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Susanne Sattler
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The Immunology of Cardiovascular Homeostasis and Pathology

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Preface

Cardiovascular immunology is a newly emerging research area based on the increasingly evident existence of several layers of crosstalk between the cardiovascular and the immune system. Nevertheless, there is still little overlap between research into cardiovascular biology and immunology. However, emerging knowledge is challenging this paradox and forcing communication between the two fields. As a result, we are now approaching a time where the immune system is rapidly being appreciated for its role other than fighting infections, particularly in the cardiovascular sciences.

For this book, we have sought to bring together experts on various aspects of cardiovascular immunology, with the aim of providing an overview of the crosstalk between the cardiovascular and the immune system under homeostasis and during disease. First, we discuss our changing understanding of the immune system and its various roles in physiological processes other than host defence. We then describe the immunological capacities and functions of the most important cardiovascular cell types, including cardiomyocytes, fibroblasts, endothelial cells, pericytes as well as resident macrophages, the most prominent cardiac immune cell population. This is followed by an exploration of areas, in which disturbance of immune regulation and aberrant activation of the immune system is causative in the development of cardiovascular disease including atherosclerosis and cardiac and cardiovascular autoimmunity. We conclude with two chapters on the crucial role of the endogenous innate and adaptive immune system in heart repair and regeneration after tissue damage.

With this comprehensive coverage of state-of-the-art knowledge on the mutual and interdependent link between the cardiovascular and the immune system, we hope to provide a valuable resource for readers with either immunology or cardiovascular background.

London, UK — Susanne Sattler
London, UK — Teresa Kennedy-Lydon

Introduction

Most textbooks still describe the immune system largely in the light of infectious disease. However, we now know that defence against invaders is only one of several roles of the immune system aiming for the maintenance or restoration of tissue integrity. Non-self-recognition and defence against infectious microorganisms even seem to be an evolutionary younger addition to the ancient mechanism of phagocytosis, which is the crucial basis for fundamental physiological processes during development and homeostasis.

As such the immune system cannot be separated from the rest of the body but is an integral part of any organ system or physiological process. To name just a few striking examples, ovulation, mammary gland development, the establishment of a successful pregnancy through fetomaternal tolerance, embryonic development through developmental apoptosis, angiogenesis, bone and brain development and of course wound healing and regeneration of adult tissues are all dependent on a variety of immune effector cells or molecules.

A crucial role of the immune system beyond the control of infectious diseases has also become evident in the cardiovascular system. Immune cells and molecules play critical roles as effectors in cardiovascular health and disease. The heart itself contains a diverse population of tissue-resident immune cells, which are crucial in the continuous maintenance of tissue integrity. Moreover, the vasculature is in intimate contact with immune effectors in the blood and thus particularly susceptible to inflammatory changes. Conversely, parenchymal and stromal cells of the heart and vasculature have a wide range of crucial immunological functions and are active players in shaping immune responses.

Although the field of cardiovascular immunology is still in its infancy, it's becoming increasingly evident that a tightly controlled interplay between the two systems is essential to maintain cardiovascular health. Taking into account the effects on both systems will have potential to significantly improve future therapeutic strategies.

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Part I
The Immune System in Tissue and Organ Homeostasis

Chapter 1
The Role of the Immune System Beyond the Fight Against Infection

Susanne Sattler

1.1 Introduction: Our Changing Understanding of the Immune System

Our current understanding of the immune system varies drastically from the view that prevailed just over 20 years ago. Early observations during infectious diseases lead to a major focus on the immune system's ability to discriminate between self and non-self and defence against pathogenic microorganisms. In its classical definition, the immune system comprises of humoral factors such as complement proteins, as well as immune cells and their products including antibodies, cytokines/chemokines and growth factors. This system of humoral and cellular factors is considered responsible for defending the host from invading pathogens.

However, the roles of immune cells and factors are not limited to host defence, but extend to development, tissue homeostasis and repair (Fig. 1.1). In addition, there are crucial immunological functions played by stromal and mesenchymal cells, which are not commonly considered part of the immune system, such as fibroblasts and endothelial cells. On top of that, it is now also appreciated that the inflammatory status of the environment is important in defining the type of response to any antigen and that the immune system is in fact crucial for the maintenance and restoration of tissue homeostasis in both sterile and infectious situations.

