 7.6×10^5 plaque-forming units of the virus, as evidenced by genome counts in bronchoalveolar fluid and nasal swabs[127]. The interim results of the phase I clinical trial in 45 healthy patients (18−55 years of age) showed that two injections of mRNA-1273, 28 days apart, induced dose-dependent antigen-binding titers[131]. Combining *in vitro* pseudovirus and wild-type virus neutralizing assays, antibody protection appeared comparable to that measured in sera from convalescent patients, especially after the second vaccination. Similar to data obtained in primates, a $CD4^+$ T_H1-biased response was confirmed by the expression of TNFα, IL-2, and IFN-γ, and low levels of spike-specific $CD8^+$ response was observed[131]. A randomized, placebo-controlled, phase III study was conducted in +30,000 patients to evaluate two 100-µg intramuscular doses of the vaccine, administered 28 days apart[132]. Over 120 days following randomization, 11 infections were diagnosed in patients that had received mRNA-1273, compared to 185 in the placebo group, hence an estimated efficacy of 94%[132]. Importantly, the

vaccine appeared to efficiently protect against the severe form of the disease which occurred in 30 participants form the placebo group, but none from the treatment arm.

In essence, preclinical[133] and clinical[128,134] evaluation of Pfizer/BioNTech's technology afforded very similar results. Intramuscular injection of the vaccine induced the production of neutralizing antibodies in mice and rhesus macaques[133], presumably by transfecting the antigen in APCs from the draining lymph nodes and the spleen. Non-human primates (2−4 years of age) were also protected against infection, upon challenge with 1.05×10^6 plaque forming units of the virus[133]. In contrast with data obtained with mRNA-1273, vaccination with BNT162b2 showed evidence of spike-specific $CD8^+$ cellular response in macaques[133] and humans[135]. In the randomized, placebo-controlled phase III trial, the regimen consisted of two 30-μg doses administered intramuscularly at a 21-day interval. Over 120 days, vaccination resulted in a 95% efficacy (*i.e.*, 8 cases of COVID-19 with the vaccines *vs.* 165 in the placebo group, +43,000 patients randomized). In the whole study, only 10 cases of severe disease were reported, with one in the vaccine group occurring >60 days after the second dose.

These very positive results led to recent regulatory milestones for both Moderna's and Pfizer/BioNTech's vaccines. In December 2020, the FDA authorized emergency use for both vaccines, while the European Medical Agency recommended conditional marketing authorizations. Broad vaccination campaigns have since then been initiated.

6. Conclusions and perspectives

Mammals have developed superb capabilities to protect themselves against microbes. When designing novel therapeutics, understanding of the complex relationships between biological functions might be valuable to predict possible inefficacies and adverse reactions. Whether the intended purpose of the technology is to deliver therapeutic payloads more efficiently or to vaccinate against pathogens, being able to foresee how the host will react to single and multiple doses is critical.

Until now, numerous efforts in the field of nanomedicine have been directed toward fighting cancer. The preclinical development of such technologies often necessitates the use of immunocompromised models which do not fully embody what occurs in immunocompetent hosts. For this reason, interactions of nanomedicines with the immune systems are sometimes overlooked. Likewise, while the clinical successes (and failures) of nanomedicines might provide some understanding on how to develop drugs that will help patients the most, many of the commercialized products are highly cytotoxic. With such agents, deconvoluting the effects of the drug from those of the carrier remains difficult.

The recent commercialization of systemic RNA-based therapies, notably patisiran lipid complexes (Onpattro®), has paved the way for the use of nanomedicines beyond cancer. Opportunities will continue to arise to encapsulate, protect and efficiently deliver drugs and other molecules for a variety of human diseases. Similarly, although it is too soon to predict whether nanosized vaccines will be our ticket out of the global COVID-19 pandemic, the rapid development of Moderna's mRNA-1273 and BioNTech's BNT162b1 have clearly consolidated interest toward mRNA vaccines. Altogether, this justifies concerted efforts toward a better understanding of the intricacies governing how nanomedicines interact with biological systems.

Author contributions

Iara Maíra de Oliveira Viana and Nicolas Bertrand wrote the article, Iara Maíra de Oliveira Viana, Sabrina Roussel, and Nicolas Bertrand prepared the figures, all authors revised and commented on the article.

Conflicts of interest

The authors declare no conflicts of interest.

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