Special Section on Drug Delivery Technologies—Minireview

The Interplay Between Blood Proteins, Complement, and Macrophages on Nanomedicine Performance and Responses

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ABSTRACT

In the blood, depending on their physicochemical characteristics, nanoparticles attract a wide range of plasma biomolecules. The majority of blood biomolecules bind nonspecifically to nanoparticles. On the other hand, biomolecules such as pattern-recognition complement-sensing proteins may recognize some structural determinants of the pristine surface, causing complement activation. Adsorption of nonspecific blood proteins could also recruit natural antibodies and initiate complement activation, and this seems to be a global process

with many preclinical and clinical nanomedicines. We discuss these issues, since complement activation has ramifications in nanomedicine stability and pharmacokinetics, as well as in inflammation and disease progression. Some studies have also predicted a role for complement systems in infusion-related reactions, whereas others show a direct role for macrophages and other immune cells independent of complement activation. We comment on these discrepancies and suggest directions for exploring the underlying mechanisms.

Introduction

Blood leukocytes (predominantly monocytes and neutrophils) and tissue macrophages in contact with blood [e.g., periportal and midzonal Kupffer cells in humans, rats, and mice; pulmonary intravascular macrophages (PIMs) in horses, pigs, and sheep; and splenic marginal zone and red pulp macrophages in many species] rapidly intercept and clear intravenously injected nanoparticles from the blood circulation (Brain et al., 1999; Moghimi et al., 2012a). Macrophages deploy an array of plasma membrane receptors (Taylor et al., 2005) to recognize spatial and temporal changes on nanoparticle surfaces, thus ensuring a recognition hierarchy phenomenon in phagocytic clearance. Dynamic changes on nanoparticle

surfaces are often brought by blood biomolecule deposition (Maiolo et al., 2015), and some of these components aid nanoparticle recognition by different macrophage subpopulations (Moghimi et al., 2012b; Tavano et al., 2018). The latter process is referred to as blood opsonization, and classic examples of blood opsonins include antibodies and two cleavage products of the third complement protein 3 (C3), namely, C3b and iC3b, which prime a surface for recognition by Fc and complement receptors (CRs), respectively, and/or in a cooperative manner (Ricklin et al., 2010). On surface deposition, blood proteins may undergo conformational changes and expose otherwise hidden epitopes, form new clusters and architectural arrangements with other biomolecules (e.g., fiber projections) that could serve as ligands for other macrophage receptors (e.g., different classes of the scavenger receptors) (Chao et al., 2012), or act as templates for subsequent binding of opsonic molecules (Moghimi and Hunter, 2001; Vu et al., 2019). Nanoparticle flow properties within different blood vessels and at bifurcations in vascular and capillary systems, and collision dynamics with circulating cells and vessel walls, may further modulate

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ABBREVIATIONS: C1, complement protein 1; C3, complement protein 3; C5, complement protein 5; CR, complement receptor; MASP, mannose-binding lectin-associated serine protease; MBL, mannose-binding lectin; mPEG, methoxy poly(ethylene glycol); PEG, poly(ethylene glycol); PIM, pulmonary intravascular macrophage.

the dynamic aspects of the biomolecule corona (Moghimi et al., 2012a).

Interfacial changes brought by blood biomolecules (including opsonic proteins) on nanoparticle surfaces are also dependent on nanoparticle characteristics such as shape, curvature, pristine surface chemistry, functionality, and presented defects (Lundqvist et al., 2008; Tenzer et al., 2013). Thus, strategies including nanoparticle shape modification (deviations from a spherical shape) (Gratton et al., 2008; Decuzzi et al., 2009; Moghimi et al., 2012a) and surface grafting/coating with hydrophilic polymers [e.g., methoxy poly(ethylene glycol) (mPEG), and block copolymers such as poloxamers, poloxamines, mPEG-poly(L-lactide), poly(oxazoline), epichlorohydrin crosslinked dextran, and hyperbranched polyglycerols] could offer protection to rapid nanoparticle extraction by blood and tissue phagocytes (Moghimi et al., 2001; Abbina et al., 2017; Tavano et al., 2018). Indeed, in many cases, nanoparticle surface modification with hydrophilic polymers has minimized blood opsonization processes (Moghimi et al., 2001), but in some studies such surface modification processes have also attracted blood dysopsonic molecules to further overcome nanoparticle recognition by macrophages (Moghimi and Patel, 1993; Moghimi et al., 1993b; Schöttler et al., 2016). Furthermore, the steric hindrance from the surface projected hydrophilic polymers could directly interfere with nanoparticle binding to macrophage receptors (Dos Santos et al., 2007; Moghimi et al., 2006). However, some phagocytic cells, depending on their phenotype and the microenvironment, can still recognize and ingest a significant fraction of injected stealth nanomaterials (Moghimi et al., 1993a, 2012b; Moghimi and Gray, 1997; Boraschi et al., 2017; Tavano et al., 2018).

Among many blood proteins, complement opsonization has long been considered as a universal defense strategy for intercepting foreign and self-effete particulate matters, and priming them for recognition by blood and tissue scavengers (Ricklin et al., 2010; Holers, 2014). As a result, complement

opsonization may compromise the performance of nanopharmaceuticals intended for therapeutic intervention outside the phagocytic cell network (Moghimi et al., 2011). Furthermore, uncontrolled complement activation could bring a number of undesirable effects including inflammatory reactions, cardiovascular distress, and promotion of tumor growth (Markiewski et al., 2008; Moghimi, 2014, 2018; Farhangrazi and Moghimi, 2016; Guglietta and Rescigno, 2016; Szebeni et al., 2018). Considering multifaceted roles of the complement system in homeostasis, this perspective examines pathways and mechanisms of nanoparticle-mediated complement activation and turnover, and their impact on immune safe-by-design initiatives in nanomedicine engineering.

