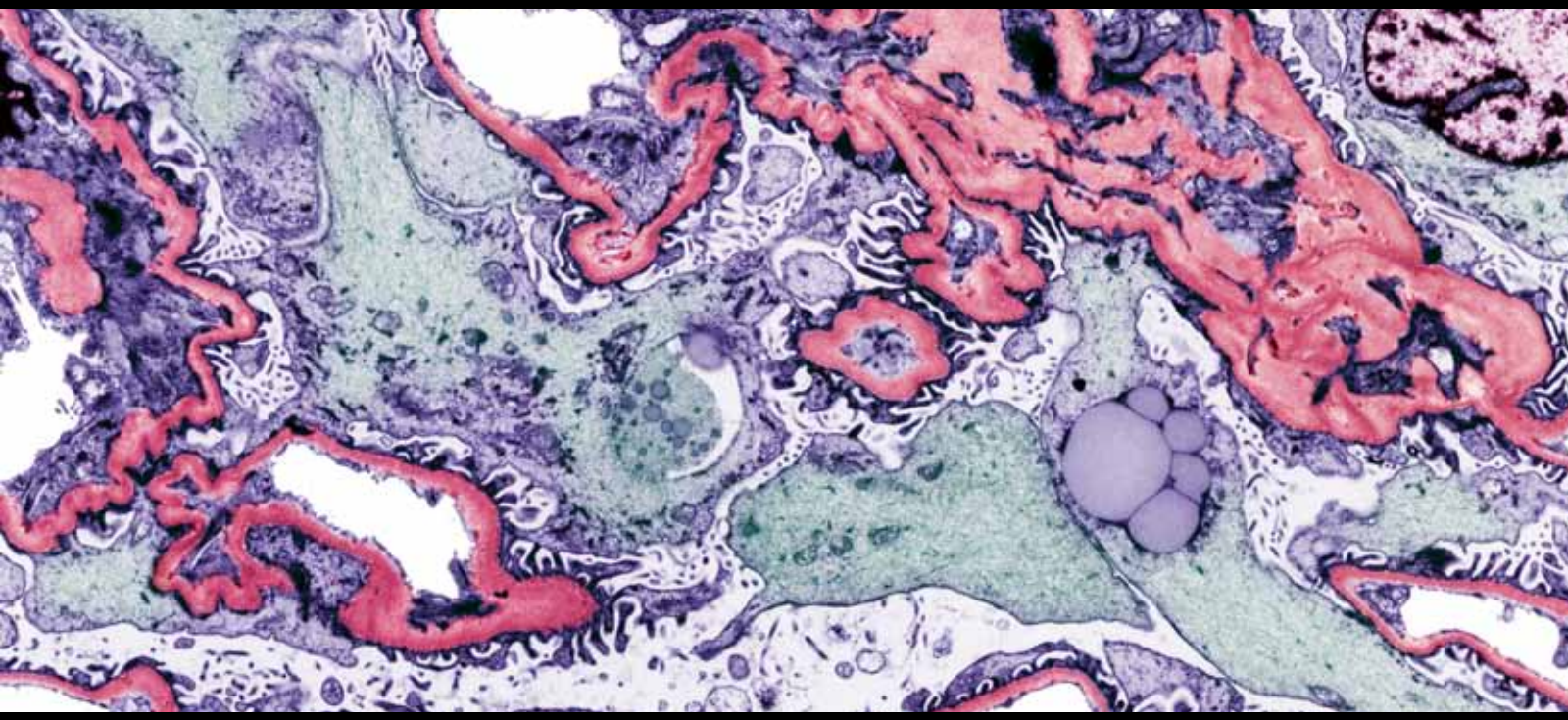
Heat-Shock Proteins in Autoimmunity

Guest Editors: Kamal D. Moudgil, Stephen J. Thompson, Fabiana Geraci, Boel De Paepe, and Yehuda Shoenfeld



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Autoimmune Diseases

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Editorial Board

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Editorial

Heat-Shock Proteins in Autoimmunity

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Heat shock proteins (HSPs), also known as "stress proteins," are among the highly conserved and immunogenic proteins shared among diverse groups of microbial agents and mammals [1]. Heat and other types of stressful stimuli can increase the cellular expression of HSPs. These proteins have been categorized into different families according to their molecular mass, for example, HSP110, HSP90, HSP70, HSP60, HSP40, HSP20-30, and HSP10 [1–3]. For uniformity, guidelines for the nomenclature of various human HSP families have been proposed [4]. Under physiological conditions, the ubiquitously distributed HSPs maintain the integrity and function of other cellular proteins in stressful conditions. However, HSPs also can become targets of immune response, resulting in immune pathology and clinical manifestations of autoimmunity. HSPs have been implicated in the pathogenesis of a variety of autoimmune diseases [3, 5–7]. Moreover, the involvement of other small HSPs (e.g., H11/HspB8) besides the canonical members of that family in health and disease is increasingly being realized. Importantly, HSPs are also capable of inducing immune responses that are immunoregulatory in nature [7–10], including resolution of the inflammatory responses [11]. Also, the concept of immune network (immunological homunculus) including HSPs as one of its components has been proposed to explain immune homeostasis in health and disease [12].