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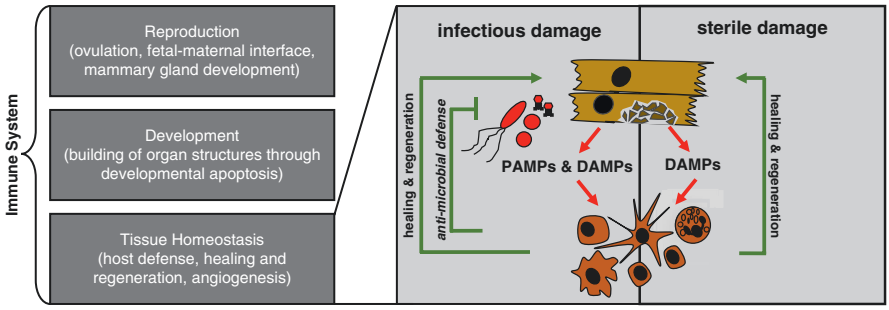

Fig. 1.1 The fundamental roles of the immune system beyond host defence: The immune system is essential for reproduction, development and homeostasis. Sterile tissue damage such as physical trauma or ischemia/reperfusion injury (e.g. myocardial infarct) induces an inflammatory reaction to initiate wound healing and/or regenerative mechanisms. The same basic immunological mechanisms will eliminate microbes if they are present due to injury at a barrier sites (e.g. skin) or primary infectious tissue damage (e.g. viral myocarditis). Necrotic cells in damaged tissue release damage/danger-associated molecular patterns (DAMPs) such as HMGB1, IL-33, ATP, heat-shock proteins, nucleic acids and ECM degradation products. Microbes are recognised by the immune system through their expression of pathogen-associated molecular patterns (PAMPs) such as LPS, flagellin, dsRNA and unmethylated CpG motifs in DNA. *ATP* adenosine triphosphate, *HMGB1* high mobility group box 1, *ECM* extracellular matrix

1.2 A Brief Historical Perspective

What is believed to be the first record of an immunological observation dates from 430 BC. During a plague outbreak in Athens, the Greek historian and general Thucydides noted that people that were lucky enough to recover from the plague did not catch the disease for a second time [1]. The beginnings of modern-day immunology are usually attributed to Louis Pasteur and Robert Koch. Pasteur, in contrast to common belief at the time, suggested that disease was caused by germs [2], and Robert Koch confirmed this concept in 1891 with his postulates and proofs, for which he received the Nobel Prize in Physiology or Medicine in 1905 [3, 4]. These very early observations were fundamental for the first identification and early characterisation of the immune system but also skewed all subsequent definitions towards a defence machinery against invading microorganisms.

1.2.1 The Traditional View of Immunity: Evolution to Protect from Infectious Microorganisms

The immune system has long been considered to have evolved primarily because it provided host protection from infectious microorganisms and correspondingly a survival advantage. Genes of the immune system have been suggested to

be under particularly high evolutionary pressure due to the need to prevent pathogenic microorganisms from harming the host. Hosts are therefore under selective pressure to resist pathogens, whereas pathogens are selected to overcome increasing host defences [5]. This process of a stepwise increase in resistance by the host and subsequent mechanisms for evasion by the pathogen is the basis for a well-established co-evolutionary dynamics, the 'host–pathogen arms race' [6].

In 1989, Charles Janeway proposed his 'Pattern Recognition Theory' [7], which still provides the conceptual framework for our current understanding of innate immune recognition and its role in the activation of adaptive immunity. Janeway proposed the existence of an evolutionary conserved first line of defence consisting of antigen-presenting cells equipped with pattern recognition receptors (PRR) which recognise common patterns found on microorganisms, which are different and thus distinguishable from those of host cells. These innate immune cells take up foreign antigens, present them to adaptive immune cells and thus determine the following adaptive immune response. Janeway's model also suggested that the innate immune system evolved to discriminate infectious non-self from non-infectious self as microbial patterns were not present on host tissues [8]. A few years later, the first family of pattern recognition receptors, the Toll-like receptors (TLRs), were indeed discovered [9]. Notably, Toll-like receptors (TLRs) are also one of several striking examples of convergent evolution in the immune system [10]. TLRs are used for innate immune recognition in both insects and vertebrates. The ancient common ancestor, a receptor gene with function during developmental patterning, subsequently evolved a secondary function in host defence. This happened independently in insects and vertebrates after the vertebrate and invertebrate lineage had separated [11].