A Brief Overview of the Complement System. The complement family comprises at least 35 soluble and membrane-bound proteins playing diverse roles in complement cascade events such as surface sensing, opsonization, and assembly of the membrane attack complex (Ricklin et al., 2010; Holers, 2014). A foreign surface may trigger complement activation through any of the following three main routes: classic, lectin, and alternative pathways (Fig. 1). Each pathway is initiated through binding of a pathway-specific sensing molecule, and subsequent activation of its zymogens, which then react with other complement proteins to form proteases that are themselves activated by proteolytic cleavage (Moghimi et al., 2011). For example, the classic pathway may be triggered through direct binding of the cationic globular head of the complement protein 1 (C1)q molecule to a surface or to the Fc region of immunoglobulins (as in the classic antigen-antibody complexes). On binding, C1q undergoes a conformational change, resulting in sequential activation of C1r and C1s in a calcium-dependent manner to form a serine protease that acts on complement proteins 4 and 2, respectively (Ricklin et al., 2010; Holers, 2014). Pattern-recognition molecules such as mannose-binding lectin (MBL), ficolins, and collectin 11 sense a range of

Fig. 1. Schematic representation of complement activation pathways. The diagram shows the role of selected complement-sensing molecules (e.g., C1q, MBL, ficolins, and nascent C3b) and antibodies in complement activation. Natural complement regulators and inhibitors are not shown. Reproduced with permission from Wibroe et al. (2014) (©American Society for Microbiology).

carbohydrate ligands on pathogens (and nanoparticles) and trigger the lectin pathway through activation of MBL-associated serine proteases (MASPs) 1, 2, and 3 (Ricklin et al., 2010; Holers, 2014). Activated MASP-1 is believed to cleave MASP-2, which in turn cleaves complement protein 4, and the resulting complex forms an assembly with complement protein 2 (Héja et al., 2012). Next, through participation of both MASP-1 and MASP-2, the bound complement protein 2 is cleaved, forming a new protease. The alternative pathway is generally triggered through autoactivation of soluble C3 that undergoes slow spontaneous hydrolysis, or when the α -chain of the nascent C3b undergoes nucleophilic attack in the presence of a surface rich with nucleophilic groups (Ricklin et al., 2010; Holers, 2014). Available evidence also suggests that MASP-1 may directly cleave C3, and trigger alternative pathway activation. MASPs may also modulate activity of other alternative pathway proteins such as factors D and B (Takahashi et al., 2010; Banda et al., 2010; Dobó et al., 2016). Direct binding of properdin, a stabilizer of the alternative pathway convertase, to a foreign surface was also suggested to trigger complement activation (Hourcade, 2006), but this process remains controversial and disputable (Harboe et al., 2017) due to the artificial nature of the former study (Hourcade, 2006).

Regardless of the activation pathway, all three pathways of the complement system converge to generate the same set of effector molecules through C3 cleavage (Ricklin et al., 2010; Holers, 2014). This not only generates the opsonic C3b and iC3b components (Fig. 2), but also the C3a anaphylatoxin (Ricklin et al., 2010; Holers, 2014). The C3b component further participates in the assembly of a convertase that triggers the cleavage of complement protein 5 (C5), and hence activation of the terminal pathway of the complement system as well as

formation of the lytic membrane attack complex (Ricklin et al., 2010; Holers, 2014). C5 cleavage also liberates a potent anaphylatoxin/chemotactic molecule known as C5a (Ricklin et al., 2010; Holers, 2014). Anaphylatoxins can also become liberated without direct complement activation. Coagulation factors FX, FX1a, and plasmin can directly act on C3 and C5, and liberate C5a and C3a, respectively (Amara et al., 2008). Thrombin also acts on C3, but not C5, to liberate anaphylatoxin (C3a) (Amara et al., 2008).

The complement system further comprises soluble and membrane-bound regulatory molecules such as C1-inhibitor, complement protein 4-binding protein, factors H and I, decay accelerating factor, and CD46 that modulate the extent of complement activation (Sjöberg et al., 2009; Ricklin et al., 2010; Holers, 2014). Noncomplement proteins such as apolipoproteins A-I and A-II also regulate complement activation apparently by minimizing the formation of the membrane attack complex (Hamilton et al., 1993; Hamad et al., 2013). In some instances, apolipoprotein B-100 has also displayed inhibitory effect on block copolymer micelle-mediated complement activation, but apolipoprotein B-100 is not a true complement inhibitor (Hamad et al., 2013). Apolipoprotein J (also known as clusterin) is another established complement inhibitor, which together with vitronectin binds to nascent C5b-9 complexes rendering them lytically inactive (McDonald and Nelsestuen, 1997).

