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Ischemia/Reperfusion Injury and its Consequences on Immunity and Inflammation

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Abstract Ischemia/reperfusion injury (IRI), an inherent component of transplantation, affects organ quality and transplant outcomes. Although the complexity of the pathophysiology is recognized, detailed mechanisms remain unclear, and strategies preventing the consequences of IRI have been challenging. Of critical significance appears to be the link between IRI, the initiation of innate immune responses, and the (potential) augmentation of adaptive immunity. An improved understanding of those complex mechanisms and interactions may pave the way for more effective treatment strategies.

Keywords Ischemia-reperfusion injury · Innate immunity · Inflammation

Introduction

Innate immune responses are a critical component of organ transplantation affecting both immediate organ function and long-term graft survival. Mechanisms linking innate and adaptive immunity are poorly understood and insufficiently addressed in current established treatment approaches.

The concept of an 'injury response' as a link between injury, immune responses, and transplant outcomes has been proposed several years ago [1] based on the notion that immune cells become activated by danger/alarm signals from injured cells rather than solely through the recognition of 'non-self' [2]. While the impact of innate immunity on transplant outcome is recognized, key players, mechanisms, and potential treatment targets require a more in-depth analysis.

Here, we provide an overview of mechanistic events subsequent to ischemia/reperfusion injury (IRI) with a focus on key components of a complex interplay between innate and adaptive immunity in addition to current experimental and clinical treatment concepts.

Ischemic Injury

Interruption of blood supply is inevitable following procurement of an organ for transplantation. Deprivation of oxygen and nutrients trigger a series of events that lead to cell injury and death. As oxygen levels fall, there is a transition from aerobic to an anaerobic metabolism, resulting in the accumulation of metabolic byproducts and cellular acidification. Metabolic demand eventually exceeds compensatory adenosine triphosphate (ATP) production, and ATP levels fall to critically low levels. Consequently, ATP-dependent membrane pumps fail, leading to an accumulation of intracellular sodium and calcium, followed by cell swelling, membrane rupture, and ultimately oncotic cell death.

Limiting ischemic times by restoring perfusion and supplying oxygen and nutrients is therefore a vital aspect of reducing ischemia-related injury. Reperfusion by itself, however, triggers a new series of detrimental cellular processes that exacerbate injury and cell death (Fig. 1).