All this seemed to strongly support the concept that the primary role of the immune system is to defend against potentially infectious microorganisms.

1.2.2 The Danger View of Immunity: Evolution to Protect from Endogenous Danger

Charles Janeway's model is still considered largely correct today, although too simplistic as it fails to explain certain aspects of immunity including sterile immune responses in the absence of infectious agents as well as the unresponsiveness to a variety of non-self-stimuli such as dietary antigens and commensal microorganisms. In 1994, Polly Matzinger proposed the 'Danger Hypothesis' [12]. Her model, again on purely theoretical grounds, suggested that the primary driving force of the immune system is the need to detect and protect against danger as equivalent to tissue injury. Importantly, in the same year, a group of scientists working on kidney transplantation discussed the possibility that in addition to its foreignness, it was the injury to an allograft which ultimately caused an

immune response and rejection [13]. Activation of innate immune events by injury-induced exposure of normally hidden endogenous molecules has since been demonstrated countless times [14, 15]. Examples for such endogenous molecules include nucleic acids [16], heat-shock proteins [17], cytoskeletal proteins [18], HMBG-1 [19], SAP130 [20], IL-33 [21] and IL-1a [22]. In addition to proteins that are normally hidden from detection by the immune system, there are small molecules released as a result of endogenous stress including high glucose [23], cholesterol [24] and ATP [25]. All these agents have been shown to contribute to sterile inflammatory responses and have been termed damage/danger-associated molecular patterns (DAMPs).

Thus, an inflammatory environment caused by tissue injury (danger hypothesis) alerts the immune system and is the prerequisite to an adaptive immune response (self versus non-self pattern recognition hypothesis).

1.2.3 The Integrative View of Immunity: Evolution as a System to Establish and Maintain Tissue Homeostasis

Considering the crucial importance of the innate immune response to tissue injury to initiate tissue repair processes and mount an effective adaptive response, the question arises if the early evolution of the immune system may have been driven by the need to maintain tissue homeostasis and the ability to deal with tissue injury rather than infection. Strikingly, the Russian developmental zoologist Ilya Metchnikoff discovered phagocytosis in echinoderms at the end of the nineteenth century and proposed the phagocyte and innate immunity as the centre of the immune response. Metchnikoff's already developed a concept of immunity as a summary of all those activities that defined organismal identity and which regarded host defence mechanisms as only subordinate to this primary function [26]. The evolutionary development of the process of phagocytosis provides a very strong argument for the immune system being more than just a defence mechanism. Evolutionary old organisms, such as amoeba, already use this ancient mechanism, albeit mainly for feeding [27, 28]. In multicellular organisms, phagocytosis is first used during embryogenesis for the removal of dying cells and the recycling of their molecules. In adults, phagocytosis continues to play a crucial role during tissue remodelling [29, 30]. Only the evolutionary appearance of the major histocompatibility complex (MHC) locus in jawed fish seems to have allowed the phagosomes to play a role in the establishment of adaptive immunity [31].

Decades of research using ever more sophisticated technologies allow the conclusion that defence against 'non-self' is only one of many layers of how the immune system protects us from disease. This is most evident in the evolutionary ancient mechanism of phagocytosis, which is still the most fundamental basis for tissue development, homeostasis and repair.

1.3 Functions of Immune Cells Beyond Host Defence

In this section, examples of non-defence functions of classical immune cells during reproduction, embryonic development, angiogenesis and post-injury repair and regeneration will be discussed.

1.3.1 Reproduction

The immune system plays a crucial role in reproduction both before and during pregnancy, and leucocytes are found in male and female reproductive tissues [32–34]. Several classical inflammatory mediators participate in the process of ovulation. Granulocytes, macrophages and T lymphocytes migrate to the ovulation site and are activated locally, suggesting an active role of leucocytes in the tissue remodelling which occurs during ovulation [35]. Mice deficient of the major macrophage growth factor, colony-stimulating factor-1 (CSF-1), show severe fertility defects, as CSF-1 is involved in feto-maternal interactions during pregnancy and has a crucial role in the development of the mammary gland [36–39]. Eotaxin, a major chemokine for local recruitment of eosinophils into tissue, also contributes to mammary gland development [40, 41].