Recent Advances in Nanoparticle-Mediated Complement Activation Processes. There is sporadic reporting and inconsistencies in complement activation by engineered nanoparticles and nanomedicines, and these have been recently reviewed (Boraschi et al., 2017). These inconsistencies may have roots in sera and plasma sources and preparations, anticoagulant type, and assay procedures, as opposed

Fig. 2. The structure of the third complement protein (C3), and nanoparticle opsonization by C3b and iC3b. The arrowheads in the α chain indicate C3 cleavage sites through the action of C3 convertases (1) and factor I (2 and 3). When C3 convertases release C3a, the remaining molecule (α' and intact β chain) is known as C3b. In C3b, the reactive thiolester moiety (located in the C3dg segment) can either form an ester or an amide bond with a reactive hydroxyl and amino moiety on the pristine nanoparticle surface (and/or its biomolecule corona), respectively. The lower portion shows interaction of C3-opsonized nanoparticles with CRs on a phagocytic cell.

to classic complement research protocols, which pay detailed attention to serum/plasma preparation and complement pathway validation (Lachmann, 2010). Nanoparticle heterogeneity is also another contributing factor, which has also been noted among clinical preparations (Wibroe et al., 2016a). Within a typical batch, nanoparticles may show heterogeneity in terms of spatial distribution of surface functional groups, morphology, size, and aspect ratio (Hamburg, 2012; Moghimi et al., 2012a; Wibroe et al., 2016a). Considering this heterogeneity, different nanoparticle subpopulations may trigger complement activation through any of the three main pathways or their combinations.

Despite these factors, a number of efforts have been directed toward unraveling the mechanistic complexities surrounding nanoparticle-mediated complement activation processes (Moghimi et al., 2006, 2011; Banda et al., 2014; Boraschi et al., 2017; Chen et al., 2017; Vu et al., 2019). The simplest way in which a nanoparticle could trigger complement activation is through direct binding of a complement-sensing molecule to the pristine surface (Moghimi et al., 2011). For example, human C1q shows high affinity for poly(2-methyl-2-oxazoline)-coated nanoparticles (but not for the corresponding uncoated nanoparticles) with a binding constant comparable to that of the natural ligands of C1q (e.g., histones and IgG), resulting in activation of the classic pathway and C3 opsonization (Tavano et al., 2018). Another example is the binding of C1qA to cardiolipin liposomes, resulting also in activation of the classic pathway (Kovacsovic et al., 1985; Bradley et al., 1999). On the other hand, C1q binding to pristine carbon nanotubes has produced extreme outcomes. In one study, C1q binding to these nanomaterials triggered complement activation through the classic pathway (Salvador-Morales et al., 2006), whereas in another study C1q deposition failed to trigger complement activation (Ling et al., 2011). These differences may have been due to different levels of impurities and differences in surface characteristics of the nanotubes in question in the two studies. Depending on the surface properties, others have shown carbon nanotube-mediated complement activation through lectin and alternative pathways (Hamad et al., 2008; Moghimi and Hunter, 2010; Andersen et al., 2013a,b; Pondman et al., 2014). For example, complement activation by poly(ethylene glycol) (PEG)ylated carbon nanotubes was predominantly through the lectin pathway (Hamad et al., 2008; Andersen et al., 2013a). Variability in C1q binding (and classic pathway activation) also applies to graphene sheets (Belling et al., 2016; Wibroe et al., 2016b), which in turn may be related to differences in surface functionality and chemistry. With respect to the alternative pathway, direct noncovalent C3 deposition on a surface, and particularly in the form of metastable C3(H₂O), may also trigger complement activation through a C3 conformational change that results in the assembly of the alternative pathway convertases (Klapper et al., 2014).

Antibodies have long been known to modulate complement activation through different pathways (Moghimi et al., 2011). They may either show specificity for some pristine ultrastructure components (e.g., antiphospholipid antibodies and antidextran antibodies, could bind to phospholipid headgroups and dextran, respectively), nonspecifically deposit, or specifically bind to exposed epitopes of deposited blood proteins on nanoparticle surfaces. However, simple

antibody binding or deposition may not necessary trigger complete activation. For example, the antidextran IgM differently triggers the complement depending on the bound antibody conformation and strain (Pedersen et al., 2010). On the surface of dextran-coated nanoparticles, 100–250 nm in diameter, IgM assumes a staple conformation with sufficient strain that triggers C1 activation, and hence the classic pathway activation. On the other hand, IgM assumes a planar conformation on larger (600 nm) dextran-coated nanoparticles, which fails to activate C1 (Pedersen et al., 2010).

Many proteomic studies attest to deposition of antibodies from human sera and plasma on different nanoparticles (Lundqvist et al., 2008; Tenzer et al., 2013), but these studies do not differentiate on the mode of antibody deposition, and their complement activation mechanism(s) if any as addressed recently (Tavano et al., 2018). For instance, IgG deposition on a surface through its Fc moiety may suppress or even prevent complement activation. Indeed, many virulent pathogens exploit this process to escape complement activation and macrophage surveillance (Sulica et al., 1979; Zhang et al., 1998). However, a recent study has unraveled a link between the generated protein corona and the antibody-mediated complement activation, where the binding of only a few antibodies to different types of nanopharmaceuticals (e.g., Feraheme, Doxil, and Onivyde) was sufficient in directly activating complement through the alternative pathway (Vu et al., 2019). This functional antibody binding was strictly dependent on the protein corona, thereby suggesting a role for the adsorbed proteins in generating necessary antigenic epitopes for antibody docking (Vu et al., 2019). This in turn indicates that the extent of antibody binding (and the resultant complement activation) is dependent on antibody isotype and titer, as well as the frequency of antigenic epitopes created by different surface bound proteins. Nevertheless, these antibodies served as targets for nascent C3b attack and formation of C3bBb-properdin convertases, where the latter amplified C3 cleavage on nanoparticle surfaces resulting in covalent binding of C3b to available reactive amino and/or hydroxyl moieties on protein corona (Vu et al., 2019). Indeed, the dependency of nanoparticle C3 opsonization on immunoglobulin binding is universal, and occurs with different nanoparticle types. Notwithstanding, C3 opsonization has also been shown to be continuous and changeable *in vivo*; complexes formed between C3 and corona proteins are released from nanoparticle surfaces and formed again on fresh protein deposition (Chen et al., 2017).

