Biomembrane Mechanical Properties Direct Diverse Cell Functions

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Abstract The shape of any cell is defined and delimited by the shape of the outermost membrane of lipid. Whether a cell's membrane is locally flat, protrusive, or invaginated at a given instant is often the resultant of forces generated by molecules within the cell as well as those attributable to external factors. For mammalian cells, major changes in cell shape are evident in processes that include cell spreading, migration, and cell division as well as differentiation and death. Such processes are illustrated here for blood cells, starting with stem cells in bone marrow plus the many different types of circulating cells, particularly RBCs whose membranes have been more deeply studied for decades compared to other mammalian cell types. A handful of the key proteins that apply or resist forces at the membrane are described here while focusing on the cortical protein meshworks that underlie membranes and contribute to properties and processes. Engulfment of particles and cells is one particular focus, with broad relevance to disease and therapy. Equally interesting is the frequently noted observation that changes in cell shape and orientation are also evident in shape and orientation changes of the cell's nucleus—which is again delimited by a membrane. A final section focuses on the physics of a sub-membranous lamina in the nucleus, which interfaces with the genome and provides insight into mechanosensing and cell fates.

Keywords Red blood cells · Hematopoiesis · Membrane skeleton · Myosin · Mechanobiology · Nucleus · Lamins · Differentiation

A cell's shape is defined by how a cell's plasma membrane is spatially arranged in its three-dimensional microenvironment. Sculpting a cell at any instant in time are the forces within a cell—including forces actively generated through energy consumption—as well as forces from outside a cell such as extracellular pressure and fluid shear. The resistance to shape change under force is variously referred to

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as stiffness, elasticity, rigidity, or plasticity; some deformations are of course fully reversible (i.e. elastic) while others are irrevesible (eg. plastic). Relatively stable cell shapes are achievable for just a few mammalian cells when isolated, and the red blood cell (RBC) with its symmetrically-dimpled "discocyte" shape is the best understood. Dynamic changes in the shapes of far more active cells are critical to cell functions that range from adhesion and migration to division, differentiation, and death. Understanding the mechanisms that underlie such essential cell processes requires clarifying the forces that locally shear, dilate, and bend membranes. This chapter seeks to introduce in broad terms a few of the basic cell biological processes that involve striking changes in cell shape, and in doing so we describe a small handful of the responsible proteins.

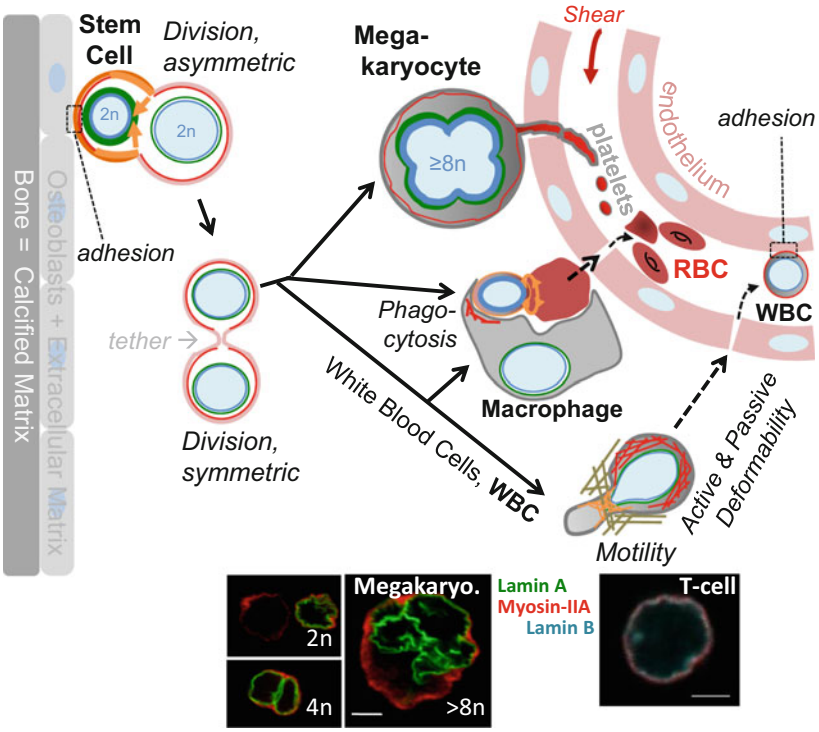

Fig. 1 Biophysical determinants of blood formation. Adhesion and contractile forces generated by MII are important in sensing matrix stiffness, which is heterogeneous in the BM microenvironment. During the cell division process, stem cells near the osteoblasts undergo asymmetric division to segregate MIIB into one daughter cell. Without MIIB, cells divide symmetrically. The other daughter cell becomes differentiated into three different lineages. Because MKs upregulate both lamin isoforms by endomitosis, they are too large to traffic through endothelial barriers. Instead, they undergo fragmentation into platelets. During erythroid differentiation, the nucleus shrinks in chromatin condensation. Condensed nuclei are too stiff to migrate through the endothelial barrier and are phagocytosed by macrophages, leading to enucleated RBCs. *RBC* red blood cell, *WBC* white blood cell, *HSC/P* hematopoietic stem cell/progenitor

An overall view of the diversity of membrane shapes and their changes within a mammalian system is perhaps best achieved by focusing on the various types of human blood cells and their ongoing development in bone marrow (Fig. 1). Human bone marrow collectively generates red blood cells, platelets, and white blood cells, each at a rate of roughly 100,000 per second. Production is matched in steady state by cell death and removal from circulation, which occurs on average after ~100 days for RBCs (red blood cells) in humans or just a few days to a week for platelets and some of the more abundant types of white blood cells. The longer life of the RBC thus explains why this is far more abundant than any other cell type in blood. The distinct multistep process by which an RBC is made and eventually destroyed provides many useful insights into biomembrane properties and forces that underlie remodeling of membrane shapes. Any terminally differentiated cell such as an RBC is derived from a less specialized cell referred to as a stem cell. Therefore, we begin by describing processes of the relevant bone marrow stem cell, focusing on the membrane and the stresses that control its shape—while. We also attempt to convey the importance of the cells and clinical motivations that every day save lives around the world.