Establishment and maintenance of feto-maternal tolerance during pregnancy has intrigued immunologists for a long time, and to date a set of anatomical, cellular and molecular regulatory mechanisms that protect the fetus from immune-mediated rejection has been uncovered [42]. The feto-maternal interface is an immunologically highly dynamic site rich in cytokines and hormones [43, 44]. During the first few weeks after fertilisation, interstitial and endovascular infiltration of trophoblast cells leads to the recruitment of maternal immune cells and the production of pro-inflammatory cytokines [45]. Maternal immune responses have been proposed to protect from trophoblast over-invasion while allowing for the acceptance of the semi-allogeneic fetal–placental unit. 40% of cells in the decidua during the first trimester are $CD45^+$ leucocytes. 50–60% of decidual leucocytes are a unique type of natural killer (NK) cells which is not present outside the context of pregnancy and has crucial trophic function by helping to remodel the spiral arterioles of the uterus that supply the placenta with blood [46]. Failure to sufficiently remodel these vessels leads to inadequate placental perfusion, intrauterine growth restriction and pre-eclampsia, two important obstetric complications [47]. The remaining leucocytic infiltrates are roughly 10% T lymphocytes, 1–2% dendritic cells (DCs) and 20–25% decidual macrophages [48]. The decidual macrophage population are subdivided into a $CD11c^{high}$ and $CD11c^{low}$ population, which are responsible for antigen processing and presentation. Depending on the macrophage subset, antigen presentation leads to either an induction of maternal immune cell tolerance to fetal antigens ($CD11c^{high}$) or homeostatic functions including the clearance of apoptotic cells during placental construction ($CD11c^{low}$) [49, 50]. Thus, besides being a potential threat to the developing fetus due to allorecognition of foetal antigens, decidual leucocytes play a crucial role in the development of the fetal–placental unit [51].

1.3.2 Development

Macrophages both initiate and respond to developmental apoptosis [52, 53]. Notably however, and a major sign of the fundamental role of the phagocytic process, non-immune cells are able to take over phagocytosis if necessary. In mice lacking macrophages due to a deficiency for the hemopoietic-lineage-specific transcription factor PU.1, the task of developmental phagocytosis is taken over by mesenchymal cells, although they are significantly less efficient than professional macrophages in recognition, engulfment and degradation of apoptotic debris [54]. Comparable roles of macrophages in developmental apoptosis have been reported in evolutionary older vertebrate species and insects. In the frog *Xenopus laevis*, macrophage phagocytosis is involved in programmed cell death of tail and body muscle during metamorphosis [55]. In the *Drosophila* embryo, the development of the tracheal system is created through migration, rearrangement and elimination of cells, which are engulfed and removed by macrophages [56].

Bone Development Bone osteoclasts are multinucleated cells that resorb bone material during development and form by fusion of mononuclear precursors of the monocyte/macrophage lineage. CSF-1 is an important factor involved in osteoclast differentiation [57]. The toothless (tl) mutation in the rat is a naturally occurring, autosomal recessive mutation in the *Csf1* gene and causes severely reduced numbers of macrophages and a profound deficiency of bone-resorbing osteoclasts and peritoneal macrophages. This results in severe osteopetrosis, with a highly sclerotic skeleton, lack of marrow spaces and failure of tooth eruption [58]. Administration of CSF-1 can correct these defects demonstrating the crucial importance of macrophages in bone development [59].

Brain Development Brain microglia are highly motile phagocytic cells that infiltrate and take up residence in the developing brain, where they are thought to provide surveillance and scavenging function [60]. They assist during embryonic development by mediating induced cell death of neurons [61]. Both CSF-1 and its receptor are expressed in the developing mouse brain, and CSF-1 deficiency induces neurological abnormalities [62]. During postnatal brain development, microglia actively engulf synaptic material and play a major role in synaptic pruning [63]. They can remove entire dendritic structures after depletion of appropriate inputs, a process termed synaptic stripping. They accumulate, through signalling mediated by the chemokine receptor CXCR3, at the lesion site, and dendritic structures are removed within a few days [64, 65]. Microglia cells may also be a source of other brain cells, as isolated microglia cells in culture have the potential to generate neurons, astrocytes and oligodendrocytes [66, 67]. Microglia also release factors that influence adult neurogenesis and glial development [68, 69]. They secrete neurotrophins of the nerve growth factor (NGF) family, suggesting that they promote development and normal function of neurons and glia [70] and have autocrine function on microglial proliferation and phagocytic activity in vitro [71].