Binding of antiphospholipid antibodies to different phospholipid headgroups in liposomes has been suggested to trigger complement activation through the classic pathway (Moghimi and Hunter, 2001; Moghimi et al., 2011). However, this requires at least two IgG molecules within 10–40 nm of each other to form a stable platform for C1q binding and C1 activation. On the other hand, others have identified β -2-glycoprotein-1 deposition as a prerequisite for antiphospholipid antibody binding to liposomes and complement activation (Jones et al., 1992). Since such liposomes also trigger the alternative pathway of the complement system (Moghimi et al., 2011), it is highly plausible that a few surface bound antiphospholipid antibodies could directly aid assembly of alternative pathway convertases and accelerate C3 opsonisation. Indeed, this was also shown to be the case with

two clinical liposome formulations (Doxil and Onivyde) (Vu et al., 2019).

A number of reports have established a modulating role for the nanoparticle polymer coat in complement activation, where changes in polymer conformation could not only affect the extent of complement activation, but also in switching complement activation from one pathway to another (Hamad et al., 2010; Yu et al., 2014; Coty et al., 2017). Generally, changing the configuration of surface projected hydrophilic polymers such as poloxamers, poloxamines, and mPEGs, and different polysaccharides from mushroom to brush has minimized the extent of complement activation, but in some instances this shifted complement activation from classic to lectin pathway or modulated the alternative pathway activation mode (Hamad et al., 2010; Yu et al., 2014; Coty et al., 2017). Such polymer conformational changes could alter display patterns of polarity and hydrophobicity, and this in turn may affect the affinity of complement-sensing proteins (e.g., C1q vs. MBL and ficolins) for the surface. This suggestion is in line with recent approaches that have shown the importance of nanoscale patterning and controlling spatial and architectural arrangements of ligand presentation in MBL-binding kinetics (Gjelstrup et al., 2012). On the other hand, conformational changes of surface projected polymers may also affect protein binding, and hence the composition (and conformation) of nonspecific blood proteins, which in turn may regulate the pathway of complement activation. For instance, changing from mushroom to brush configuration may affect exposure of the glycosylated regions of adsorbed blood proteins (such as apolipoproteins) for MBL and ficolin binding (Hamad et al., 2008).

Species Variation in Complement Activation and C3 Opsonization. Rodent models are widely used in nanomedicine research, and particularly with respect to nanoparticle opsonization processes and macrophage clearance. However, there are disparities in nanoparticle-mediated complement activation and C3 opsonization processes between rodents and humans (Banda et al., 2014; Inturi et al., 2015; Wang et al., 2016; Tavano et al., 2018) as well as in vitro-in vivo correlations (Wang et al., 2017). For instance, dextran-coated superparamagnetic iron oxide nanoworms differently trigger complement activation in mice and humans, but C3 opsonization is necessary for nanoworm uptake by both murine and human blood leukocytes (Inturi et al., 2015; Wang et al., 2016). In human sera, nanoworms predominantly activate complement through the antibody-mediated alternative pathway, but moderately via the lectin pathway, whereas in mouse complement activation by nanoworms occurs mainly through binding of MBL-A/C (the lectin pathway) (Banda et al., 2014). Crosslinking the dextran coat of nanoworms with epichlorohydrin blocks lectin pathway activation and C3 opsonization in mice, but neither this strategy nor modifications of sugar alcohols of dextran with alkylating and acylating agents can overcome nanoworm-mediated complement activation, and C3 opsonization in human sera (Wang et al., 2016). Thus, these observations support the notion that human complement activation is independent of dextran modification of nanoworms, and driven through alternative pathways via antibody deposition on protein corona.

Another interesting example is C1q-mediated complement activation by poly(2-methyl-2-oxazoline)-coated nanoparticles in human sera, whereas these nanoparticles do not activate mouse complement, and moreover resist rapid ingestion by

murine macrophages (Tavano et al., 2018). Indeed, in support of in vitro observations with murine macrophages, poly(2-oxazoline)-grafted liposomes also show long circulatory behavior in rodents (Zalipsky et al., 1996). There are even disparities in immune handling of nanoparticles among rodents. In mice, intravenously ganglioside GM1 liposomes are long circulatory (Gabizon and Papahadjopoulos, 1988), whereas in rats the hepatic Kupffer cells rapidly clear these liposomes from the blood circulation due to the presence of naturally occurring anti-GM1 antibodies, which through complement activation promote liposome opsonization with C3 (Liu et al., 1995). Collectively, these observations suggest the importance of testing nanoparticle opsonization and susceptibility to macrophage clearance with human-derived plasma and phagocytic cells, rather than relying on non-human sources.