The main focus of this special issue is on the role of different HSPs in the pathogenesis of autoimmunity through induction/propagation as well as regulation of the disease-related processes; on the impact of physiological and disease-related metabolic processes on the induction of HSPs; on the mechanistic basis of the effector functions driven by HSPs; and on the immunomodulatory role of HSPs. Thirteen excellent papers describing new original results and the most recent developments in the field on these topics are presented in this special issue. These papers cover the role of HSPs in antigen cross-presentation, induction of autoimmunity, and immunotherapy of autoimmunity/cancer (R. Binder et al., Y. Kato et al., and S. Calderwood et al.); the role of HSPs in the pathogenesis of the autoimmune components of diverse diseases including atherosclerosis (A. Kilic and K. Mandal); systemic lupus erythematosus (H. Shukla and P. Pitha); Behcet's disease (J. Shimizu et al.), uveitis (A. Commodaro et al.), and diabetes (C. Blasi et al.); the relationship between infection, particularly *M. paratuberculosis*, and autoimmunity (C. Dow); the role of small heat-shock protein H11/HspB8 and its homologous proteins in human disease (L. Aurelian et al.); the impact of exercise and metabolic disorders on HSPs (E. Noble and G. Shen); the immunosuppressive activity of HSP70 (P. Stocki and A. Dickinson); and HSP-induced regulatory T cells and their role in control of autoimmunity (E. Brenu et al.). These papers highlight results obtained from studies in animal models as well as patients with autoimmune or metabolic disorders.

HSPs are highly conserved in nature, and they are also quite immunogenic. These attributes may render these proteins as initiators of immune response as well as targets of autoimmune attack. Foreign (e.g., microbial) HSPs may prime cellular or humoral immune responses that might

be cross-reactive with the corresponding self-HSPs or other self-antigens leading to the induction of autoimmunity. "Molecular mimicry" and unveiling of the previously cryptic (hidden) determinants are among the different mechanisms involved in the induction of autoimmunity [3]. Interestingly, foreign-self mimicry may not always be pathogenic; instead, it may be immunoregulatory in nature [13, 14]. Further, defined pathogenic versus regulatory T- or B-cell epitopes have been identified within the same HSP, for example HSP60/65. Accordingly, during the course of autoimmunity, epitope spreading or diversification of response can lead to induction of new T-cell/antibody responses, which in turn may aggravate or downmodulate autoimmunity depending on the antigenic epitopes targeted in the process. Several papers in this special issue discuss the pathogenic role of HSP-induced immunity. The role of particular HSPs in specific autoimmune disorders is highlighted in the papers on atherosclerosis (A. Kilic and K. Mandal), uveitis (A. Commodaro), lupus (H. Shukla and P. Pitha), and Behcet's disease (J. Shimizu et al.). A viewpoint on the role of *M. paratuberculosis* HSP65 in the induction of autoimmunity via molecular mimicry is presented by C. Dow. Two papers in this issue have addressed the relationship between HSPs and metabolic disorders with components of autoimmunity (E. Noble and G. Shen; C. Blasi et al.).

HSPs are known to play critical roles in innate immunity as well as adaptive immunity. HSPs can activate specific toll-like receptors (TLRs) and influence activation of antigen-presenting cells (APC) and thereby other immune cells including T cells and B cells. These proteins also play a major role in cross-presentation of extracellular antigens such as microbial antigens or tumor antigens in parenchymal cells via the class I pathway resulting in induction of CD8+ cytotoxic T-lymphocyte (CTL) responses [15, 16]. Cross-presentation of HSP-mediated presentation of self-antigens might also play a role in the pathogenesis of autoimmunity. The mechanistic basis of the role of HSPs in cross-presentation of antigens and the impact of this process on immune response in cancer and autoimmunity are elaborated in this issue in papers by S. Calderwood et al., R. Binder et al., and Y. Kato et al.

HSPs are induced by a variety of stressful stimuli, and they aid in controlling the physical and metabolic integrity of the cells under stress. Metabolic disorders and exercise can induce HSPs and activate other heat-shock factor-1- (HSF-1-) mediated effector pathways. These in turn can enhance the generation of mediators of inflammation. E. Noble and G. Shen discuss in this issue the above-mentioned associations, including their dual role in inflammation. However, C. Blasi et al. report that an effective control of type 2 diabetes was not accompanied by a reduction in serum levels of HSP60 and antibodies to HSP60, while it lowered the levels of the proinflammatory cytokine IL-6.

As elaborated above, HSPs are involved in the induction as well as regulation of immune responses. How the same proteins can mediate opposite outcomes is a dilemma for both basic researchers and physician investigators. This dual role of HSPs has been revealed in a wide variety of disorders, including autoimmune diseases and tumors, as well as in immune responses associated with organ transplantation [7, 8, 10, 13, 15, 17, 18]. It is becoming clear that the pro- versus anti-inflammatory activities of HSPs are contextual and affected by multiple factors including the concentration of HSP, the timing of exposure to HSP, and the overall physiopathological milieu at the target site. Another challenge regarding HSPs and immune responses lies in the fact that autoimmunity and tumors present with opposite requirements for control of the disease processes [2, 17]. Autoimmunity involves a breakdown of self-tolerance and induction of anti-self immune responses. Accordingly, an effective control of autoimmunity requires suppression of autoreactive immune responses. On the contrary, tumors survive and grow in the body owing to an ineffective antitumor response in part because of immune-evasive strategies adopted by tumor cells. A deliberate induction of potent antitumor immunity is required to control cancer. HSPs are being exploited differentially to control autoimmunity and cancer. The disparate roles of HSPs in tumor immunity and autoimmunity and the molecular and cellular mechanisms involved therein are discussed here by S. Calderwood et al., R. Binder et al., and L. Aurelian et al.