1 Division: Fission Forces at the Membrane for Cell Differentiation

The long-term health of any tissue invariably requires that death or turnover of mature cells be balanced not only by differentiation from the relevant stem cell but also by self-renewal of the stem cell. These processes seem optimized in specialized tissue microenvironments called niches. Hematopoietic stem cells and progenitors (HSC/P) reside in bone marrow niches that are formed from extracellular matrix proteins and other cells. The latter include mesenchymal stem cells (MSCs) and MSC-derived lineages, namely, bone cells and fat cells [1]. Decades of clinical success in transplantation of HSC/Ps into patients [2] have motivated the exploration of mechanisms that underlie the balance between stem cell self-renewal, differentiation, and trafficking of mature cells from the marrow and into the blood circulation. Soluble factors and cell–cell contacts regulate these biological processes, but it is only recently appreciated that stem cells can generate and resist physical forces, while matrix stiffness and external stresses such as shear flow impact stem cell adhesion and intracellular signaling [3]. Structural proteins just below the cell membrane and even the nuclear membrane are major determinants.

Asymmetric division is one evolutionarily conserved mechanism that explains how stem cells both self-renew and differentiate [4]. A parent stem cell divides asymmetrically to give rise to one daughter cell that maintains stem cell characteristics and another that is committed to differentiation. Asymmetric segregation of proteins during division has been well documented in invertebrates [5], and in

HSC/Ps these include integral membrane proteins such as CD34 (a "surface marker" that might not be crucial to function and simply helps identify these cells) as well as proteins that contribute to asymmetric division [3]. While extrinsic adhesive cues from neighboring cells have been implicated in asymmetric division of some white blood cells [6], the requisite polarization of a cell is inextricably linked to its cytoskeleton. In blood cells, the actin-myosin cytoskeleton is just below the plasma membrane as a cortex (Fig. 1), with its forces and/or crosslinking helping to break the symmetry of the doublet of daughter cells to induce a polarized distribution of molecules. Microtubules maintain the stability of the polarization [7], but the stiffness of microtubules could also feedback and help direct the contractile activity of the actin-myosin cytoskeleton [8].

Myosin-II (MII) proteins are ATP-consuming motor proteins that assemble into bipolar filaments and apply both contractile forces to and active crosslinking of actin filaments which, in turn, link to a diverse set of membrane proteins. In comparison to the non-muscle MII in blood cell types and other non-muscle cells, cardiac MII in heart muscle striates the cytoplasm of heart cells and contracts rhythmically to pump blood out of the heart. Skeletal muscle MII likewise striates the cytoplasm of elongated muscle cells, and its neuron-triggered, voluntary contractions drives body movements. Non-muscle MII is more closely related to the most evolutionary ancient form of MII that is found even in some single-cell organisms. Among its many cellular functions in non-muscle cells, MII confers the cell-intrinsic cortical tension that both stabilizes the plasma membrane [9, 10] and drives cell division (cytokinesis). The latter is achieved through a coordination of forces in the constriction ring and at the opposite poles of the daughter cells [11]. Cortical tension is also modulated at least locally by adhesion to stiff extracellular matrix [12, 13], which provides a mechanism by which MII regulates the ability of cells to sense physical properties of the matrix such as matrix elasticity, E. A simple intuition into mechanosensing is obtained by considering that actin polymerization drives cell spreading at a near constant rate, $v_{\text{polymer}} = A$, whereas MII pulls back on the actin network at a rate $v_{\text{retract}} = B\,/\,(K + E)$ which is a hyperbolic decrease (with constants B, C) with resistance set by the extracellular load E (as with muscle, low speed at high loads). The extent of cell spreading relates to a steady state $v_{\text{polymer}} - v_{\text{retract}} = A - B\,/\,(K + E)$ that yields minimal cell spreading for $E \ll K$ and maximal spreading for $E \gg K$ as observed for spreading cells [12]. A typical value of $K \sim 5$ kPa [12], in units of stress or pressure, should be multiplied by a typical cell-generated strain of ~5% [12] and then related through the well-known law of Laplace to an effective tension ~ 0.1 mN/m in a typical protrusion curvature radius of ~1 μm. A cortical tension of ~ 0.1 mN/m has indeed been measured for HSC/Ps, and this tension decreases dramatically with MII inhibition [10].

For at least some stem cells, matrix mechanosensing can regulate cell differentiation [12, 13]; inhibition of MII also limits the proliferation of HSC/P when these cells adhere to a highly flexible or soft matrix [13]. However, in order for daughter cells to migrate away from each other, actin seems necessary while MII is not needed; such migration can help stretch and break a lipid tether or membrane nanotubule that is often the final vestige of the cytokinetic furrow [8]. Other membrane

factors in stem cells are also likely to contribute to cleavage and repair of the nanotubule.

Cell force generators have evolved to be fine-tuned by slightly more specialized molecules, which has come to reveal how important the proper forces and dynamics need to be for cell function. In particular, humans express three myosin-II genes (MIIA, MIIB, and MIC) with different properties. MIIB appears especially key to asymmetric division of HSC/Ps [3]. MIIB motors make notably stable assemblies with actin filaments and can be seen to polarize within cells due to external stiffness or perhaps stresses such as fluid shear [3]. The latter forces could be relevant to triggering blood formation in early embryos [14]. Soft microenvironments do not sustain or transmit high stress, which limits myosin-IIB forces and polarization, and since bone marrow is very soft compared to rigid bone, polarization is more likely on the latter. External cues and spontaneous intracellular fluctuations in the cell cortex [15] could cause polarization during cell division of adult HSC/Ps, but it is very clear that MIIB segregates asymmetrically to the cortex of the one daughter cell that maintains stemness (i.e., CD34 high cells). The other nucleated daughter cell with less MIIB is thereby differentiated, and MIIB is similarly reduced per division in differentiation—at least in differentiation toward white blood cells and cells called megakaryocytes that make platelets. Asymmetric segregation of MIIB likely establishes an asymmetric cortical tension that drives differentiation, although physical differences still need to be measured.

As MIIB is downregulated in differentiating cells, MIIA is activated by dephosphorylation. This change favors MIIA assembly into filaments that again localize to the cortex. However, soft matrix favors MIIA phosphorylation. Soft microenvironments thus maintain early HSC/Ps by suppressing a switch of myosin-II's from high to low levels of MIIB and from deactivated to activated MIIA. Stiff microenvironments that resist stress have the opposite effect and initiate asymmetric division and differentiation.