Nonactivating Surfaces. Considering the multifaceted roles of the complement system in nanomedicine stability, accelerated clearance by macrophages, inflammatory reactions, and disease progression, there have been concerted efforts in engineering of targetable nanomedicines that do not trigger complement activation (Gbadamosi et al., 2002; Sou and Tsuchida, 2008; Wu et al., 2011; Andersen et al., 2013a; Wibroe et al., 2015; Azmi et al., 2016; Guan et al., 2018). Recent findings on the role of protein corona in antibody-mediated triggering of alternative pathway (Vu et al., 2019), and the exchangeable dynamics of C3 opsonization (Chen et al., 2017), make design of surfaces that repel natural antibodies a grand challenge. At first instance, such developments may rely on availability of biocompatible superhydrophobic polymers that repel blood proteins under shear flow (Moghimi and Simberg, 2017). Alternatively, precision surface patterning with available polymers (and functional groups) may overcome complement activation. For instance, since PEG can minimize protein adsorption, keeping PEG chains 10–15 Å apart may generate ideal spacing for optimal protein exclusion effect (Moghimi and Simberg, 2017). This approach has shown some degree of success in minimizing complement activation by single wall carbon nanotubes coated with poly(maleic anhydride-alt-1-octadecene) bearing two molecules of mPEG5000 (Andersen et al., 2013a). However, precision PEG patterning may not be applicable to many nanoparticle self-assemblies such as liposomes, but void filling through polymer pairing, as in combinations of short, medium, and long chain polymers, may offer a viable solution, and such procedures are known to modulate immunity (Gbadamosi et al., 2002; Moghimi, 2006; Dai et al., 2014). Nonetheless, there are some empirical approaches that have yielded nanoparticles that avoid complement activation in human blood. A recent example is a hexosome formulation from citrem and glyceryl monooleate, where the lack of complement activation was suggested to arise from recruitment of complement regulatory protein factor H (Wibroe et al., 2015). Indeed, there are structural similarities between the terminal citric acid moiety of citrem and sialic acid (Fig. 3). Thus, this approach is analogous to some virulent bacterial pathogens that bypass complement activation through factor H recruitment by virtue of surface expression of sialylated glycans and polysialic acid (Langford-Smith et al., 2015). Indeed, the amino acid residues in the CCP20 domain of factor H have been suggested to bind to the glycerol side chain and carboxyl moiety of sialic acid (Blaum et al., 2015). Thus, the citric acid moiety of citrem in

Fig. 3. Structures of glycerol (glyceryl) monooleate, citrem, and sialic acid. Circles in sialic acid structure indicate moieties that interact with factor H.

concert with the glycerol component of glyceryl monooleate may form dynamic platforms for factor H binding. Subsequent studies have also shown that other citrem-containing lamellar and nonlamellar liquid crystalline nanoparticle self-assemblies including vesicles, cubosomes, and hexosomes also behave as poor complement-activating entities (Azmi et al., 2016). Similarly, other nanoparticles with high surface density of carboxylic acid (Sou and Tsuchida, 2008) and sulfonate functionality (Montdargent et al., 1993) have also shown resistant to complement activation, which presumably is due to factor H recruitment. Similarly, heparin-coated nanoparticles also exhibit poor complement-activating properties (Chauvierre et al., 2004). In contrast to these observations, sulfonation and carboxymethylation of cross-linked iron oxide nanoparticles proved unsuccessful at decreasing complement activation, and hence factor H recruitment (Wang et al., 2016). This underscores the notion that such modifications may not be universally applicable to all nanoparticle types.

There are many examples of mammalian cell and naturally evolved pathogen-based strategies that evade complement and some of these have been translated to nanoparticle surface engineering. For instance, surface enrichment with a phage peptide that recruits factor H is one successful example that overcomes complement activation (Wu et al., 2011). Others peptidomimetic approaches have included surface functionalization with designer peptides that attenuate IgM deposition (Guan et al., 2018). There are also efforts focusing on enriching nanoparticle surfaces with naturally derived immunoregulators such as CD47, CD55, and CD59 (Sims et al., 1989; Yin et al., 2008; Ricklin et al., 2010). One such approach is cloaking nanoparticles with platelet membranes, which was suggested to overcome complement activation and C3 opsonization (Hu et al., 2015). However, the validity of complement activation assessment and interpretation in this study was questioned by us (Moghimi et al., 2016), and we have now established that cloaking of polystyrene

nanoparticles with either platelet or leukocyte membrane does not overcome complement activation in human plasma, and conversely accelerates complement activation through alternative pathways (S. M. Moghimi, unpublished data). Cloaking of solid nanoparticles with biomembranes can induce unfolding and denaturation of membrane proteins (as well as flipping of the inner-membrane components), and this may trigger binding of natural antibodies, resulting in complement activation.

Increasing attention is being placed on the use of exosomes as potential vehicles for experimental drug delivery. Some evidence suggests that nonmalignant cell-derived exosomes are poor complement-activating entities due to expression of complement regulatory proteins such as CD59 and decay-accelerating factor (Clayton et al., 2003). On the other hand, one study has shown more potent activation of the human complement system by both metastatic and nonmetastatic malignant cell-derived exosomes (Whitehead et al., 2015). This is interesting, since intratumoral complement activation by exosomes may promote tumor growth (Markiewski et al., 2008), and complement activation is also known to play a modulatory role in endothelial cell retraction (Kerr and Richards, 2012), a process that may aid metastatic spread.