HSPs may facilitate regulation of effector responses under appropriate conditions [3, 7–9]. This can be achieved in part via increasing the production of anti-inflammatory cytokines so as to deviate the cytokine balance from a proinflammatory to an anti-inflammatory type. In addition, HSPs may induce different types of regulatory T cells, including CD4+ Tr1 cells that secrete interleukin-10 (IL-10) and CD4+CD25+ Forkhead-box-P3- (Foxp3-) expressing Treg that produce transforming growth factor-b (TGF-β) and IL-10. Treg may also mediate their effect via cell-to-cell contact with the target cells. In this issue, the immunosuppressive activity of HSP is outlined by P. Stocki and A. Dickinson, while HSP-induced regulatory cells are described by E. Brenu et al.

HSPs are being exploited for immunotherapy of autoimmune diseases and cancer [2, 15, 19, 20]. The current approaches under development or those being tested in clinical trials harness the immunoregulatory properties of HSPs. For example, purified HSPs or their peptides containing defined epitopes are being tested in clinical trials in type 1 diabetes [19] and rheumatoid arthritis [20] patients with the hope of developing immunotherapeutic approaches for these debilitating diseases. The use of HSP-induced regulatory T cells is an emerging area of potential promise in this regard [9].

We hope you enjoy reading the diverse collection of outstanding papers in this special issue.

Acknowledgments

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Review Article

Heat Shock Proteins and Regulatory T Cells

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Heat shock proteins (HSPs) are important molecules required for ideal protein function. Extensive research on the functional properties of HSPs indicates that HSPs may be implicated in a wide range of physiological functions including immune function. In the immune system, HSPs are involved in cell proliferation, differentiation, cytokine release, and apoptosis. Therefore, the ability of the immune system, in particular immune cells, to function optimally and in unison with other physiological systems is in part dependent on signaling transduction processes, including bidirectional communication with HSPs. Regulatory T cells (Tregs) are important T cells with suppressive functions and impairments in their function have been associated with a number of autoimmune disorders. The purpose of this paper is to examine the relationship between HSPs and Tregs. The interrelationship between cells and proteins may be important in cellular functions necessary for cell survival and expansion during diseased state.

1. Introduction

Optimal cellular function is regulated by several molecules including heat shock proteins (HSPs). These proteins have chaperone properties and are important in both stressed and unstressed cells. HSPs can be categorized into six diverse highly or less-conserved families. These include HSP10, HSP40, HSP60, HSP70, HSP90, and HSP100 [1–4]. HSP60 is found in the mitochondria [5]. HSP70 is implicated in protein transport assembly and synthesis. It has anti-apoptotic properties that are implicated in intrinsic and extrinsic apoptotic pathways. HSP70 interacts with the mitochondria through death receptor signaling where it binds to death receptors DR4 and DR5 impeding TNF-related apoptosis inducing ligand (TRAIL) [6]. Importantly, HSP70 can bind to either the unphosphorylated C terminus of protein kinase C or Akt prompting rephosphorylation and kinase stabilization [7]. ATP-dependent HSP90 regulates cell survival by stabilizing kinases such as Akt and suppressing apoptosis by inhibiting caspases [8, 9]. The exact structural domains for all human HSPs remains to be determined; however, HSP70 and HSP90 have been well characterized. HSP70 is comprised of an N-terminal nucleotide-binding domain with ATPase activity and a C terminal containing a substrate-binding domain [10–12]. HSP90 on the other hand has three characterized structural domains including an N-terminal nucleotide-binding domain, a middle segment and a C terminus [13–16]. Interdomain interactions occur by a conserved linker [17].

HSPs are found in intracellular and extracellular spaces as well as in the circulation. Intracellular HSPs including HSP27, HSP70, and HSP90 have direct roles in preventing protein aggregation, induction of cell death pathways, cellular rescue and maintaining receptor interactions [18]. These HSPs may be overexpressed in most cancers promoting the growth and survival of tumor cells. The downregulation of their expression results in a substantial decrease in tumor cells [3, 19–21]. Extracellular HSPs, HSP70, HSP90, and gp96 are peptide carriers, inducers of cytokines, and stimulants for immune cells during stress [18]. These HSPs may be either membrane bound to the plasma membrane or released

into the circulation [9]. In general, there are two sources of extracellular HSPs, pathogen- and human-derived HSPs. Pathogen derived HSPs are found in the extracellular space as a consequence of infection while human-derived HSPs are released into the extracellular space in the event of intracellular traumas such as apoptosis or necrosis [8]. Extracellular HSPs may be attached to the plasma membrane. Additionally, extracellular HSPs induce the maturation of dendritic cells and present peptide molecules to antigen-presenting cells (APCs) thus, linking the innate immune and adaptive immune systems. Cell-free or circulating HSPs, for instance, HSP70, are released into the circulation by glial cells, B cells, PBMCs, or following necrosis [22–25]. These HSPs can be found in the serum and plasma. Cytokines, interferon (IFN)-γ and IL-10, can cause the release of HSP70 from exosomes [26]. Thus, HSP70 may serve as molecular markers of diseases such as acute myocardial infarction where these HSPs are unduly expressed in the circulation [27].

In unstressed cells, HSPs are chaperone proteins that maintain protein configuration and transport. The presence of HSPs is advantageous especially during cell stress owing to the versatility in their functional attributes, encompassing the inhibition of protein processing, regulation, and production [28, 29]. In the incident of stress, the heat shock functional domain and heat shock transcription factors (HSFs), including HSF-1, HSF-2, HSF-3, and HSF-4, are activated ensuing in the stimulation of the heat shock response (HSR) [30]. HSF-1 translocates into the nucleus oligomerizes, phosphorylates, and binds to heat shock elements causing the release of RNA polymerase [31, 32]. The heat shock functional domain is comprised of a nucleotide-binding domain and a peptide-binding domain. Hydrolysis of ATP to ADP occurs when it binds to the adenine nucleotide-binding domain causing a structural change and detachment of substrates. The peptide domain interacts with the hydrophobic substrates [33].