1.3.3 Angiogenesis

The formation of blood vessels is essential for tissue development and tissue homeostasis in all vertebrates. Monocytes and macrophages are known to be involved in the formation of new blood vessels and are involved in all phases of the angiogenic process. They are capable of secreting a vast repertoire of angiogenic effector molecules, including matrix-remodelling proteases, pro-angiogenic growth factors (VEGF/VPF, bFGF, GM-CSF, TGF-α, IGF-I, PDGF, TGF-β) and cytokines (IL-1, IL-6, IL-8, TNF-α, substance P, prostaglandins, interferons, thrombospondin 1) [72]. The expansion of the blood vessel network during angiogenesis starts with sprouting and is followed by anastomosis. Vessel sprouting is induced by a chemotactic gradient of the vascular endothelial growth factor (VEGF), which stimulates tip cell protrusion to initiate vessel growth [73]. Macrophages are crucial for the fusion of tip cells to add new circuits to the existing vessel network by physically bridging and guiding neighbouring tip cells until they are fused [74].

1.3.4 Tissue Homeostasis, Regeneration and Repair

The immune system is crucial in wound healing and regeneration after tissue damage. There is a wealth of information available about the involvement of immune cells in the repair of all major organs including the skin [75, 76], skeletal and heart muscle [77–82], kidney [83, 84], liver [85], brain [86, 87] and gut [88]. If damage to blood vessels is involved, the activated coagulation system initiates the first stages of healing with the release of chemical mediators that promote vascular permeability and leucocyte adhesion and recruitment. Coagulation activates platelets which produce growth factors such as transforming growth factor-β (TGF-β) and platelet-derived growth factor (PDGF), which activate fibroblasts and act as chemoattractants for leucocytes [89]. However, even without activation of the coagulation cascade, alarmins released from necrotic cells recruit leucocytes. Infiltrating neutrophils and macrophages remove dead cells and secrete chemokines and cytokines, including tumour necrosis factor (TNF) and interleukin-1 (IL-1), which further upregulate leucocyte adhesion molecules to increase immune cell recruitment and induce the production of additional growth factors and proteases such as matrix metalloproteases. Matrix metalloproteases degrade the extracellular matrix which allows for tissue remodelling. Fibroblast growth factor (FGF), PDGF, prostaglandins and thrombospondin-1 promote new blood vessel growth, fibroblast proliferation and collagen deposition. Tissue remodelling is accompanied by parenchymal regeneration or regrowth of the epithelial cell layer with resolution of the healing process [90].

Recently, several innate-type lymphoid cell (iLC) subsets have been identified and characterised that seem to play a particularly important role in sterile inflammatory settings. These cell types include lymphoid tissue-inducer cells (LTi), innate type 2 helper cells and γδ T lymphocytes [91]. They rapidly express effector cytokines that

are commonly associated with adaptive T-helper lymphocyte responses such as IL-17, IL-13, IL-4 and IL-22 production [92, 93]. Their role in wound healing and regeneration is strongly mediated by the cytokines they produce. LTi cells play a central role in promoting appropriate thymic regeneration in sterile inflammatory settings, an effect which is mediated largely through the cytokine IL-22 which promotes epithelial repair and tissue regeneration [94]. Further, the endogenous alarmin IL-33 has profound effects on innate type 2 helper cells and thereby plays a central role in driving type 2 immunity under sterile and infectious settings [95, 96]. Tissue repair processes following injury are dominated by type 2 immune cells producing cytokines such as IL-4, IL-5, IL-10 and IL-13. Many type 2 processes promote the 'walling off' of large invaders through granuloma formation and matrix deposition, which are the same mechanisms employed to close open wounds [97]. Shifting the inflammatory type 1 response towards a type 2 response is beneficial for quick wound healing, which likely was the evolutionary most cost-effective approach to deal with large parasites or insect bites, although this may come at the cost of fibrotic repair and long-term loss of tissue functionality [80, 98]. Intense research efforts in the field of regenerative medicine are trying to find the right balance between pro-inflammatory and reparative immune responses to prevent scarring and fibrotic repair and boost regenerative healing instead.