Controversy on the Role for Complement in Infusion Reactions to Nanomedicines. Infusion reactions to nanomedicines are idiosyncratic and non-IgE-dependent, where typical symptoms (e.g., facial swelling, skin rash, cough, chest pain, and cardiovascular distress) vary from mild to severe depending on the individual, nanomedicine dose, and rate of infusion (Moghimi, 2018). Prediction of infusion reactions to nanomedicines has proven unsuccessful with standard allergy tests (Moghimi, 2018). Instead, the porcine model has been promoted for nanomedicine prescreening, since many symptoms of acute infusion reactions (e.g., skin rash and cardiovascular distress) in humans are outwardly reproducible in pigs (Szebeni et al., 2012). Equivocal investigations have further indicted a role for the complement system in infusion

reactions to nanomedicines, where the extent of nanoparticle/nanomedicine-mediated complement activation in human serum (at 45 minutes of incubation) is inappropriately being correlated to cardiopulmonary responses in pigs, which occur within minutes of injection (Mészáros et al., 2018). Many nanoparticles/nanomedicines, including those in the study by Mészáros et al. (2018), are very mild activators of both human and pig complement compared with potent complement-activating agents such as endotoxins and zymosan (Moghimi, 2018), and within the first 5 minutes of contact with human serum/plasma these nanoparticles hardly trigger complement activation (Wibroe et al., 2017). Also, the magnitude of cardiopulmonary responses (such as rise in pulmonary arterial pressure and drop in systemic arterial pressure) induced with nanoparticles and some nanomedicines is similar to low-dose zymosan; however, at this low dose, zymosan is far more potent in activating human and pig complement (Moghimi, 2018; Wibroe et al., 2017). Thus, the validity of the porcine model in global nanomedicine safety assessment has recently been questioned (Moghimi, 2018; Wibroe et al., 2017; Skotland, 2017; Moghimi and Simberg, 2018). It has long been known that unlike humans, cloven-hoof animals (such as pigs) have resident intravascular macrophages in their lungs (known as PIMs) (Brain et al., 1999). PIMs immediately respond on robust nanoparticle clearance, irrespective of complement activation, and release arachidonate metabolites, which initiate cardiopulmonary distress (Wibroe et al., 2017). Indeed, many studies have shown that rapid nanoparticle extraction from the blood by PIMs correlate with peak periods of cardiopulmonary distress (Miyamoto et al., 1988; Longworth et al., 1992; Schneberger et al., 2012; Wibroe et al., 2017), see Table 1. Furthermore, pharmacologically mediated PIM destruction or strategies that transiently delay nanoparticle sensing by PIMs (e.g., rod- or disk-shaped carboxylated polystyrene nanoparticles as opposed to their spherical counterparts, and spherical polystyrene nanoparticles bound to erythrocytes), all overcome infusion/injection reactions in pigs irrespective of complement activation (Fig. 4A; Table 1), and trigger desensitization (Gaca et al., 2003; Wibroe et al., 2017). On PIM destruction, cardiopulmonary distress to intravenously injected PEGylated liposomes and potent complement-activating agents such as endotoxins and zymosan also disappears (Sone et al., 1999; Gaca et al., 2003; Wibroe et al., 2017). In clinical practice, slowing down the nanomedicine infusion rate usually overcomes adverse reactions in patients (Moghimi, 2018). This is in line with experimental observations that a transient delay in nanoparticle (as in rod/disk-shaped nanoparticles compared with their spherical counterparts) presentation to PIMs overcomes cardiopulmonary distress in pig (Wibroe et al., 2017). Therefore, macrophages (and perhaps other immune cells such as dendritic cells) play a vital role in the infusion reaction, where the rate of nanoparticle presentation to responding macrophages, rather than the complement activation per se, modulates downstream processes. Still, different nanoparticle types, depending on their physicochemical properties (including shape and PEGylation), may modulate macrophage responses differently. Accordingly, batch-to-batch and in-batch nanoparticle heterogeneity (as in surface properties and morphology, and exemplified with Doxil and follow-on products) (Wibroe et al., 2016a) may account for

interindividual variations in adverse reactions to nanomedicine infusion (Moghimi, 2018).

As to the location of responsive macrophages in humans, a number of studies have demonstrated the presence of induced PIMs in pulmonary circulation of patients with liver dysfunction/disease, hepatopulmonary syndrome, and inflammatory lung disease (Dehring and Wismar, 1989; Miot-Noirault et al., 2001; Moghimi, 2018). Accordingly, patients that have shown infusion reactions to nanomedicines may have induced PIMs in their lungs, but to date there is no published study investigating this possibility. Furthermore, reactive macrophages may not necessarily reside in patients' lungs, and responses may arise from a subpopulation of Kupffer cells and/or splenic macrophages, or even other immune cells such as lung dendritic cells (Dams et al., 2000; Moghimi, 2018). Recently, we demonstrated that a significant fraction of intravenously injected liposomes, regardless of their composition, rapidly accumulate in F4/80⁺ skin phagocytic cells (Griffin et al., 2017). Therefore, it is plausible that responsive skin macrophages could play a role in adverse cutaneous reactions as well as desensitization to nanomedicines.