A set of HSPs known as small HSPs have also been identified to have an involvement in the immune system; nonetheless, this paper is limited to HSPs and not small HSPs.

2. Heat Shock Proteins and the Immune System

The immune system is an intricate network of cells and proteins, and bidirectional communication between different components of the immune system is necessary for optimal homeostasis. HSPs are implicated in both the adaptive and innate immune systems. In the innate immune system, HSPs stimulate dendritic cells and macrophages, as these are APCs, they consecutively stimulate adaptive immune cells [34, 35]. HSPs are important in NK cell function as they are known to increase cytotoxic function and cell proliferation [36]. In particular, membrane-bound HSP70 on various cancer cells is recognized by cluster of differentiation (CD)94 on the NK cell, initiating effective cytolysis of the tumor cells [37, 38]. HSPs may induce the secretion of either anti- or proinflammatory cytokines thereby monitoring the immune response [39, 40].

HSPs may serve as immunogens released in response to an inflammatory episodes which associates with particular surface receptors to induce adaptive immune reactions [39, 41–44]. Antigenic binding of HSPs occurs via interactions between hydrophobic residues such as the V436 in DnaK and bound peptides hence, mutations in these residues may obscure adaptive immunity as a consequence of loss in binding abilities of the peptide [45]. Similarly, HSPs increase the effectiveness of cross-presentation between antigens and APCs in the extracellular milieu, perpetrating in the presentation of peptides to major histocompatibility complex class one (MHCI) or MHCII molecules on T cells. CD91, an HSP receptor, is a requisite for this process and increases T-cell-mediated responses related to T helper (Th)1, Th2, and Tregs [9]. Thus, in the presence of tumors, concomitant relations between the extracellular HSPs and the APCs following internalization of the tumor peptides via CD91 pathway generate both anti-and proinflammatory immune response mediated by T cells [46].

Incidentally, extracellular HSPs may have potent cytokine-related properties necessary for immune response. They act via the association with pattern recognition receptors (PRR) including toll-like receptors (TLRs) and CD14. CD14 is a lipopolysaccharide membrane protein receptor lacking a transmembrane or an intracellular domain [47]. CD14 is a necessary stimulant for HSP60 and HSP70. Following stimulation, these extracellular HSPs are endocytosed causing calcium influx and phosphorylation [48]. Myeloid differentiation primary response gene (MYD) 88 associates with the cytoplasmic domain of the TLR while interleukin (IL)-1 receptor-associated kinase (IRAK) is recruited, phosphorylated, and released. IRAK interacts with the tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6). This is sequentially followed by the stimulation of transforming growth factor-β-activated protein kinase 1 (TAK1) [49]. TAK1 stimulates IκB Kinase (IKK) and this phosphorylates IκB prompting the movement of nuclear factor kappa-light-chain enhancer of activated B cells (NFκB) to the nucleus where it binds to target genes. NFκB modulates the transcription of cytokine genes including TNF-α, IL-1β, IL-6, and IL-12 [50]. The generation of NFκB elicits sequences of events altering the expression of cytokines, chemokines, cell adhesion molecules, growth factors, anti-apoptotic proteins, and immune receptors [51]. HSP may repress NFκB successively decreasing TNF-α [52]. This occurs via mitogen-activated protein kinases (MAPK) pathway originating in the phosphorylation of c-Jun, which sequentially stimulates activator protein (AP)-1 and upregulates proinflammatory IL-18. Similarly, excess secretion of IL-18 is regulated by HSR which suppresses IL-18, by inhibiting JNK/MAPK signaling [53].

The multifaceted nature of HSPs incorporates the regulation of reactive oxygen species (ROS) and some chemokines from stimulated monocytes, macrophages, and dendritic cells. Equally, heightened HSP antigen presentation in APCs is correlated with an increase in CD86, CD40, and MHC molecules [9, 54]. HSP60 expressively inhibits chemotaxis and in combination with TLRs upregulates anti-inflammatory reactions while altering B-cell activity [55,

56]. Involvement of HSPs in the mechanism of danger-activated molecular pattern (DAMPS) is controversial as they have been described as DAMPs while elsewhere they have been implicated in the dampening of DAMPS owing to their interactions with TLRs thus, inducing proinflammatory responses [57, 58]. DAMPS are intracellular endogenous molecules secreted following necrosis with the ability to induce nonspecific adaptive immune responses following dendritic cell activation. DAMPS may also interact with pattern recognition receptors (PRRs) resulting in the presence of inflammatory cytokines [59]. In mice, macrophages stimulated with LPS release high mobility group box 1 (HMGB1) sequestering a proinflammatory response which effectively prompts apoptosis [60]. In the presence of HSP, HMGB is not translocated to the nucleus averting induction of the apoptotic pathway; thus, cell death is aborted [61]. Similarly, HSF-1 binds to the promoter regions of cytokine genes such as TNF-α and IL-1β in mice, inhibiting TNF-α expression in macrophages [62]. TNF-α is involved in the TRAIL death receptor pathway and perhaps obscures their production preventing nonspecific cell death that may be harmful to the immune response or arouse an overreactive immune response that activates autoimmunity.

In autoimmune diseases, HSPs may be important in regulating T-cell-related cytokine dominance from a primarily proinflammatory to an anti-inflammatory state [40, 63–65]. High incidence of HSP70 decreases endotoxin-induced protein and mRNA levels of TNF-α in heat-induced peritoneal macrophages [66] suggesting an influence of HSPs on the transcription of these genes. However, overexpression of HSP70 by peripheral blood macrophages decreases LPS-induced TNF-α, IL-1β, IL-10, and IL-12 [67]. Additionally, HSP70 alters proinflammatory cytokine production increasing endotoxin tolerance and survival [68].