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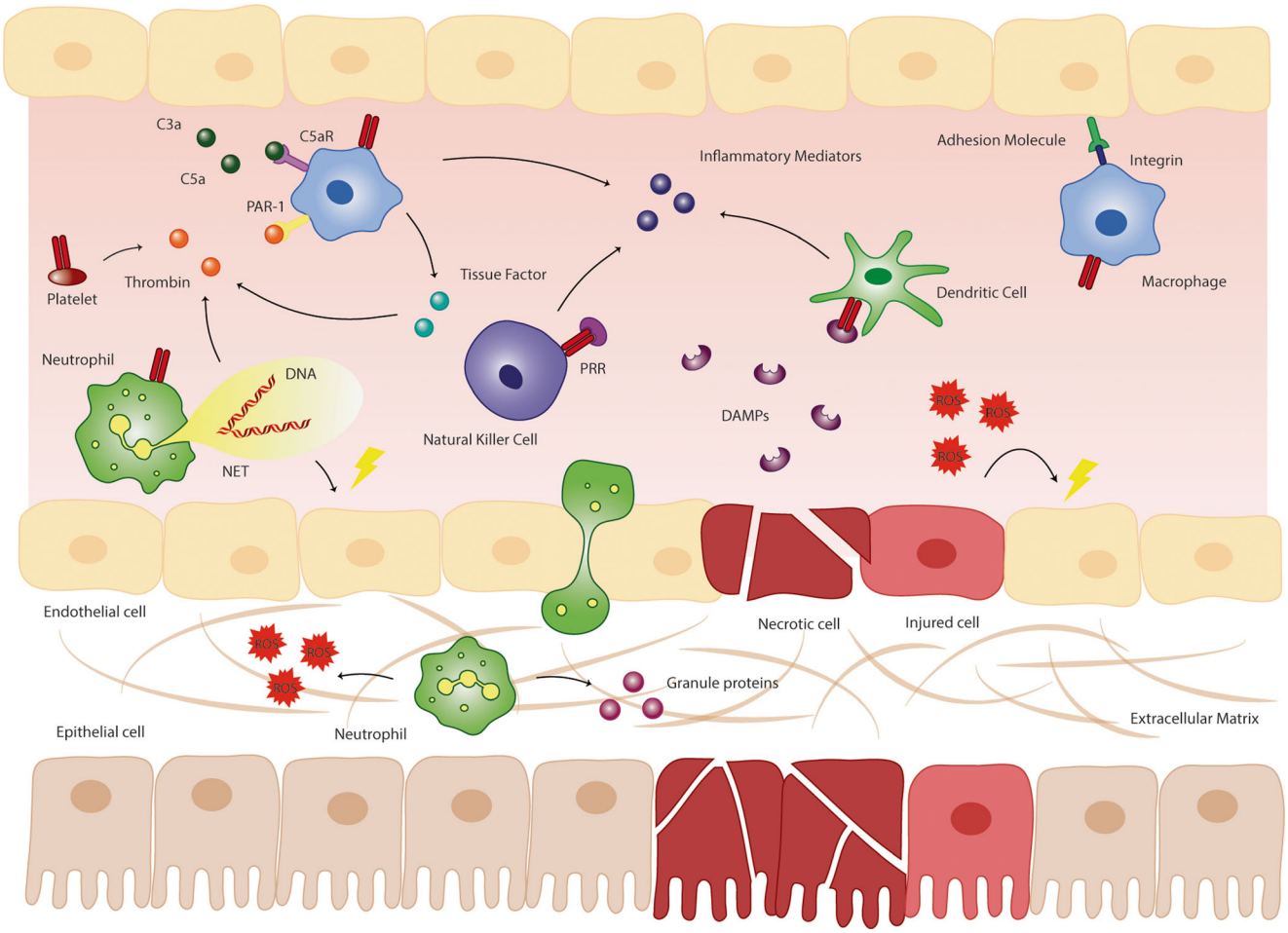

Fig. 1 Damage-associated molecular patterns (DAMPs), released from necrotic and injured cells, activate pattern recognition receptors (PRRs) expressed on innate immune cells and epithelial and endothelial cells. Consequently, inflammatory cytokines and chemokines are produced. Injured cells produce reactive oxygen species (ROS) that are cytotoxic. Activated endothelial cells upregulate expression of adhesion molecules, thereby facilitating leukocyte adhesion and transmigration. Neutrophils release neutrophil extracellular traps (NETs) that are both pro-thrombotic and cytotoxic. Neutrophils also engage in the respiratory burst and release their granule proteins. Macrophages and neutrophils release microvesicles containing thrombin and platelets express PRRs that mediate the generation of thrombin. Thrombin activates innate immune cells through proteinase-activated receptor 1 (PAR-1) ligation resulting in the production of inflammatory cytokines and chemokines. The complement system is activated, providing a chemotactic gradient as well as an activating cue for innate immune cells through their cognate receptors

Reperfusion Injury

Reperfusion subsequent to ischemia generates reactive oxygen species (ROS), mitochondrial failure, endothelial dysfunction, and sterile inflammation. Under physiological conditions, mitochondria generate small quantities of ROS through the leakage of electrons. When exposed to ischemia, however, mitochondrial complexes are damaged, resulting in an excessive generation of ROS beyond antioxidant-scavenging capacity upon reperfusion [3]. ROS, in turn, cause damage to membrane lipids, proteins, and nucleic acids that can lead to cell death [4]. Additionally, the mitochondrial permeability transition pore (mPTP), a non-specific pore formed in the inner mitochondrial membrane allowing entry of molecules <1.5 kDa, is activated subsequent to exposure to ROS and increased mitochondrial calcium levels. This phenomenon results in a disruption of the electrochemical gradient, uncoupling of oxidative phosphorylation, and ATP depletion. Moreover, an augmented osmotic pressure is linked to mitochondrial swelling and membrane rupture, resulting in necrosis or apoptosis [5]. Finally, ROS contributes to a pro-inflammatory environment by promoting the formation of the NLRP3 inflammasome [6], a multiprotein complex that, in turn, activates caspase-1. Subsequently, caspase-1 cleaves the precursor pro-interleukin (IL)-1β, forming the pro-inflammatory cytokine IL-1β [7].