Notwithstanding, two key factors could play critical roles in macrophage responses. The first is the nature of the responding macrophage receptor, and its associated signaling threshold, that may regulate the transitional link between robust internalization and kinetics of bioactive molecule release from macrophages (Escribese et al., 2017; Moghimi, 2018). For example, a plausible role for low-affinity Fc-receptor-mediated response to nanomedicine anaphylaxis has been proposed (Moghimi, 2018). Indeed, Fc receptors (FcγRI, FcγRIIA, FcγRIIB, FcγRIIC, FcRIIIA, and FcγRIIIB) show different affinities for antibodies and exhibit different expression patterns and downstream signaling (Strait et al., 2002). Fc receptors also contribute to desensitization (Strait et al., 2002; Escribese et al., 2017). Nevertheless, the location of responding macrophages, their number, and accessibility to nanoparticles play a role as a rate-limiting factor. Whether natural antibody binding could serve as a broad biomarker of nanoparticle clearance (within the context of IgG-mediated alternative pathway activation), and infusion-related reactions, awaits future studies. We may also consider whether complement opsonized nanoparticles could contribute to macrophage responses. For example, iC3b opsonized nanoparticles are multimerized ligands for the third complement receptor (i.e., CR3), and induce CR3 clustering. In monocytic cell lines, CR3 clustering induces NF-κB activity through Toll/interleukin-1 receptor family-like signaling cascade, which results in the expression of proinflammatory genes (Shi et al., 2001). Whether this applies to primary human macrophages awaits future studies.

The second factor is particle size (or particle volume within a three-dimensional context), where the magnitude of cardiopulmonary responses to larger nanoparticles could be more severe (Mészáros et al., 2018). Indeed, macrophages are known to ingest larger particles more efficiently (and with faster kinetics) than their smaller counterparts (Moghimi et al., 2012a). Smaller particles may also trigger adverse cardiopulmonary reactions on aggregation and formation of large clusters in the blood. Our own efforts (S. M. Moghimi, unpublished data) have shown more severe PIM responses to larger nanoparticles than their smaller counterparts

TABLE 1

The effect of PIM manipulation in cloven-hoof animals on adverse reactions to nanoparticle administration

Species	PIM Manipulation	Test Nanoparticle	Remarks	Reference
Sheep	None	Liposomes ^a (200–300 nm)	PAP elevation; TxB ₂ elevation at PAP peak	Miyamoto et al., 1988
	Indomethacin	Liposomes ^a (200–300 nm)	PAP rises completely blocked by indomethacin	
	Thromboxane synthase inhibitor	Liposomes ^a (200–300 nm)	PAP rises blocked by 75%	
Newborn lamb	None (newborn lambs do not have PIMs in their lungs)	Liposomes ^b ; Monastral blue nanoparticles (45–65 nm)	No pulmonary hypertension; no net pulmonary production of TxB ₂	Longworth et al., 1992
	None (2 wk after birth)	Liposomes ^b ; Monastral blue nanoparticles (45–65 nm)	PIMs appear 2 wk after birth; pulmonary hypertension and elevated TxB ₂ on liposome and Monastral blue nanoparticle administration; nanoparticle deposition to the lungs was confirmed (more nanoparticle deposition to the lungs in older animals due to more PIM populations)	
Lamb	None	Endotoxin	Pulmonary hypertension; increased lung microvascular leakiness	Sone et al., 1999
	PIM depletion by clodronate-encapsulated liposomes	Endotoxin	No pulmonary hypertension; >90% attenuation in early and late rises in lung lymph flow; recurrence of pulmonary hypertension on PIM recovery after 2 wk	
Pig	None	Endotoxin	Pulmonary hypertension; elevated serum levels of IL-6, TNF- α , and thrombin	Gaca et al., 2003
	PIM depletion by clodronate-encapsulated liposomes	Endotoxin	None/very mild pulmonary hypertension; significantly lower serum IL-6, TNF- α , and thrombin	
Pig	None	Carboxylated polystyrene nanospheres (500 and 750 nm); sulfated polystyrene nanospheres (750 nm); PEGylated liposomes (200 nm); Zymosan	Pulmonary hypertension; TxB ₂ elevation at peak PAP	Wibroe et al., 2017
	PIM depletion by clodronate-encapsulated liposomes	Carboxylated polystyrene nanoparticles (500 and 750 nm); sulfated polystyrene nanospheres (750 nm); PEGylated liposomes (200 nm); Zymosan	>80% attenuation in pulmonary responses and TxB ₂ generation	
	None	Carboxylated polystyrene rods (450 $\times$ 120 nm); carboxylated polystyrene disks (250 $\times$ 75 nm)	No cardiopulmonary distress; no TxB ₂ elevation (shape effect; slower nanoparticle presentation rate to PIMs compared with nanospheres)	
	None	Carboxylated or sulfated polystyrene nanospheres (500 and 750 nm) attached to erythrocytes	>70% attenuation in pulmonary responses and TxB ₂ generation compared with free nanospheres (due to a slower nanosphere presentation rate to PIMs when bound to erythrocytes)	

IL-6, interleukin-6; PAP, pulmonary arterial pressure; TxB₂, thromboxane B₂; TNF, tumor necrosis factor.^aLiposome composition: egg phosphatidylcholine:cholesterol:phosphatidylserine (mole ratio 6:4:1).^bLiposome size, not indicated; liposome composition: egg phosphatidylcholine:cholesterol:phosphatidylserine (mole ratio 6:4:1).