1.4 Concluding Remarks

Both evolutionary development and functional variety in current day organisms strongly support a notion of the immune system as an all-encompassing machinery to ensure system integrity. Protection from disease caused by invading pathogenic microorganisms is, although the most easily observed, only one manifestation of the workings of this machinery. Instead, the immune system is essential for development, surveillance, protection and regulation to maintain or if necessary re-establish homeostasis.

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Part II
Immune Functions and Properties of Resident Cells in the Heart and Cardiovascular System

Chapter 2
Paying for the Tolls: The High Cost of the Innate Immune System for the Cardiac Myocyte

Anne A. Knowlton

The cardiac myocyte, which continuously contracts and relaxes to deliver blood throughout the body, differs markedly from the specialized cells of the immune system. The adaptive, or acquired, immune system with the production of distinct antibodies in response to specific threats was long considered the mainstay of protection against infection; however, the production of antibodies and the full immune response against a threat takes 4–7 days, a long period for an infectious agent to propagate without response. Janeway hypothesized the existence of a simpler, less specific, but more rapid immune response, which he termed innate immunity [1]. In contrast to the specialized immune system found in advanced organisms, innate immunity is widely expressed and found in both more primitive life forms and in humans. Furthermore, the innate immune response and its receptors are found in cell types and tissues that were long viewed as non-immunologic. The persistence of innate immunity is essential for rapid protection against infections, given the long time needed to produce antibodies, but the flip side is that inadvertent activation of innate immunity by proteins, RNA, and other endogenous ligands can lead to cell and tissue inflammation/damage. Unfortunately a number of essential and otherwise innocuous molecules activate different TLRs leading to an inflammatory response, which can be deleterious leading to myocyte death/injury and to organ dysfunction. Predominantly TLR4 has been shown to have a significant role in cardiac injury, with other TLRs including TLR2, having lesser roles [2–7]. In this chapter we will focus on TLRs and the cardiac myocyte. Subsequent chapters will address other aspects immunity and the heart.

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Innate Immunity and TLRs Innate immunity includes epithelial barriers to invading organisms; phagocytic cells, such as macrophages and dendritic cells; the complement system; and the TLRs. The Toll receptor was first identified as an essential receptor for dorsal ventral patterning in the embryonic *Drosophila*, but subsequent work has demonstrated that it has a role in defending against fungal infections in the adult Drosophila [8, 9]. Ten TLRs have been identified in humans and 13 in mice (Table 2.1). TLRs 1–10 are expressed in humans and 1–13 in mice, but TLR10 is inactive in the mouse. TLRs 1, 2, 4, 5, 6, 10, 11, 12, and 13 are expressed on the cell surface, and TLRs 3, 7, 8, and 9 localize to the membranes of intracellular organelles, including endosomes and the endoplasmic reticulum. TLRs recognize pathogen-associated molecular patterns (PAMPs) and alarmins, which are endogenous molecules that signal cell and tissue damage and lead to enhanced injury and self-damage. Lipopolysaccharides (LPS) and flagellin are examples of PAMPS, while alarmins include HMGB1 and heat shock proteins (HSPs), which are an endogenous, protective response. PAMPs and alarmins are both types of DAMPs (damage-associated molecular patterns), which in cardiac myocytes includes proteins released after ischemia/reperfusion injury. Key ligands for the TLRs are summarized in Table 2.1.

Heat Shock Proteins (HSPs) Heat shock proteins are well known as protective proteins that make cells resistant to stress-induced cell damage [35–38]. Among the HSPs, HSP60 is highly conserved intracellular protein that is expressed both constitutively and under stress conditions and that serves as a molecular chaperone to facilitate mitochondrial protein folding [39–41]. Although the HSPs are protective and the endogenous increase in HSPs in response to injury reduces cell damage, they can lead to inflammation and even to apoptosis, in other words, a paradoxical deleterious response [42, 43]. HSP70, which has four isoforms, including the