3. The Role of Heat Shock Proteins in Regulatory T Cell Function

Regulatory T cells (Tregs) are a subset of $CD4^+$ T cells with suppressive functions. Two main groups of Tregs have been characterized based on their site of development, that is, in the thymus (natural Tregs (nTregs)) and in the periphery (inducible Tregs (iTregs)) [69]. NTregs are derived from bone marrow progenitor cells transported to the thymus where they differentiate into nTregs following negative and positive selection. Following maturation, these cells migrate to the periphery [70]. NTregs can be differentiated from other T cells owing to the exclusive expression of forkhead box P3 (FOXP3) which is necessary for nTreg-suppressive function [71, 72]. In mice nTregs can be differentiated from iTregs owing to the presence of high levels of neuropilin-1 on mice nTregs [73]. Other essential effector and costimulatory molecules that are expressed by these cells include CD39, CD73, cytotoxic T-lymphocyte antigen 4 (CTLA-4), lymphocyte-activation gene 3 (LAG-3), CD28, CD80/86, CD40, OX40 (CD134), and 4-IBB (CD137) [74–76]. ITregs are generated from naive $CD4^+$ T cells subsequent to induction by IL-10 and TGF-β resulting in two populations of iTregs, type 1 Tregs (Tr1), and T helper 3 (Th3) cells, respectively [77–79]. Suppressive function of these cells occurs via IL-10 and TGF-β. Peripheral Tregs can also be generated through interactions between IL-4 or IL-13 and the IL-4Rα [80]. Although, FOXP3 is a characteristic marker of nTregs, Th3 cells can also be induced to generate FOXP3 [81–83]. Upon activation of the T cell receptor, Tregs suppress dendritic cells, B cells, macrophages, osteoblasts, mast cells, NK cells, NKT cells, $CD4^+$ T, and $CD8^+$ T cells [84].

Tregs suppressive mechanisms transpire through cytokine secretion, cytolysis, metabolic destruction, and altering of APCs function. The secretion of inhibitory cytokines such as IL-10, TGF-β, and IL-35 suppresses immune function. The inhibitory effects of IL-10 occur via its association with APCs to suppress inflammation hence, in the absence IL-10-secreting Tregs there is an increase in inflammation [85]. IL-35 on the other hand suppresses the expansion of T cells into other T helper subsets, as well as B cells and macrophages [86, 87]. TGF-β is necessary for the survival of Treg subsets [88]. Tregs may suppress the function of other cells via granzyme-mediated killing following the release of granzymes into the target cells [89, 90]. Similarly, metabolic disruption involving induction of adenosine and the production of cyclic adenosine monophosphate (cAMP) may be a vital mechanism for suppressing overreactive cells [91]. The versatility in Treg effector function allows them to modulate innate immune cells in particular APCs. This entails the engagement of surface molecules such as CTLA-4 and LAG-3 with MHCII molecules on the APCs conferring inhibitory responses that avert the stimulation of other conventional T cells [92].

As HSPs regulate an extensive component of the immune system, it is likely that they have a role in the optimal function of most immune cells. Importantly, the chaperoning effects of HSPs are necessary for the induction of certain T-cell phenotypes, importantly, Th1, Th2, and Tregs. This presupposes that HSPs are important in Treg function. To date, the following HSPs have been investigated in relation to Tregs, HSP60, HSP70, and HSP90. HSPs are important in the induction, proliferation, suppressive function, and cytokine production of Tregs.

HSP60 employs TLR2-signaling pathway in regulating Treg function. TLR2 is expressed on the surfaces of Tregs [93] hence, association between the TLR2 on the Tregs and the HSP stimulates a sequence of events that affect the functional properties of Tregs. Incidentally, increasing levels of HSP60 are correlated with proportional elevations in the intensity of $CD4^+$ $CD25^+$ Treg-directed suppression on the production of TNF-α and IFN-γ [94]. An increase in HSP60 increases ligand binding of the HSP and the TLR2, thus, increasing suppression. This may represent an autoreactive inflammatory response causing autoimmunity [95]. HSP60 also causes an increase in Treg secretion of TGF-β and IL-10 [94]. HSP60 enhances the differentiation of cord blood cells into $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs [96]. Similarly, costimulatory signals from p277 also increase the activity of $CD4^+$ $CD25^+$ Tregs [94]. Therapeutic administration of HSP60 increases the presence of nTregs, and this is often correlated with a decrease in atherosclerotic plaques, the

generation of Tregs, and an increase in the production of TGF-β [97, 98]. The concentration of HSP60 affects Treg suppression and proliferation. Hence, with respect to TLR2 on Tregs, strong ligand binding results in Treg proliferation while relatively low levels or interactions of ligands and TLR2 on the Treg result in an increase in Treg suppression [99].