(irrespective of complement activation) when injected at the same rate to pigs on an equivalent particle number basis (schematically demonstrated in Fig. 4B). Interestingly, a higher dose of smaller nanoparticles, which triggered adverse cardiopulmonary distress, failed to desensitize the animals, and cardiopulmonary distress occurred on injection of corresponding larger nanoparticles (S. M. Moghimi, unpublished data). These experiments suggest that nanoparticles of different sizes (but of identical morphology and surface chemistry) are presumably recognized by different macrophage receptors (and/or internalized through different routes) (Gratton et al., 2008).

Complement activation by the solubilizing agent Cremophor EL has also been blamed for adverse infusion reactions to Taxol (Szebeni et al., 2001). In contact with blood, Cremophor EL micelles interact with lipoproteins and undergo structural transformation. Upon loss of their hydrophobic components to high- and low-density lipoproteins, the hydrophilic components of Cremophor EL micelle remnants form large droplets (Szebeni et al., 2001). Therefore, robust clearance of these large droplets by responsive macrophages could account for the observed reactions in responsive patients (Moghimi, 2018).

Particles such as Albunex (air-filled albumin microspheres) and Intralipid (a 20% intravenous fat emulsion) have also

Fig. 4. Schematic representation of polystyrene nanoparticle shape and volume effects on cardiopulmonary distress in pigs. (A) Spherical polystyrene nanoparticles can trigger responses from PIMs and induce cardiopulmonary distress. Nonspherical polystyrene nanoparticles (poor activators of the porcine complement) and spherical nanoparticles bound to erythrocytes (complement activators) do not trigger PIM responses on intravenous injection. (B) Effect of commercially available model polystyrene nanoparticle size (or volume) in cardiopulmonary distress. Larger nanoparticles induce more potent cardiopulmonary responses compared with their smaller counterparts on the basis of equivalent particle number. This is regardless of nanoparticle surface chemistry at least with respect to OH, COOH, and NH₂ functionalities as well as mPEG grafting. See Wibroe et al. (2017), for general experimental protocols and methodologies.

induced cardiopulmonary distress in pigs (and sheep) (Mckeen et al., 1978; Østensen et al., 1992; Bedocs et al., 2014), but have shown no such deleterious reactions in human subjects, and have reached the market (Moghimi and Simberg, 2018). Again, the observed animal responses are in line with nanoparticle organ distribution. Indeed, biodistribution studies with radiolabeled Albunex have shown species differences in organ accumulation, where lung retention accounted for 90% and 5% of the injected dose in pigs and rats, respectively (Walday et al., 1994; Skotland, 2017). Another example is citrem-based hexosomes, which show no complement activity in either human or pig blood, yet generate identical cardiopulmonary distress (e.g., rapid drop in systemic arterial pressure and rise in pulmonary arterial pressure) in pigs as

potent complement-activating Pluronic F127-stabilized hexosomes (Wibroe et al., 2015; P. P. Wibroe, A. Yaghmur, and S. M. Moghimi, unpublished data). Thus, considering pig's natural physiologic responses to many nanoparticles (i.e., PIM-induced responses), global nanomedicine safety assessment in pigs, and within the context of anaphylaxis, may be considered inappropriate and scientifically questionable. Therefore, compulsory nanomedicine response tests in pigs should not be advertently promoted and imposed on the pharmaceutical industry. For testing whether complement plays any role in human-related infusion reactions, and considering the disparity of complement activation processes and responses between preclinical models and humans, the answer may rely on development of complement inhibitors and their direct assessment on

human volunteers (Szebeni et al., 2018). However, these inhibitors should include a panel of anaphylatoxin receptor antagonists as well as complement pathway selective inhibitors (Woodruff et al., 2011; Barnum, 2017; Huang, 2018), and particularly inhibitors of the alternative pathway, since the alternative pathway turnover plays a dominant role in complement activation by many nanomedicines (Vu et al., 2019). Such combinations may further allow discrimination between anaphylatoxin release triggered responses and complement opsonized nanoparticle-mediated signaling effect.

Conclusions

Considering the multifaceted roles of complement in bridging innate and adaptive immune responses, we are still in need of better mechanistic understanding of interfacial factors that regulate the interplay between nanoparticles, complement systems, and innate immunity as whole (Boraschi et al., 2017). Among these factors, nonspecific protein adsorption plays at least a global role in antibody-triggered alternative pathway activation (Vu et al., 2019), where such uncontrolled and random complement activation may affect nanoparticle pharmacokinetics, promote inflammation, and help disease progression. A clear understanding of underlying physicochemical and pathophysiological factors that regulate complement activation and responses could lead to immune-safe-by-design innovations not only in nanomedicine, but also in biomaterials and biomedical engineering. As to mechanism(s) of infusion reactions, we are still in need of systematic studies to identify reliable biomarkers for patient selection, risk mitigation, and therefore a patient-centric approach to effective nanomedicine therapy. Here, development and approval of human complement inhibitors may at least resolve the role of complement (if any) in infusion-related reactions. More importantly, we should start studying the immunobiology of induced PIMs in human lungs and assessing the role of other suspect resident macrophages (and other cells such as dendritic and natural killer cells) in infusion-related reactions and desensitization mechanisms to nanomedicines. Notwithstanding, through concerted bioinformatics and big data analysis and inception of genome association studies we may eventually identify the underlying genomic, proteomic, and immune cell factors responsible for mild, moderate, and severe infusion reactions to nanomedicines, and within the context of the patient's medical history.

Authorship Contributions

Wrote or contributed to the writing of the manuscript: Moghimi, Hunter, Yaghmur, Simberg, Skotland.

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