RBCs have much higher levels of MIIB than platelets and white blood cells. Although myosin-II's in mature RBCs are probably vestigial in not contributing greatly to discocyte-shaped RBCs, they do contribute to the loss of nuclei during formation of RBCs. In particular, MIIB helps to polarize the nucleated RBC progenitor (called an erythroblast) and to expel the cell's nucleus in a final step of asymmetric division [16]. The expelled nucleus is rigid [3] compared to the nascent RBC, which is called a reticulocyte. As in cytokinesis, the nucleus remains tethered by a membrane nano-tubule to the reticulocyte [17]. Eventually, the tether is severed as the nucleus is engulfed by white blood cells called macrophages that reside in bone marrow as well as all other tissues [18]. Engulfment might be accompanied by macrophage-facilitated expulsion of the reticulocyte from the marrow into the blood vessels flowing through the marrow. The latter speculation is based on an absence of any data showing that reticulocytes can move or crawl. Megakaryocytes serve as a useful comparison since they actively extend pseudopods into the bloodstream via membrane-protrusive polymerization of actin and microtubules (as myosin is phospho-inhibited). The pseudopods then fragment under shear to make pre/pro-platelets [19] that are about half the size of a mature RBC. Once in the circulation,

these giant pre/pro-platelets divide to make the small and abundant platelets, and division uses MIIA that is normally activated by fluid shear unless mutations in MIIA keep it aggregated [20]. The latter mutations give rise to *MYH9*-related diseases (i.e., MIIA related diseases) that lead to "macro-thrombo-cytopenia," which refers to giant platelets that are few in number. RBCs appear relatively normal in number and shape in these patients even though a recent study of mature RBCs [21] concluded that pharmacological inhibition of myosin-II could generate a very small fraction of 'elliptocytic' RBCs. Platelet maturation thus finishes within the circulation to a much greater extent than with RBCs. What remains in the marrow from the megakaryocytes after production of many pre/pro-platelets is just a giant nucleus unable to cross the small micro-pores into the sinusoids. This nucleus that is stuck in the marrow is engulfed by macrophages. The same occurs with RBC nuclei after generation of a reticulocyte.

As with platelets, RBC maturation also finishes within the circulation: the reticulocyte sheds membrane and volume (15% and 10%, respectively [22]) within hours to become the discocyte-shaped RBC. In this final remodeling, more lipid is observed to be lost than cortical cytoskeleton, and this cytoskeleton is particularly enriched in the actin-crosslinking protein spectrin which forms a two-dimensional spectrin-actin network that attaches below the lipid bilayer. The relative loss of lipid thus increases the overall stoichiometry of 2D cytoskeleton to 2D lipid membrane. The spectrin network has been observed to dilate strongly at the tip of an RBC projection that is aspirated into a micropipette (Fig. 2) [23, 24], but in the absence of such external forces the state of stress in the "at rest" spectrin-actin network remains a matter of uncertainty. The cortical network could be under constant tension and tending to pull away from the bilayer [25], or the network could be stress free or else compressed. The cited loss of lipid relative to cytoskeleton is consistent with the network being compressed by the more limited overlying lipid bilayer, and in this case the lipid bilayer is under an equal and opposite tension which smooths and suppresses fluctuations of the lipid bilayer. Such a balance of membrane forces does not set global cell shape as the forces are too weak. Rather, the flaccid discocyte shape results from volume regulation and the relatively fixed cell area set by the near incompressibility of the lipid bilayer.

The RBC cytoplasm lacks all other organelles and is predominantly a highly concentrated fluid of hemoglobin protein (with chelated iron that makes RBCs red) plus many other macromolecules and counterions that cannot permeate the lipid bilayer. Water readily permeates the few-nanometer-thick bilayer, despite the oiliness of the core of the lipid bilayer, so that the entrapped solutes attract and retain hydrating water in strict relation to the osmotic pressure outside the cell. Thus, to first approximation, RBC volume is not regulated by membrane physical properties but could affect membrane state—such as rupture and lysis in hypo-osmotic media. Importantly, the shear resilience of an increasingly compressed spectrin-actin network would tend to resist further rapid fragmentation of the RBC, so that a cell circulates for another ~100 days after quickly shedding 15% of its initial plasma membrane and losing a smaller fraction of its cytoplasmic solutes and membrane skeleton. Old RBCs have ~3–6% less hemoglobin mass relative to

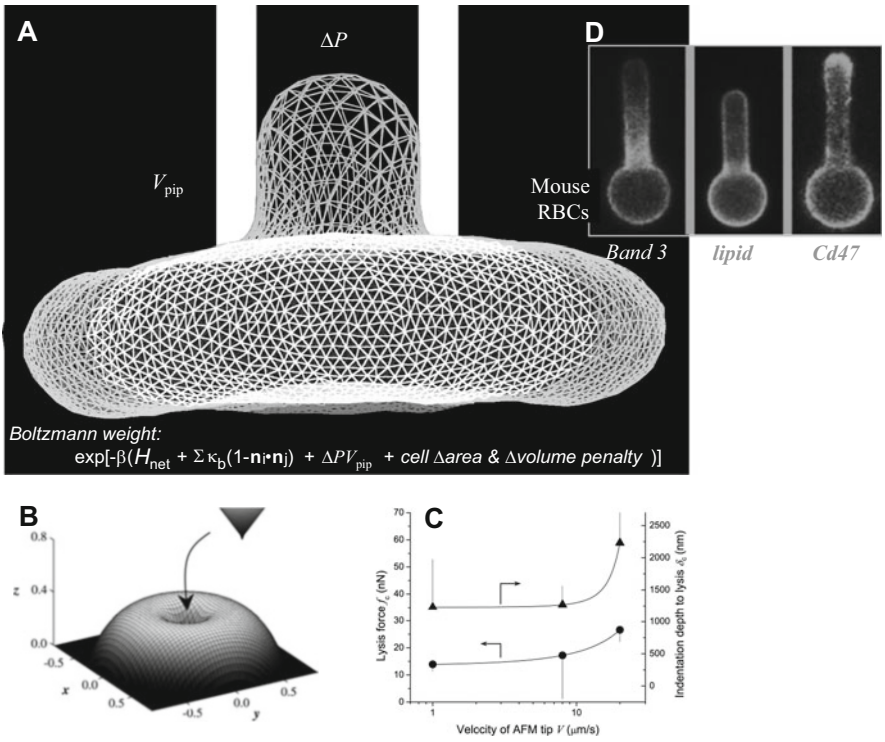

Fig. 2 (**a**) Simulation of a near full-scale RBC under aspiration. The surface of the cell is triangulated with 6110 vertex nodes that represent the spectrin-actin junction complexes of the erythrocyte cytoskeleton. Mouse RBCs have about two-thirds the surface area of human RBCs and are estimated to have about 18,000 spectrin-actin nodes, so that the simulation is about one-third scale. The volume of the cell is 0.6 times the fully inflated volume, and the snapshot is from simulation of a stress-free meshwork. (**b**) AFM indentation in a continuum calculation of an axisymmetric shape with isotropic tension and elastic dilation of the membrane, but no bending resistance. (**c**) With increasing velocity, v, of the AFM tip, the lytic force, f_c, needed to penetrate the tensed RBC membrane increases exponentially as does the indentation depth until lysis. (**d**) On mouse RBC membranes, the membrane protein Band 3 appears largely connected to the cytoskeleton, whereas another membrane protein Cd47 appears completely mobile, squeezing out to the tip of the aspirated membrane. Lipid is uniformly distributed with equal entrance and cap intensities