Table 2.1 TLRs in human and mouse

TLR	Ligand	
TLR1	Triacyl lipopeptides	[10]
TLR2	Lipoprotein, lipopeptides, atypical LPS, HSP70	[11–13]
TLR3	Double-stranded RNA	[14, 15]
TLR4	LPS, HMGB1, HSP60, HSP70?	[2, 11, 16–20]
TLR5	Flagellin	[21]
TLR6	Diacyl lipopeptides, lipoteichoic acid	[22, 23]
TLR7	Single-stranded RNA Imidazoquinoline compounds imiquimod and R-848	[24–26]
TLR8	Single-stranded RNA	[25]
TLR9	CpG DNA	[27]
TLR10	Negative regulator of MYD88-dependent and MYD88-independent signaling	[28, 29]
TLR11	Profilin, flagellin	[30] Mouse, not humans
TLR12	Profilin	[31]
TLR13	Bacterial 23 s ribosomal RNA	[32]

Humans have TLR1–10. Mice have TLR1–9 plus TLR11–13. TLR10 in the mouse is nonfunctional as it is disrupted by retroviral insertions, but the rat has been found to have the complete TLR10 sequence [33, 34]

constitutive (HSC)70, an inducible HSP70 (HSP72) and a mitochondrial HSP70, is the most ubiquitous and protective of the HSPs having many cellular functions including folding proteins, targeting irreversibly denatured proteins for degradation, and binding newly synthesized peptides at the ribosome, so that they do not interact with the abundant proteins in the surrounding cytosol [44]. HSP60 is primarily a mitochondrial protein, where it is critical in combination with HSP10, with which it forms a barrel, for folding of proteins imported into the mitochondria. HSPs have been considered to be intracellular proteins; however, HSPs have been found in blood samples at levels of 1–100 μg/ml, and this is a problem [45–47].

Extracellular Heat Shock Proteins 60 and 72 as Mediators of Injury Intracellular HSPs are protective proteins with many key functions that maintain cellular functions, remove or refold denatured proteins, and protect the cell when exposed to a wide range of injuries [38, 48–52]. However, when HSP60 and HSP72 are extracellular, they can be injurious with one mechanism being activation of TLR4 and TLR2, respectively (Fig. 2.1), resulting in the activation of NFκB and production of cytokines, including TNFα [16, 53]. Antibodies to HSP60 can pull down TLR4 from isolated the membrane fraction of cardiac myocytes after 30 min of incubation

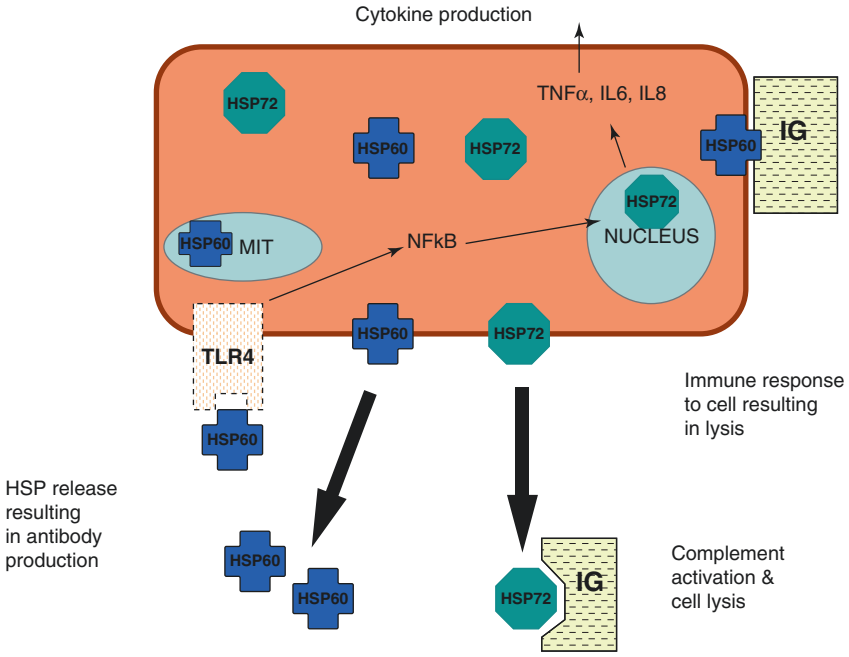

Fig. 2.1 Endogenous ligands and the immune system. Heat shock proteins, although normally protective, can produce injury through several pathways as shown. Both HSP60 and HSP72 have been found in the plasma membrane with injury/stress. Both can be released from the cell, and both have been found in human serum. HSP60 and HSP72 can bind to TLR4/2, respectively, and activate NFκB and cytokine production as shown. Both can elicit antibody response and activation of complement, and both can potentially lead to cell destruction when expressed on cell membrane. Both can also bind to TLRs on monocytes/macrophages leading to a greater inflammatory response