The graft microvasculature is particularly sensitive to IRI [8]. Subsequent to reperfusion, blood flow to ischemic tissue is not immediately restored completely. This process, termed the ‘no-reflow phenomenon’, is thought to be linked to endothelial cell swelling and intravascular plugging by leukocytes, fibrin, or platelets, all contributing to intravascular obstruction

[9]. In the context of IRI, damaged and activated endothelial cells also lose barrier function, becoming more permeable and allowing the transmigration of leukocytes. Moreover, endothelial cells are sensitive to biomechanical forces generated by blood flow. Flow-mediated shear stress induces the vasoprotective transcription factor KLF2, which suppresses the IL-1β-mediated endothelial induction of E-selectin and vascular cell adhesion molecule 1 (VCAM-1) [10]. Cessation of blood flow in the context of organ preservation has been shown to trigger endothelial dysfunction mediated by a decay of KLF2 [11]. Interruption of blood flow therefore primes the endothelium for activation by pro-inflammatory cytokines and, once activated, the endothelium promotes leukocyte adhesion and transmigration.

After restoring perfusion, a sterile inflammatory response is induced through the release of endogenous molecules from necrotic and injured cells. Inflammation is an important process required for tissue repair and regeneration through the clearance of dead cells and the release of growth factors and chemokines that induce cell proliferation and angiogenesis. However, the release of ROS, proteases, and fibrosis-promoting factors, all aspects of inflammation, are detrimental. In IRI, inflammation becomes excessive and injury and repair become unbalanced, with innate immune cells playing a critical role in mediating injury responses.

Innate Immune Responses Subsequent to Ischemia/Reperfusion Injury

The innate immune system is an evolutionary conserved first line of defense, capable of acting rapidly upon infection through recognition of repeating structures expressed by microbes, termed pathogen-associated molecular patterns (PAMPs) that are recognized by pattern recognition receptors (PRRs) [12]. All cells of the innate immune system, including neutrophils, monocytes, macrophages, dendritic cells (DC), and natural killer (NK) cells express PRRs and therefore contribute to a pro-inflammatory environment that is established following reperfusion. Additionally, non-immune cells such as endothelial [13] and epithelial cells [14] express PRRs. This heterogeneous group of receptors includes Toll-like receptors (TLRs), NOD-like receptors (NLRs), C-type lectin receptors (CLRs), and RIG-I-like receptors (RLRs) located on cellular membranes, intracellularly, or secreted in soluble form. PRRs also respond to endogenous molecules, so-called danger-associated molecular patterns (DAMPs), that are released by injured and necrotic cells in response to IRI. DAMPs include high mobility group box-1 (HMGB1), heparan sulfate, ATP, nuclear DNA, mitochondrial DNA, and RNA [15]. Ligation of PRRs results into the activation of the inflammasome [16] in addition to the induction of nuclear factor-κB (NF-κB) and mitogen-activated protein kinases (MAPK) pathways. As a result, pro-inflammatory cytokines and chemokines, including IL-1, IL-6, tumor necrosis factor-α (TNF-α), monocyte chemoattractant protein 1 (MCP-1), and IL-8 are induced [15, 17] and major histocompatibility complex (MHC) and co-stimulatory molecules are upregulated [18]. Interestingly, oxidative stress has been reported to upregulate TLRs in macrophages [19], augmenting the inflammatory response. Experimental knockout (KO) models have shown that TLR4- [20] and TLR2-deficient mice [21] are protected from IRI while exposure to an administered TLR agonist augments acute rejection of cardiac allografts [22]. Thus, at least in theory, IRI forms an important link between innate and adaptive immunity. Indeed, alloimmunity was enhanced through the activation of the innate immune system subsequent to TLR ligation in experimental transplant models [23–26]. Although all cells of the innate immune system are capable of sensing DAMPs, specific cells have been shown to play distinct roles.