the bulk population [26], which indicates that the membranes of these cells rarely rupture under shear or else they rapidly reseal. Regardless, old RBCs are removed from circulation as they are engulfed or "phagocytosed" by macrophages located near slit-like blood vessels within the spleen.

Some additional physical aspects of the prototypical RBC membrane should be highlighted before proceeding to the next section on phagocytosis, with a focus on membrane-mediated interactions and cytoskeleton-driven adhesion. First, the simplicity of the RBC membrane has lent itself to a rapidly increasing number of

molecular and multiscale simulations that increasingly capture the fine structure and 2D elastic properties of the spectrin network as well as the resistance of the bilayer to both dilation and bending. One such free energy expression used in whole cell simulation (Fig. 2) has employed a "Hamiltonian" for a triangulated meshwork of spectrin chains that have both steric exclusion (as $\sim c$ / *Area* for each triangle, with c being a constant) and worm-like chain stretching energy of the spectrin chains (as fraction x of the maximum contour length):

$$H_{\text{net}} = \Sigma_{\text{triangles}} c/\text{Area} + \Sigma_{\text{bonds}} k x^2 \,(3 - 2x) \,/\, (1 - x)\,, \tag{1}$$

where k quantifies spectrin's stiffness when stretched. A change in position of a node in a triangle due to a thermal fluctuation or external force such as aspiration into a micropipette or fluid shear costs energy, which should—as a goal—reach a global minimum for the discocyte shape under stress [27]. As a function of pressure, a linear increase in length of aspirated membrane for a flaccid RBC reveals the apparent shear elastic modulus of the spectrin-actin network, which is in the range of 0.006–0.009 mN/m. This is tenfold softer than the MII-dependent cortical tension of ~0.1 mN/m typically measured for cells including HSC/Ps [10].

Membrane proteins bind to the spectrin network, so that dilation of the deformed spectrin meshwork that is evident in simulation is also seen in experiments to cause a similar density gradient of attached membrane proteins (e.g., Band 3). The steric bulk of such proteins excludes mobile membrane proteins (e.g., mouse-CD47), whereas lipid remains uniform [23]. In this way at least, the local membrane density of both mobile and immobile membrane proteins is mechanosensitive. Futhermore, unfolding of the spectrin protein when stretched has been demonstrated in intact RBC membranes [28], which provides a means for the ubiquitous spectrin network to contribute to membrane mechanosensitivity of many cell types.

Beyond the small elastic dilation limits of the bilayer, poration of RBCs with loss of hemoglobin can occur physically as well as upon addition of various chemicals that insert and disrupt the lipid membrane. Even a simple tool such as the sharpened tip of an atomic force microscope cantilever can indent adhesively attached RBCs to stress the membrane and rupture it [29, 30]. Differential geometry is useful for calculating the continuous, equilibrium shape of an indented cell up to rupture. Regardless, the rate dependence of such a process is an expected signature of stochastics in failure [31].

2 Phagocytosis and Adhesion: Microbes, Colloids, and Key Molecular Pathways for "Self" Cells

Phagocytosis was already mentioned above as important to both the removal of nuclei from the progenitor cells that make RBCs or platelets and also the clearance from circulation of old RBCs (platelets, and other cells also). Biophysical factors

and molecular factors both determine whether a macrophage engulfs an entire cell or just a nucleus. A "macro*phage*" is a large cell that *devours*, with principal "targets" for engulfment being microbes that constantly cross beach tissue barriers. The occasional serious pathogen might include malaria parasites which are notable for having a blood stage in which this microbe enters an RBC to digest hemoglobin and replicate. Additional targets of phagocytosis are now well appreciated to include all types of injected colloids, including nanoparticles, and also dying cells (or nuclei) in the same tissue, but there are also exciting efforts to make macrophages eat cancer cells. Multiple features of a target influence eating by a macrophage, including surface molecules that promote adhesion and eating, but also at least one molecule that inhibits eating as elaborated below. In addition, physical properties such as target shape and target rigidity impact phagocytosis efficiency of the target.

Phagocytosis is undoubtedly an ancient evolutionary development that provided sustenance to some of the first amoeboid cells. With soft plasma membranes rather than the rigid cell walls of bacteria, ancient amoeba (like modern amoeba typified by *Dictyostelium*) could wrap around their target to engulf it and digest it within a phagosome [32]. Fast forward eons to organisms like humans that gain nutrition through a highly differentiated and multicellular digestive tract, and we find that phagocytosis within humans is a highly efficient process used primarily by specialized white blood cells of the mononuclear phagocyte system. Microbes (in and on us) remain major targets as they not only outnumber and outproliferate our own cells but also invade through any and all compromised tissue barriers [33]. Principal cell types of the mononuclear phagocyte system are macrophages which reside in every tissue and monocytes that circulate out of the bone marrow to enter a tissue and differentiate to macrophages [34]. These cells as well as the highly phagocytic neutrophils must—for the health of the organism—choose to devour "foreign" targets rather than devour human "self" cells or extracellular matrix that generally surrounds the phagocytic cell. Phagocytosis thus evolved for engulfment and destruction of "foreign" strictly for protection of the organism.