Equally, HSP70 in Tregs promotes heightened suppressive function in Tregs [100]. HSP70 confers its activity via TLR4 pathway inducing a surge in the regulatory activities of Tregs. The TLR4-signaling pathway is important in Treg function, and this may be important for FOXP3 induction and suppression of inflammatory reactions [101]. TLR4 interactions with HSP70 may also augment effector T cell suppression by Tregs as this has been confirmed in coculture experiments with other ligands [102]. Additionally, the type of Tregs present following HSP administration may be dependent on the type of inflammatory response occurring at the time. For example in the mice model of arthrosclerosis, immunization with HSP70 produces a significant amount of $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs [97]. Similarly, adoptive transfer of HSP70 peptide epitopes such as B29 induces antigen-specific $FoxP3^+$ or LAG-3^+ $CD4^+$ $CD25^+$ Tregs that are effective in either aborting or suppressing arthritis in mice [103]. B29 is highly immunogenic peptides with conserved sequences that are presented to T cells by MHC II molecules. Immunization with HSP70 increases IL-10 producing Tregs [104]. HSP70 derived from mycobacterium tuberculosis stimulates the proliferation of Tregs by acting through dendritic cells causing a surge in IL-10 while dampening TNF-α release [105]. Additionally, HSP70 has anti-inflammatory properties including down-regulating inflammatory cytokine production, increasing cell and tissue tolerance of cytokine-related cytotoxicity, and influencing the permeability of the epithelial barrier [106].

HSP90 is important for conserving proteins involved in signal transduction, via a multichaperone complex [107]. HSP90 can be regulated by histone deacetylases (HDACs) such as HDAC6, and hypoacetylation of HSP90 occurs in the presence of excessive HDAC6 [108]. HDAC6 belongs to the Class II family of HDACs that are necessary for the removal of acetyl from histones and are found in the nucleus and cytoplasm [109]. In Tregs, the removal of HDAC6 results in the overexpression of HSP90 acetylation resulting in an increase in HSF1-related genes instigating an increase in the suppressive function of Tregs [100]. This may be important in treating patients with colitis. Mice deficient in HDADC6 are more likely to have increased levels of Treg suppression due to the presence of HSP90 and excess Foxp3 [100]. Similarly, mice deficient in HDAC9 have an increased expression of Foxp3 [110]. The presence of HDAC9 has been observed to decrease Foxp3 via deacetylation and incidentally Treg function. HSP70 not only acts via the TLR4 to regulate Tregs, but also may inhibit HDAC9 ultimately enhancing the release of Tregs and effective Treg repression [111]. Acetylation is a necessary posttranslational modification process for protein production. Hence, increased acetylation of Foxp3 may avert ubiquitination, increase its regulatory effects, stability, and promote DNA binding [112, 113]. Therapeutic strategies involving the use of HSPs to enhance the availability of $Foxp3^+$ Tregs may be important in autoimmune diseases while in diseases like cancer it may be necessary to inhibit Foxp3 acetylation [112].

4. Conclusion

In summary, despite the limited amount of research on Tregs and HSPs, the available literature suggests an involvement of HSPs in the suppressive function and cytokine production of Tregs. HSPs may indirectly or directly stimulate Tregs, via acetylation, TLR, ligation or act as costimulatory molecules via the induction of other cells or molecules to stimulate the Tregs. These may involve cytokines, antigens, and APCs. Hence, HSPs acting are therefore essential in inducing Foxp3 expression, cytokine secretion, and mediating Treg suppressive effects. Additionally, peptides such as B29 may have therapeutic potential as they are able to suppress inflammation and maintain tolerance. Thus, the therapeutic advantage of HSPs relates to their potential use in diseases where the function of Tregs is impaired, importantly, in the management of autoimmune diseases.

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Review Article

The Immunosuppressive Activity of Heat Shock Protein 70

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Heat shock protein 70 (HSP70) has previously been described as a potent antitumour vaccine. The mechanism relied on the ability of tumour derived HSP70 to associate with antigenic peptides, which, when cross presented, elicited a T cell mediated antitumour response. Subsequently, HSP70 was incorrectly described as a potent adjuvant of innate immunity, and although mistakes in the experimental approaches were exposed and associated with endotoxin contamination in the recombinant HSP70 specimen, questions still remain regarding this matter. Here we review only publications that have cautiously addressed the endotoxin contamination problem in HSP70 in order to reveal the real immunological function of the protein. Accordingly, "endotoxin free" HSP70 stimulates macrophages and delivers antigenic peptides to APCs, which effectively prime T cells mediating an antitumour reaction. Conversely, HSP70 has potent anti-inflammatory functions as follows: regulating T cell responses, reducing stimulatory capacity of DCs, and inducing development of immunosuppressive regulatory T cells. These activities were further associated with the immune evasive mechanism of tumours and implicated in the modulation of immune reactivity in autoimmune diseases and transplant-related clinical conditions. Consequently, the role of HSP70 in immune regulation is newly emerging and contrary to what was previously anticipated.

1. Tumour-Specific Antigens and Heat Shock Proteins

Numerous recent advances in the field of HSP-mediated immune regulation were inspired by the work of Pramod Srivastava. Srivastava advanced initial observations from the field of tumour immunisation and contributed greatly to the development of HSP-mediated tumour-specific immunotherapy. However, original research dates as far back as 1905 when Clowes and Baeslack observed that serum from a mouse, which had spontaneously recovered from a transplanted tumour, could give rise to antitumour immunity when injected into another inbred mouse with the same tumour [1]. Since then scientists have been searching for "the immune substance," as a potential antitumour therapy. In 1943 Gross investigated immune resistance against tumours using the methylcholanthrene induced sarcoma (MC-Sa) mouse model, which he called "sarcoma 1" [2]. Gross intradermally injected a cell suspension of "sarcoma 1" into an inbred strain of mouse [2] and those mice, which spontaneously recovered from the transplanted tumour, gained resistance to subsequent attempts of tumour "sarcoma 1" induction [2]. However, the immunised mice were only resistant to "sarcoma 1" and were not resistant to spontaneous mammary tumours [2]. In 1957 Prehn and Main used a similar model to Gross [2] and generated a number of mice with MC-Sa sarcomas [3]. In a comparable set of experiments they showed that mice, which had spontaneously recovered from a tumour, gained resistance to the same MC-Sa tumour, but not to other MC-Sa tumours [3]. Some years later, in 1960, Klein et al. reported that irradiated tumour cells could be used to induce immunity but only to the same tumour from which the cells were taken [4]. In addition, the same authors showed that the immunisation was also effective when used in unrelated mouse lines [4].