Neutrophils

Neutrophils are the largest circulating fraction of leukocytes and first to arrive at the site of injury [27]. Their involvement in transplantation has been shown in experimental models, in which the inhibition of neutrophil chemotaxis prolonged allograft survival [28]. Neutrophils are recruited by signals provided by chemokines that are produced by tissue-resident macrophages and endothelial cells [29]. Rolling adhesion and then tight adhesion are initiated through the upregulation of adhesion molecules on endothelial cells. Once neutrophils transmigrate through the endothelium, they release the content of their granules containing proteases and then engage in a process termed the ‘respiratory burst’ involving the release of ROS. Additional leukocytes are recruited through the production of cytokines and chemokines by neutrophils. Moreover, neutrophils have the capacity to produce neutrophil extracellular traps (NETs) by releasing nuclear chromatin and granule proteins [30]. NETs primarily capture and kill microbes during infection but have recently also been shown to be involved in cardiac IRI. Indeed, KO mice incapable of producing NETs were protected from the consequences of ischemia [31]. Similarly, decreased NET levels in TLR4 KO mice ameliorated damages subsequent to hind-limb IRI [32].

Mononuclear Phagocytes

Mononuclear phagocytes comprise a group of myeloid-derived monocytes, macrophages, and DCs [33]. Circulating monocytes are recruited through chemotaxis. Upon arrival at the injury site, they differentiate into tissue macrophages or DCs. Macrophages demonstrate a high degree of plasticity and assume a pro-inflammatory or anti-inflammatory phenotype depending on micro-environmental cues [34]. Similar to

the T-helper (Th)1/Th2 polarization framework established in T cells, macrophages can undergo M1 and M2 polarization. TLR ligation and stimulation with interferon (IFN)-γ promote M1 activation, whereas IL-10, IL-4, and IL-13 promote M2 skewing [35]. After exposure to the inflammatory environment subsequent to IRI, macrophages assume a M1 polarization and promote inflammation through cytokine production. Depletion of macrophages prior to IRI has been shown to ameliorate injury in an experimental murine kidney IRI model. In contrast, when macrophages were depleted days after injury, impaired tubular proliferation and repair were observed linked to the depletion of M2 macrophages [36•].

DCs form a critical link between innate and adaptive immunity. Once activated, they orchestrate the adaptive immune response through maturation and migration to lymphoid tissues [37]. DCs that originate from donor organ tissue or circulating recipient DCs have the capacity to activate PRRs following IRI [38, 39]. However, DCs are also capable of modulating peripheral T cell tolerance [40] and may play a protective role in IRI [41]. It has been proposed that donor resident DCs, but not circulating DCs, are protective during hepatic IRI [42]. In a murine kidney IRI and transplant model, loss of donor tissue resident DCs has been linked to progressive T cell recruitment [43]. These data demonstrate dual roles for DCs in IRI, presumably depending on location and microenviromental cues.

Natural Killer Cells

Natural killer cells are cytotoxic lymphocytes that are capable of distinguishing between self and non-self by activating and inhibiting surface receptors [44]. MHC-1 expression forms an inhibitory signal for NK cells while downregulation of MHC-1 enables NK cell activation and cytolysis, a paradigm known as the missing-self hypothesis [45]. In transplantation, allogeneic cells lack recipient MHC-1 alleles, leading to NK cell activation and a more rapid rejection [46]. However, others have reported on a tolerance-promoting role for NK cells by targeting donor antigen-presenting cells (APCs) [47]. The role of NK cells specific to IRI has not been well-characterized. NK cells were shown to be recruited through tubular epithelial cell (TEC) production of the chemokine CCR5 following TLR2 ligation by DAMPs in a renal IRI model [48]. Subsequently, NK cells contribute to IRI through upregulation of CD137L, stimulating additional chemokine production by epithelial cells and recruitment of neutrophils [49]. IL-17 production by NK cells has been implicated as an immune-activating mechanism in a hepatic IRI model [50]. Moreover, NK cells have also been reported to directly target signaling stressed epithelial cells by inducing apoptosis. In a renal IRI model, expression of reninoic acid early inducible (Rae-1) was upregulated following IRI, thereby providing a signal activating the NK cell receptor NK group 2 member D (NKG2D) that induces apoptotic cell death [51].