A variety of molecular cues and sensor assemblies must be used by our phagocytic cells to distinguish and destroy "foreign" amidst "self." Many decades of work have elaborated a list of biochemical entities, soluble and/or surface bound, which activate macrophages (we will hereafter ignore the distinction from monocytes) to initiate engulfment of a target. One of the most important classes of molecules that is described below in context are immunoglobulin-G (IgG) antibodies which diffuse and bind to a target surface so that when a macrophage contacts the target, the constant fragment (Fc) of the IgG binds the macrophage membrane receptor FcγR and (for some classes of FcγR) activates the macrophage to eat the opsonized target (Fig. 3). Importantly, while it is commonly presumed that our "self" cells simply lack such surface "opsonization" by activating molecules such as IgG, it is now clear that "self" recognition is *not* simply the absence of a "foreign" signal. Instead, a dominating and passivating interaction occurs between a "Marker of Self" CD47 membrane protein on a candidate target and the macrophage membrane receptor CD172a (also known as SIRPA, signal-regulatory protein alpha). Controlling the balance of "eat me" cues (e.g., IgG-FcγR interaction) and "don't eat me" signals

(CD47-SIRPA) is currently an active area of translation to the clinic for anticancer therapy [35] and has begun to be exploited on nanoparticles in preclinical models [36]. However, the decision-making process within the macrophage remains a topic in need of deeper insight.

An explosion of efforts to make a broad range of injectable and implantable particles or devices for therapy and diagnostics has also revealed phagocytes to be a major impediment to delivery. Make a nanoparticle, inject it into the bloodstream of a mouse or man, and one invariably finds that most of the particles have been eaten by macrophages of the spleen and of the liver (the latter are called Kupffer cells). Based on several decades of work on a diversity of nanoparticles, such clearance can be delayed but never eliminated [37–40]. Studies of macrophages in conventional static culture where nanoparticle diffusion and buoyancy can dominate have questioned whether evident uptake of small nanoparticles occurs by phagocytosis [36] or not [41]. In vivo, however, blood-borne nanoparticles flow into contact with macrophages that line the spleen and liver vasculature, where these cells constantly and actively filter out dying and energy-depleted blood cells (e.g., after blood storage) to maintain blood homeostasis. A leaky vasculature at a site of injury or disease such as an infarct in the heart or a tumor can allow sufficiently small particles to permeate tissue and perhaps be retained [42]. However, when macrophages in damaged and disease sites are examined, they prove to be major consumers of permeating nanoparticles [43]. Macrophages are indeed resident if distinct in every tissue in the body [34], and at damaged and disease sites they will at least be involved in clearing dead and dying tissue. A large implant (or even a splinter) also damages tissue and causes a "foreign body response" that starts with serum protein deposition and soon recruits phagocytes to the site, but phagocytosis is frustrated for large implants and somehow triggers macrophage–macrophage fusion to a "foreign body giant cell" that encases the implant [44]. Physical size is thus a factor in macrophage function, but the focus below is on targets including cells that are cell-sized or smaller, with attention to additional properties such as target rigidity and shape as well as surface signaling (Fig. 3). Despite the decades of work on synthetics, there is nothing more biocompatible than a properly matched blood transfusion, but the reasons why continue to be elaborated.

"Eat me" signals can be weak or strong but are unavoidable. IgGs are well known for high-affinity interactions (∼nano-Molar) between epitopes and their antibody fragment (Fab) domains, but they are also large glycoproteins of ∼150,000 Daltons with considerable surface area to mediate nonspecific interactions. They are among the most abundant proteins in normal serum at ∼100 μM. Antibodies and other serum proteins physisorb in vivo to red blood cells (RBCs) [45, 46], to viruses [47], and even to particles coated with PEG (polyethylene glycol) which otherwise delays adsorption and in vivo clearance from minutes to many hours [48, 49]. Autologous IgG binding to autologous RBCs in humans and dogs in vivo increases up to sevenfold toward the end of the cell's ∼100-day life span. Aged human RBCs lack additional "eat me" signals such as exposure of the negatively charged lipid phosphatidylserine [46, 50]. IgG opsonization is increased in blood diseases including sickle cell anemia and malaria among other conditions, where