In the following years research focused on the identification of "the immune substance," which is currently referred

to as tumour-specific antigens (TSAs). In 1982 DuBois et al. isolated TSA, which when used for immunisation, induced resistance to subsequent tumour transplantation. This protein of 75 kDa was purified from two distinct mouse MC-Sas, Meth A and CI-4 [5]. Antibodies raised against the 75 kDa TSA purified from Meth A were shown to also recognise the 75 kDa TSA from CI-4 [5]. Interestingly, the 75 kDa TSA isolated from Meth A induced protection against the Meth A tumour but not against CI-4 or other tumours [5]. In the same way the 75 kDa TSA purified from CI-4 induced resistance only to the CI-4 tumour [5]. Although it has never been confirmed, the size of the protein and its characteristics indicate that the 75 kDa TSA was in fact HSP70. Later, Srivastava et al. identified a glycoprotein of 96 kDa (gp96) as another TSA [6]. After it became apparent that gp96 is a very conserved protein, and could not induce a tumour-specific response by itself, the idea that gp96 works as an antigen carrier emerged [7]. A few years later, in a similar set of experiments, HSP70 was shown to give rise to antitumour immunity and that activity was solely dependent on the associated peptides and not the protein itself [8]. The process, from which the antitumour response was developed by HSP immunisation, was further attributed to cross presentation [9]. Accordingly HSPs, which induce such a response, had to be bound to MHC epitopes or epitope precursors upon purification. Indeed, our analysis of HSP70 associated peptides revealed the presence of MHC class I and class II epitope precursors bound to purified HSP70 [10, 11]. In accordance with our observation that HSP70 associates not only with MHC class I but also with class II epitopes, were previous observations that showed the importance of $CD8^+$ and $CD4^+$ cells in HSP70 mediated antitumour response [12].

2. Tantrum about Proinflammatory Functions of HSP70

By the early 2000s the mechanism by which tumour purified HSP70 with associated peptides can elicit an antitumour response was relatively well understood [13]. However around this time a very controversial article about the "danger signal" properties of HSP70 was published in a very prominent journal [14]. The phenomenon was very attractive because it packaged two important properties of an antitumour vaccine: antigen carrier and immune adjuvant, in one-HSP70. Many additional publications reporting on this mechanism appeared shortly after the initial paper. Unfortunately they overlooked that the a recombinant specimen of HSP70 (same HSP70 specimen that was used by Asea et al.—from StressGen, now Enzo) only induced an immune response when endotoxin levels were high and was completely abrogated after removal of the contamination [15, 16]. Disregarding this fact, the proinflammatory functions of HSP70 were still promoted in many reviews [17–21]. Since then the situation has not much changed and even the most recent reviews by prominent scientists describe unjustifiably all HSPs, including HSP70, as damage-associated molecular patterns (DAMPs) that facilitate proinflammatory responses following tissue injury or cell death [22, 23]. Controversy surrounds many HSPs and their proinflammatory roles, and mainly results from endotoxin contamination artefacts which seems to be commonly overlooked due to a dogmatic approach adopted by some of the scientific community. Many reviews were written and argued for or against mammalian HSP70 proinflammatory functions; however the fact remains that in those publications where the striking "danger signal-like" proinflammatory activity of HSP70 was shown, researchers used the endotoxin contaminated recombinant protein provided by Stressgen Biotechnologies [16, 25, 26]. Therefore in this paper we dismiss any data from studies where HSP70 endotoxin contamination was neglected and we will concentrate only on reports where HSP70 purified from a nonbacterial origin was used or studies where endotoxin contamination was stringently controlled.

3. Occam's Razor-Endotoxin-Free HSP70 and APC Modulation

So when one excludes those reports investigating the role of HSP70 using the recombinant specimen, whilst neglecting the possibility of endotoxin contamination, what are they left with? In fact there are quite a number of publications which may reveal the true function of HSP70. In the studies where HSPs were purified from a nonbacterial origin it was shown that HSP70 could stimulate macrophages to secrete cytokines and induce maturation of DCs [27]. However, one has to be aware that the HSP70 mediated DC activation profile was not only minor but also partial, and different from that influenced by LPS or gp96 [27]. In addition, the experiment indicating HSP70 induction of DC maturation was $n = 1$, which makes it impossible to interpret and questions reproducibility [27]. Later on, the same group tested the responses of primary murine macrophages as well as human myeloid cell lines and were able to show that exposure to HSP70 or gp96 can elevate nitric oxide secretion [28]. Of note, the experiments regarding DC maturation were only shown for gp96 and not for HSP70, which was possibly due to a lack of such activity [28]. Most recently it was shown that a 20 hour exposure of mouse macrophages to HSP70 at a concentration of approximately 100 μg/mL, induced secretion of a unique selection of cytokines, mainly IL-1β and IL-6 but not TNFα [29]. Accordingly these experiments have shown that HSP70 does have some influence on immune response; however the patterns did not resemble those typical of DAMPs or endotoxins, and these responses seemed to mainly come from macrophages and not other antigen presenting cells such as DCs. Accordingly, the lack of DC maturation upon exposure to HSP70 was reported by others using concentrations as high as 200 μg/mL [30]. Regarding DC maturation as an innate immunity activation measure there are also three additional publications that used "endotoxin free" recombinant HSP70 and showed no stimulatory activity towards monocyte derived DCs [15, 31, 32]. Moreover, it was also observed that the lack of this adjuvant property of HSP70 did not affect its ability to deliver antigenic peptides for effective cross presentation to T cells [15, 31, 32]. We also tested the proinflammatory capacity of HSP70 purified from two tumour cell lines, K562 and