Activating the Complement Cascade

The complement system can be initiated by three pathways: the classical, alternative, and lectin pathways. These pathways are composed of circulating precursor proteins that can be enzymatically activated. While the activation of different pathways is distinct, they converge with the activation of C3 convertase, leading to the formation of the membrane attack complex (MAC), which is capable of forming a lytic pore into the pathogen's cell wall [52]. Other functions of activated complement components include chemotaxis and opsonization [53]. All three pathways are thought to be activated in IRI [54]. Lysis of parenchymal cells by the MAC [52], C3a- and C5a-induced recruitment of leukocytes through chemotaxis, and production of inflammatory cytokines and chemokines [55] have been implicated as injury-mediating mechanisms in IRI. The critical role of the lectin activation pathway by mannose binding lectin (MBL) in renal IRI has been demonstrated in several experimental studies [56–58]. Absence of MBL was shown to be protective against the adverse effects of renal IRI with significantly less tissue damage, while reconstitution of MBL aggravated injury. Emerging evidence indicates that conventional routes of the lectin pathway may be bypassed in IRI. In a cardiac IRI model, inhibition of complement activation through the lectin pathway was shown to reduce tissue injury independent of C4 activation [59]. Moreover, therapeutic inhibition of MBL by a monoclonal antibody against MBL-A (P7E4) was protective against renal IRI in a rat model. Interestingly, the MBL-mediated tubular injury was shown to be completely independent of complement activation as interference with C3 or C5 was not protective against renal IRI, suggesting a direct cytotoxic effect of MBL [60].

Coagulation and Platelet Activation

Historically, coagulation, platelet activation, and hemostasis have been viewed as being separated from immune responses. Recent work, however, has suggested that innate immunity and thrombosis act in concert in a process that has been coined immunothrombosis [61]. In this conceptual framework, innate immune cells induce thrombosis while platelets and coagulation pathways activate immune responses. A number of findings support this hypothesis and also provide a link to IRI. Monocytes and neutrophils are known to contain microvesicles with tissue factor (TF) [62•]. When activated, these microvesicles are released and promote coagulation through the extrinsic pathway, while NETs induce coagulation through the intrinsic pathway [61]. Protease-activated receptors (PARs), expressed on all cells of the innate immune

system, in turn, are activated by coagulation proteases such as thrombin leading to induction of pro-inflammatory cytokines [63]. Moreover, platelets release thrombin in response to DNA released by necrotic cells, mediated through TLR2 and TLR4 ligation [64]. Serotonin release from platelets promotes recruitment of neutrophils [65]. Experimental data indicate that these processes may also play a role in IRI. In a renal IRI model, inhibition of TF ameliorated kidney injury [66], possibly through the reduction of microthrombus formation. PAR-1 deficiency protected against renal IRI [67] and treatment with activated protein C protected from cardiac IRI through inhibition of PAR-1 [68].

Clinical Implications and Novel Therapies

Improvement of organ preservation and allocation are both effective strategies to limit IRI. It is well-established that cold ischemic times (CIT) are an important determining factor for the degree of organ injury and thus transplant outcomes. In a retrospective study in kidney transplantation patients, DGF was clearly linked to prolonged ischemia [69]. Moreover, rates of acute rejection were not only linked to prolonged ischemia [70], but CIT exceeding 18 h was associated with a decreased graft survival [71]. Marginal, nowadays mostly called extended criteria donor (ECD), organs seem particularly sensitive to prolonged ischemia [72]. Moreover, when CIT had been minimized, outcomes of ECD and standard criteria donor (SCD) transplantations were comparable [73•]. The Eurotransplant Senior Program was established with the aim of allocating older kidneys with minimal ischemia times to older recipients, termed by some as the 'Old for Old' program. Of note, outcomes of older organs transplanted within this program were comparable with those of younger organs [74]. Optimizing allocation schemes and reducing CIT is therefore a strategy that remains relevant, particularly in times when utilization of ECD organs continues to increase.

In most transplant centers, cold static preservation is currently the standard method of organ preservation. However, clinical trials on machine perfusion have recently shown superiority [75, 76]. The benefits of machine perfusion involve the ability to supply nutrients and oxygen to the organ while washing out metabolic and toxic waste products [77]. In addition, maintaining a protective vascular phenotype mediated by the flow-responsive endothelium may be a mechanism by which machine pulsatile perfusion provides protection [11, 78]. Preclinical and clinical trials are currently in the process of evaluating outcomes for extrarenal organs.