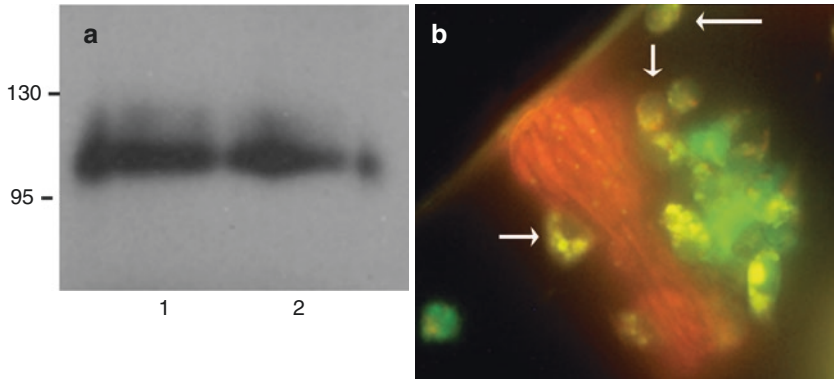

Fig. 2.2 (**a**) Anti-HSP60 immunoprecipitates TLR4 from adult cardiac myocyte plasma membrane fraction. Adult cardiac myocytes were treated with low-endotoxin HSP60 for 30 min at 4 °C, cross-linked, and then fractionated. The plasma membrane fraction was immunoprecipitated with anti-HSP60 and processed for western blotting and developed with anti-TLR4 antibody. Two different immunoprecipitations are shown. (**b**) Monocytes/lymphocytes isolated from the blood were labeled with FITC and added to adult rat cardiac myocytes, labeled with Texas *Red*, and treated with fibronectin. The right pointing *arrow* at the *bottom* left points to a monocyte and the two *arrows* at the *top* identify two lymphocytes based on nuclei characteristics. The monocytes are far smaller than the cardiac myocyte, and any attempt to ingest the larger myocyte can lead to the release of pro-inflammatory factors

with HSP60 at 4 °C (Fig. 2.2a). HSP72 has been reported to bind TLR4, but we have not found this to be the case in cardiac myocytes [2, 54, 55]. Both HSP60 and HSP72 are present in the serum and plasma of humans, even though both are intracellular proteins, and neither are known to be secreted nor to have an extracellular function [45]. HSP60 was present in the serum of diabetics at 6.9 ± 1.9 ng/ml, and similar levels were found in the serum of trauma patients [56, 57]. In contrast, plasma levels of HSP60 were1 μg/ml or more in 26% of diabetics and 10 μg/ml or more in 7% of diabetics [58]. Similarly 20% of British civil servants had HSP60 plasma levels of 1 μg/ml or more [45]. Anti-HSP60 antibodies were present in the serum of diabetics at titers of 1:100 and 1:250 in 76.5% and 58.8%, respectively [56]. We have reported HSP60 and HSP72 are released in exosomes by cardiac myocytes in the absence of necrosis, and these exosomes are quite stable, not releasing HSP60 under pathophysiologic or physiologic conditions [59, 60]. Whether HSP60 in serum and plasma samples is always present in exosomes is not clear. Many studies of serum/plasma HSPs have used blood samples, which have been stored at −80 °C before analyzing. Freeze/thaw will rupture lipid bilayers, and this would be expected to occur with exosomes. If safely encased in exosomes, then HSP60 and 70 would be unable to bind TLR4 and TLR2.

There are other mechanisms by which the HSPs can activate the immune system. Antibodies to HSPs have been found in human serum, most often to HSP60, but antibodies for HSP72, HSP90, and other HSPs have been reported [61, 62]. 34.4% of patients with *Helicobacter* (*H.*) pylori had antibodies to HSP60 at a 1:1000 titer compared to 0% in *H. pylori*-negative controls ($p < 0.001$), and the same difference was seen for anti-HSP72 antibodies in these patients ($p < 0.001$) [61]. HSP72 alone can activate complement, another component of the innate immune system (Fig. 2.1), and HSP60 complexed with antibody can do the same [63, 64].