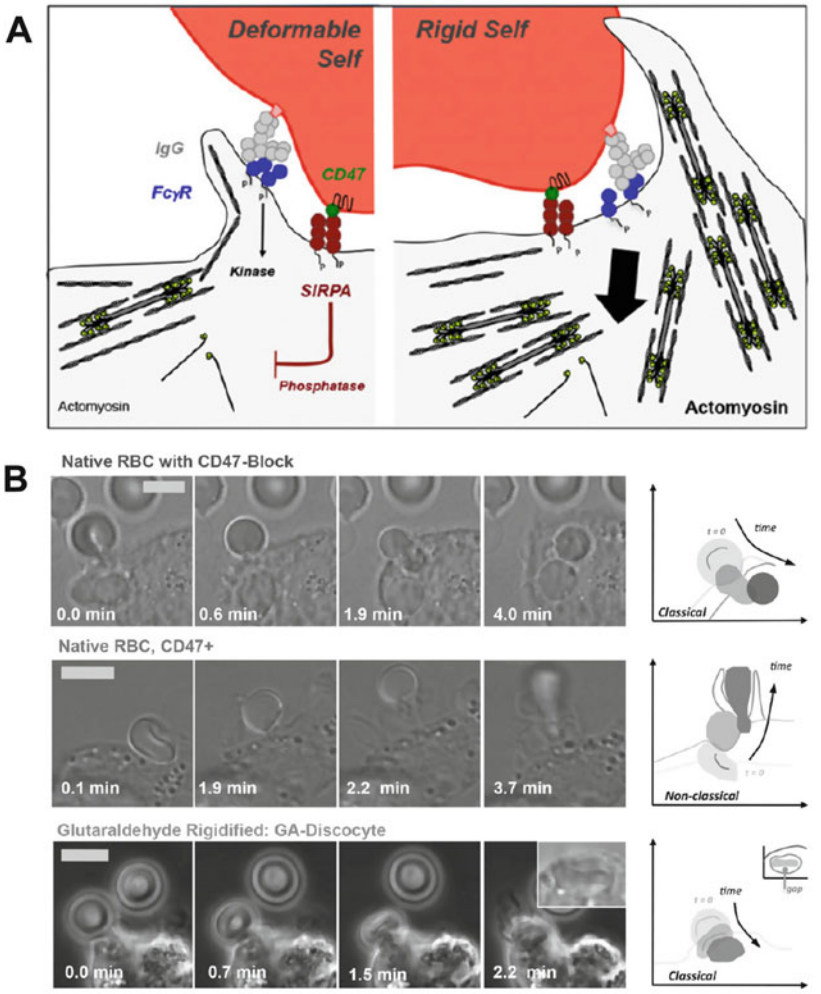

Fig. 3 (**a**) On both rigid and native flexible RBCs, SIRPA binds CD47. Downstream of FcgR binding of IgG, kinases phosphorylate multiple cytoskeletal proteins, including myosin-II, which drive assembly of the phagocytic cup and promote uptake. CD47-SIRPA signaling leads to activation of SHP-1 phosphatase that can deactivate myosin-II. Because substrate rigidity initiates assembly and polarization of myosin-II in many cell types, phagocytic target rigidity is expected to counterbalance CD47-mediated inhibition of the motor. Our working hypothesis is that with flexible self-cells (left), CD47-initiated inhibition can overcome myosin-II activation, whereas with rigid self-cells (right), the myosin-II driven cytoskeleton is not diminished by CD47-SIRPA self signals. (**b**) Phagocytic uptake of opsonized RBCs is faster with CD47 inhibition but fastest for rigid RBCs. Human-derived THP-1 macrophages were incubated with human RBCs that were opsonized with anti-hRBC antiserum and also blocked with anti-CD47, native RBCs with active CD471, or rigidified as GA discocytes. Time-lapse imaging in DIC and phase contrast begins with initial adhesion between macrophage and RBC targets and ends on complete engulfment (scale bar, 8 μm). At the right of each time-lapse series, silhouettes of the target RBC clarify the changes in RBC morphology over the course of engulfment and RBC position relative to the macrophage boundaries, as indicated by the sketched lines

phagocytosis and cell clearance are also increased (tabulated recently in [51]). When IgG-opsonized RBCs and particles are phagocytosed in vitro, uptake is hyperbolic and saturable versus IgG, which is consistent with specific activation of the FcγR phagocytosis pathway. Because macrophages and phagocytic dendritic cells can also function as antigen-presenting cells to the white blood cells of the "acquired immune system," it seems sensible that engulfment can promote immunogenicity in vivo even to foreign polymers [52].

Binding of the IgG's Fc domain to the macrophage FcγR receptor triggers phagocytic cup formation in a coordinated process of adhesion, pseudopod extension, and eventual internalization with phagosome closure. The surface interactions initiate Src family kinase phosphorylation of immunoreceptor tyrosine-based activating motifs (ITAMs) that then propagate a phosphorylation cascade [53]. Phosphopaxillin and F-actin [54, 55] accumulate in minutes or less together with other structural components at this dynamic phagocytic synapse. The process is highly analogous to adhesion formation upon integrin binding to rigid extracellular matrices, wherein the nascent adhesion matures to a focal adhesion only when the F-actin cytoskeleton is mechanically organized through pulling by non-muscle myosin-II (MII) phosphoprotein [56, 57]. At the phagocytic synapse, MIIA accumulates to greatly help with pulling targets into a macrophage, including IgG-opsonized targets as small as 100 nm nanoparticles (perhaps smaller) and at least as large as opsonized RBCs [36, 58, 59]. Engulfment of such targets is greatly decreased by inhibiting MIIA motor activity with the drug blebbistatin which also blocks MII localization without affecting F-actin or phosphopaxillin. Uptake increases linearly with MII activity based on its knockdown and overexpression [58]. At least above a low baseline level of uptake in vitro, MII makes phaogocytosis efficient for our macrophages if not for ancient amoeba like *Dictyostelium* [32].

As an IgG-opsonized target contacts a macrophage and adheres intimately via FcγR, the parallel presence on the target of an appropriate form of CD47 can lead to binding to the macrophage phagocytosis inhibitory receptor SIRPA, which accumulates in the synapse [59]. The latter complex somehow phosphorylates SIRPA's cytoplasmic immunoreceptor tyrosine-based activating motifs (ITIMs), which activates the immunomodulatory phosphatase SHP-1 (Src homology region 2 domain-containing phosphatase-1) [60] to regulate multiple proteins by dephosphorylation [61], including deactivation of MIIA [36, 58]. SIRPA-null macrophages engulf IgG-opsonized mouse RBCs more readily than wild-type macrophages [61] and show no major differences in phospho-FcγR nor the downstream effectors phospho-Syk or phosho-Cbl, which suggests that regulation of proteins even further downstream is key. Inhibition of downstream actomyosin contractility at the phagocytic synapse [36, 58] could indeed explain various observations that CD47 partially blocks engulfment of not only mouse RBCs—which started the expanding "Marker of Self" field [62] but maybe also cancer cells [63–65] and opsonized polystyrene beads (100 nm to 6 μm) that display CD47's binding domain in parallel with IgG [36, 58]. The effectiveness of CD47 with small nanobeads is surprising because pulling in *large* particles with MII forces seems more understandable

than pulling in *small* particles. Nonetheless, CD47's mechanism of inhibition is ultimately: just don't pull it in!

CD47 and SIRPA arose in evolution at the same time in amniotes and are not found even in amphibians [66]. CD47 and SIRPA are thus more recent inventions than the ancient actomyosin cytoskeleton found even in amoeba such as *Dictyostelium*. CD47 is found on all cells in man and mouse while the expression of SIRPA is more restricted. CD47 knockout mice have one-quarter to one-half the life spans of normal mice (at least for some strains) and also show evidence of anti-RBC antibodies as well as anemia [67]. This is consistent with the idea that a modest level of opsonization exists in vivo, which tilts the balance toward engulfment and possibly even an acquired immune response with antibody generation.

Saturable binding of SIRPA, CD47, and/or CD47-derived "Self" peptide to beads as well as to living cells shows that an intermediate strength interaction (sub-micro-Molar) has evolved to be largely species specific [36, 58, 68]. The molecules also differ between strains of mice. NOD-SCID strains of mice uniquely express a SIRPA variant that binds human CD47 with similar affinity as human SIRPA, which partially explains why these are the best mouse choice for engraftment of human stem cells [69]. Species specificity in vivo is a critically important issue because human-specific blocking antibodies have been injected intravenously together with opsonizing IgG to impede growth and even shrink tumors of human cancer cell lines in mice. As emphasized by others [70–72], injection of any reagent that binds human-CD47 would bind to every cell membrane in the body, even if cancer cells have severalfold more of this ubiquitous protein [73, 74]. On the other hand, CD47 is far from the most abundant protein on cells ($\sim$250 molecules/μm^2 on RBCs which is 10–20-fold less than Glycophorin-A), so that blocking CD47 even with IgG is not expected to drive strong phagocytosis unless an additional and far more abundant "eat me" cue is also bound to a candidate target. The half-max density for inhibition by CD47 on beads is independent of particle size and is $\sim$20 molecules/μm^2, which is consistent with the minimum density of CD47 on circulating RBCs from patients with anemia [36, 58]. For senescing neutrophils, CD47 is somehow downregulated from the surface and the needed cue to drive macrophage engulfment seems to be surface-exposed calreticulin (from the endoplasmic reticulum) rather than IgG [75]. Some of the above ideas are currently being put to the test in the clinic [35]. Safety is of course the first question of concern for systemic injection of any entity that limits macrophages from recognizing "self", and loss of RBCs from circulation is readily expected to be observed when patients are injected with anti-humanCD47 IgG.