Ex vivo conditioning of the donor organ could provide another avenue of interest. Currently, the EMPIRIKAL [An Investigation into the Efficacy of Mirococept for Preventing Ischaemia-Reperfusion Injury in the Kidney ALlograft] trial is assessing the efficacy of donor kidney treatment with the complement inhibitor Mirococept (APT070) for the prevention of IRI (ISRCTN49958194). Mirococept is a membrane-localizing complement regulatory protein derived from human complement receptor type 1 (CR1) that inhibits C3 and C5 convertases of both the classical and alternative pathways. Moreover, Mirococept has been identified as a co-factor for the degradation of C3b and C4b. In experimental models, donor treatment with Mirococept was linked to enhanced graft function and survival [79]. Ex vivo tacrolimus treatment of marginal liver grafts is currently being evaluated clinically in the TOP (Tacrolimus Organ Perfusion) study (NCT01564095). Prior to implantation, livers are rinsed with 1,000 mL rinse solution containing 20 ng/mL of tacrolimus. In an experimental liver transplant model, ex vivo treatment with tacrolimus ameliorated IRI possibly through preservation of glutathione homeostasis [80].

Ischemic conditioning is based on inducing a protected state in the donor organ by delivering short periods of ischemia either before (pre-conditioning) [81] or after (post-conditioning) [82] the onset of ischemia. The beneficial effects may also be achieved by using remote conditioning, in which the conditioning stimulus is submitted by inducing periods of ischemia at a remote area such as the upper or lower extremities [83]. The mechanisms of action are not completely understood, but are thought to include preservation of mitochondrial function and inhibition of ROS generation, upregulation of antioxidants, production of protective heat shock proteins, and inhibition of apoptosis [84]. Remote ischemic preconditioning is currently being evaluated in kidney transplantation using a blood pressure cuff on the arms of both donor and recipients 24 h prior to transplantation [REPAIR (Renal Protection Against Ischaemia-Reperfusion in Transplantation) trial; ISRCTN30083294] and on the leg of the recipient right before reperfusion of the kidney (Context trial; NCT01395719).

Current immunosuppressive treatments in transplantation target the adaptive immune system while innate immune responses are getting increasingly recognized as a potential target for immunosuppression. Experimental TLR inhibition has been proven to be effective in ameliorating IRI. A phase I trial was recently published evaluating the safety of a monoclonal antibody against TLR2 [85]. A follow-up phase II trial is currently in progress in renal transplant patients at high risk of delayed graft function (NCT01794663). Another phase I study is currently determining safety, tolerability, and distribution of a monoclonal antibody against TLR4 (NCT01808469). Tolerogenic and immunomodulating capacities of innate immune cells can potentially be exploited with cell-based therapies. A pilot study evaluated the effect of administrating regulatory macrophages (M regs) to two living-donor renal transplant recipients [86]. While adverse effects were absent, patients could be kept on a low-dose

maintenance immunosuppressive regimen. The ONE Study has been established with the aim to test several cell based therapies, including administration of M regs and tolerogenic DCs in kidney transplant recipients [87].

Conclusion

IRI affects all organs utilized for transplantation. Accumulating evidence suggests that organ injury is linked to allo-immune-independent and -dependent immune responses in a complex interplay of innate and adaptive immunity. Pulsatile machine perfusion, ischemic conditioning, and ex vivo pharmacological conditioning are promising optimizations that can limit IRI. Furthermore, directly targeting innate immune responses and exploiting tolerogenic innate immune properties could supplement current immunosuppressive treatments that focus largely on adaptive immunity. These treatment modalities have already shown encouraging results in experimental models and are currently being evaluated clinically. As we are just beginning to comprehend the complex interactions of injury and immune responses in the context of transplantation, developing a more thorough mechanistic understanding may lead to more efficacious future therapies that will ultimately improve transplant outcomes.

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Compliance with Ethics Guidelines

Conflict of Interest Bendix R. Slegtenhorst, Frank J.M.F. Dor, Hector Rodriguez, Floris J. Voskuil, and Stefan G. Tullius declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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