Rigid cells and particles drive phagocytosis, but shape and size of a target can also frustrate engulfment. A relatively new principle in cell biology that applies to many cell types is that adhesion-induced activation of myosin-II is maximized by adhesion to a substrate that is rigid (like glass or plastic) rather than flexible like most soft tissues [12]. Bacteria such as *E. coli* and fungi such as yeast have cell walls as rigid as some plastics [76]. For the RBC precursor erythroblasts that interact with macrophages, the effective cell stiffness relative to mature RBCs has been measured by micropipette aspiration to be 50-fold higher, and senescence is

also higher in senescence and in diseases ranging from inherited anemias to malaria (tabulated in [51]). With spherical microparticles made of hydrogels and opsonized by IgG, engulfment is proportional to stiffness, which was also shown to drive focal adhesion protein assembly at the synapse [77]. Cell stiffness changes occur with cancer and with chemotherapy [78, 79]. Soft cancer cells might thus escape anticancer efforts aimed at inhibiting CD47–SIRPA interactions [63, 64]. However, a clear relation of cell or particle stiffness to CD47 signaling and to cells with more complicated shapes had been untested until recently.

With normal human RBCs, controlled stiffening could be achieved within seconds by the addition of a protein crosslinker (divalent and covalent) that does not compromise binding of CD47 to SIRPA [22]. Measurements showed that RBC Stiffness $\sim$ exp(crosslinker concentration/a). The stiffened RBCs that were highly opsonized by IgG were engulfed faster and in greater number according to "Self" Phagocytosis $\sim$exp(crosslinker concentration/b). A ratio (a/b) ~ 0.5 was determined so that we can eliminate crosslinker concentration to arrive at:

$$\text{“Self” Phagocytosis} \sim \text{Stiffness}^{0.5}, \tag{2}$$

which is a power law typical of mechanosensitive pathways dependent on myosin-II [80]. When CD47 is not on the target as is typical in past studies of polymer beads [77], estimates of bead stiffness based on the amount of crosslinker used (per [12]) suggest a stronger exponent of $\sim$1 in Eq. (2) that is generally consistent with "Self" inhibition of phagocytosis.

Rigid RBCs showed active myosin-II at the synapse [51], which suggests that CD47 cannot signal effectively even though it binds SIRPA. As expected, the MII inhibitor blebbistatin blocked MII accumulation and RBC engulfment. Injection of rigid RBC discocytes into the circulation of a mouse also confirmed equal clearance by splenic macrophages independent of whether CD47 was blocked or not with antibody. Synthetic polymer discs resembling RBCs and that lacked any CD47 or other RBC proteins were also shown to be removed from circulating blood far more rapidly when they were stiff rather than soft [81]. Mechanistically, stiff cells and particles become stuck in narrow splenic slits [82], which could facilitate probing and clearance by splenic macrophages [83].

The results which show rigid RBC *discocytes* are engulfed independent of CD47 present a paradox for the field in that rigid, *spherical* beads that display CD47 are reproducibly capable of signaling self and thereby impeding engulfment both in vitro and in vivo [36, 58]. *Shape* is an additional target factor that also modulates phagocytosis and resolves the apparent paradox. Polystyrene microbeads distorted into diverse shapes are always equally rigid but have been shown to be engulfed more readily as spheres than as non-spheres when IgG opsonized [84]. In many studies of other cell types, the topography of a substrate affects cell attachment and is sometimes referred to as "contact guidance." For macrophage engulfment, the dependence on target shape seems relevant to the diverse shapes of bacteria and fungi that are invariably rigid as noted above. Flexible PEG-based filaments also persist in the in vivo circulation many days longer than spherical particles of the

same type of polymer, with particles always eventually cleared by spleen and liver macrophages [38].

With normal human RBCs, the rapid and controlled stiffening approach [51] was used to make rounded, cup-shaped RBC "stomatocytes" that signal "self" much better than rigid RBC discocytes. The problem with a discocyte is that its rigid concavities cannot contact and signal "self" to the macrophage which builds up a high cortical tension when activated by IgG via Fc receptor. In the same studies, native and flexible human-RBC discocytes with the requisite IgG opsonization were seen in video microscopy to be greatly distended by the human macrophage, with myosin-II turned off by CD47 signaling but actin polymerization driving protrusions as if pushing the cell away in recognition of "self." Thus, since rigid but rounded cells do signal "self"—even if not as efficiently as flexible RBC—one can ultimately understand the success in delaying clearance of CD47-nanobeads that then enable better tumor imaging and drug delivery. However, it seems that a greater advantage might be achieved with flexible beads that avoid the intrinsic activation of myosin-II.

There are some important implications of phagocytic interactions for the production of RBCs within bone marrow. RBC stiffness is one among many factors including shape that changes in erythropoiesis. A plausible mechanism is that the macrophage senses both a rigid nucleus *and* a soft nascent reticulocyte, which is a cue for the macrophage to help pull the erythroblast apart provided that CD47 on the reticulocyte effectively signals "self." Target size (e.g., small nucleus versus large retic) *does not* affect uptake provided molecular densities are properly calculated [36], but additional polarized "eat me" factors might signal the macrophage to pull on a nucleus for its rapid engulfment. With mouse erythroblast nuclei, phosphatidylserine (PS) flips within minutes to the outer leaflet to promote engulfment, although PS flipped only "when cultures were subjected to weak physical stress" [85]. CD47 might be present at low levels on nuclei isolated from erythroblast cultures [86], and we had shown CD47 levels as low as ~10% of levels on RBCs still impede eating, consistent with the lowest levels of CD47 on RBCs reported in the human population [59]. Nuclear rigidity thus seems a major "eat me" cue.

3 Nuclear Membrane Properties and Mechanosensing in Brief

Although mammalian RBCs have only the outer plasma membrane, the RBC progenitor has abundant internal membrane systems, including endoplasmic reticulum which is continuous with the double bilayer of the nuclear envelope. A meshwork of proteins again forms as a sub-membranous cortical layer or lamina, and an understanding of the complex physical properties of this nuclear lamina is just emerging. Unlike the actin filaments and actin-crosslinking proteins spectrin and myosin-II that are main constituents of plasma membrane cortices discussed above

for RBCs and the other hematopoietic cells, the nuclear lamina meshwork is formed from self-assembling intermediate filament proteins, called lamins. The lamina lies just inside the nuclear envelope and interacts at least indirectly with chromatin, but the lamina also has protein linkages to nuclear membrane proteins that interact with the cytoskeleton outside the nucleus. The nucleus is thus embedded within and also linked to the stress-generating cytoskeleton, which deforms the nucleus, perhaps affecting gene expression as well as genome integrity. As suggested by the various types of differentiated cells sketched in Fig. 1, nuclear shape and orientation tend to track with cell shape and orientation—which has long been noticed in various nucleated cells (e.g. marrow derived MSCs [87]).

In humans and mice, the main forms of lamin protein are expressed from three genes: lamin-A, lamin-B1, and lamin-B2. Like other intermediate filament proteins, such as keratin in skin, fingernails, and horns, the lamins form coiled coil parallel dimers that assemble into higher-order filamentous structures that fulfill important structural roles [88]. The B-type lamins are distinct in being permanently modified by a greasy farnesyl group that promotes avid interactions of these filaments with the innermost leaflet of the nucleus' double lipid bilayer and these lamins also interact with lamin-B receptor, which is a transmembrane protein [89]. Such interactions will tend to act as effective physical crosslinks of a meshwork of lamin-B filaments, even though there no reported lamin crosslinker proteins.

Lamin-A was found in a proteomic study of diverse tissues to be almost unique among hundreds of abundant proteins of the cytoskeleton and nucleus, in that lamin-A concentration increased 30-fold as a function of increasing stiffness of tissue, E_{tissue}, from soft bone marrow and brain tissue to stiff muscle and rigid bone [80, 90]. Tissue stiffness is defined of course by how resistant the tissue is to stress and thus relates to the stress in functional tissue: bone sustains and resists high mechanical stress whereas marrow is protected by bone from stress, as is brain, and both shear very easily. Such mechanical differences between tissues are largely attributable to the amount of collagen fiber in the extracellular matrix, which is abundant in bone and minimal in marrow and brain [80]. The finding for lamin-A thus suggests that the nucleus adjusts its levels of this protein to the mean stress σ in most cells and their nuclei in a given tissue, whereas B-type lamins remain nearly constant. Importantly, the stress σ likely tracks the active (and passive) stiffness of a cell, especially contractile activity of myosin-II inside a cell that increases with matrix stiffness In terms of the tissue-type dependent stoichiometry (A:B), an experimental correlation emerges from measurements of composition and mechanics:

$$\text{lamin-A} : \text{B} \sim E_{\text{tissue}}^{0.6} \sim \sigma_{\text{tissue}}^{0.6}. \tag{3}$$

As a relevant aside given this chapter's broad focus on the sculpting of cell membrane shapes, the only other protein discovered in the same proteomic study to exhibit such a strong and clear scaling relationship across diverse tissues is a membrane-interacting protein called cavin-1. Cavin-1 is known to regulate the morphology of mechanosensitive plasma membrane invaginations called caveolae

[91, 92]. Such a scaling relationship with stress is an indicator of mechanosensing by such proteins, and since lamin-A is also a filament-forming structural protein, it should directly affect nuclear mechanics.

Micropipette aspiration has once again been used to quantify and clarify the rate-dependent deformation of nuclei under controlled pressures [93, 94]. Because of the log range in lamin-A:B stoichiometry, the characteristic contributions of A- and B-type lamins to nuclear properties have become reasonably clear, with nuclei having low lamin-A:B responding in seconds, whereas nuclei having high lamin-A:B take many minutes to deform when similarly stressed. The B-type lamin filaments that strongly associate with the nucleus' inner lipid bilayer (via lamin-B farnesylation) understandably contribute mostly to the elastic response of the nucleus, whereas lamin-A contributes most strongly to nuclear viscosity consistent with the flow physics of filamentous polymers [90, 95, 96]. Thus, when lamin-A dominates, the nucleus response is akin to a balloon filled with honey, whereas when lamin-A is very low the nucleus behaves merely as a balloon filled with water. While the importance of A-type lamins in maintaining nuclear structural integrity and cell viability has been appreciated for a long time [94, 97], the influence of lamin-A on cell motility has been more recent. A rate-limiting role for the nucleus and its lamina in migration of a given cell type through small pores is now clear for cancer cells invading nearby tissue [95] and also for hematopoietic cells that either remain within the marrow or else escape from it through small micro-pores in the endothelial lining that separates marrow from blood (Fig. 1).

4 Conclusions

In terms of future directions, a deeper understanding of the biophysical similarities and differences between a nucleus and an RBC should be pursued. Indeed, one caveat to the analyses above is that it undoubtedly applies to a flaccid nucleus in which the chromatin is highly hydrated within a relative nuclear volume that is roughly similar to that of an RBC discocyte (e.g., Fig. 2). The ~2 meters of DNA folded as chromatin inside each and every nucleus contributes most clearly to nuclear rigidity only when the genome becomes condensed with loss of water and nuclear volume [94]. Mobile nuclear proteins including DNA repair factors and transcription factors might be lost or have limited access to dense DNA [98], thereby suppressing gene expression, but the high nuclear rigidity of such a dehydrated nucleus will also tend to stimulate local contractility of myosin-II (especially MIIB) in driving an asymmetric process of "cytokinesis" or nuclear expulsion (per erythropoiesis in Fig. 1). The rigidity of an expelled nucleus will also tend to favor its phagocytosis by bone marrow macrophages regardless of whether the "Self" marker CD47 is present or not (Fig. 3), whereas the nascent RBC reticulocyte is highly flexible in being filled with a solution of hemoglobin. Severing of the lipid membrane tether between nucleus and reticulocyte and loss of lipid membrane blebs in maturation to an RBC also raise issues of limits on nuclear membrane integrity.

This is an exciting topic with increasing evidence of curvature-dependent rupture of the lamin-B meshwork that is somehow modulated by lamin-A and followed by loss of integrity of the double bilayer [99]. Within the marrow, such a stepwise process of nuclear rigidification, gene expression shutdown, nuclear expulsion, and engulfment with severing of the tether between nucleus and flexible reticulocyte are the final stages in the marrow for generating an RBC—with rich biophysics to be perturbed and more deeply understood.

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