CCRF-CEM, on human monocyte derived immature DCs (mo-iDCs). Using a range of concentrations up to 10 μg/mL we were unable to observe any stimulation of mo-iDCs maturation unlike with recombinant HSP70 (unpublished data). We also used a HSP70 specimen provided by Britta Eiz-Vesper and again failed to observe any significant expression changes in the tested DC maturation markers, that is CD83, CD80, CD86, or MHC class II at a concentration of up to 80 μg/mL of HSP70 [26]. However we observed that a 24 hour exposure of mo-iDCs to HSP70 was able to visibly change their cluster formation pattern, which we commonly associate with the maturation status of mo-DCs [26]. We also noticed that mo-iDCs, even when left in a media without any stimuli, would still spontaneously mature as measured by CD83, CD80, CD86, or MHC class II expression as well as cluster formation ability (unpublished data). We therefore speculated that HSP70 could delay this spontaneous process rather than actively reprogramming the cells resulting in a reduced stimulatory capacity of mo-iDCs towards T cells [26]. It is also important to note that these observations were only consistently reproducible when serum-free medium was used and further work is needed to draw a conclusion from these data.

In conclusion, it can be summarised that HSP70, in high concentrations, can moderately stimulate a unique pattern of responses in macrophages but HSP70 fails to activate DC maturation. In contrast, even at low concentrations, HSP70 can reduce the stimulatory capacity of DCs [26]. Consequently, it has to be clearly stated that HSP70 does not qualify as a DAMP [33].

4. Endotoxin-Free HSP70 and T Cell Modulation

Recently HSP70 was also shown to improve survival of a neuroblastoma cell line upon heat stress and stimulated intracellular calcium flux in a monocytic leukaemia cell line [34]. It was also able to moderately induce cytokine secretion of mouse splenocytes and selectively changed their profile by increasing representation of $CD4^+$ and $CD11^+$, but not $CD8^+$, by approximately 10–15% after 4 day incubation with 100–200 μg/mL of HSP70 [34]. Also it was recently suggested that 7 day incubation of HSP70 at a concentration of 10 μg/mL, can promote a unique T cell cytokine secretion profile; however the results are difficult to interpret since no statistical significance was reached [35]. One fact in agreement with the previous study by Zheng et al., was that proliferation of $CD4^+$ cells upon the extended exposure to HSP70 was increased by approximately 15% ($P < 0.01$) followed by a 4% ($P < 0.01$) improvement of cytotoxic activity towards K562 and B-LCL [35]. No significant change in granzyme B secretion or production was found in any of the tested T cell subpopulations upon the extended HSP70 incubation. Of note, some of the anticipated HSP70 activities in T cell induction were found when additional cytokines were added alongside the protein, either IL-2 or a cocktail of IL-7, IL-12, and IL-15 [35]. However it has to be remembered that the addition of HSP70 to the media can improve cell viability in tissue culture conditions [26, 34]. Thus it can be speculated that changes in cell representation, proliferation, and cytokine secretion might have originated from the improved survival rather than the proinflammatory function, especially if extended exposure had taken place. In addition, the minor changes in certain experimental readouts in both of the studies reviewed above, although statistically significant, cannot be considered physiologically relevant without further studies. In our own study we used an HSP70 specimen provided by Britta Eiz-Vesper that was also used by Figueiredo et al. as described above. Surprisingly we found that use of the same protein sample, although in a different experimental setup, produced somewhat contradictory results. We examined both activated (whole $CD3^+/CD25^+$ population) and non-activated ($CD3^+/CD25^-$, $CD4^+/CD25^-$ and $CD8^+/CD25^-$ populations) T cell proliferative responses upon stimulation with HSP70-preincubated mo-iDCs. We observed that this pre-treated mo-iDCs had lower stimulatory capacity towards T cells [26]. However the additional supplementation of the media with HSP70 during the proliferation assay resulted in further reduction of T cell proliferation, indicating a direct effect of HSP70 on not only mo-iDCs but likely also on T cells. This was further confirmed when the activated T cell proliferation induced by IL-2 was reduced by HSP70. Also upon PHA stimulation the proliferative and secretory responses of activated and non-activated T cells ($CD3^+$, $CD4^+$ and $CD8^+$) were significantly reduced by HSP70 [26].

In summary, the results obtained to date cannot be considered conclusive since even using the same specimen of HSP70 produced opposing results in differently arranged *in vitro* experiments. Again, our results showed a strong phonotype in T cell response reduction and these were common to all T cell subpopulations, while the opposing studies showed rather minor increases in T cell responses; however these were more specific to some T cell subpopulations in particular. Accordingly, in order to remain fair to currently available data, the role of HSP70 in influencing T cell responses requires independent verification. Importantly however, some relevant data supporting the reduction in T cell responses by HSP70 are coming from the studies examining the function of membrane bound HSP70 or using microbial HSP70 and are discussed below.

5. HSP70-Mediated Immunosuppressive Regulation

Our observations regarding the immunosuppressive activity of HSP70 were unexpected, given the common, yet incorrect association of HSPs with proinflammatory activity. However there is great body of supporting evidence coming from the field of microbial HSP70 where it has been used to treat different autoimmune diseases. It was shown that microbial HSP70 mediates anti-inflammatory reactions in different chronic inflammatory diseases models. The mechanism, although not entirely clear, points towards development of immune regulatory T cells (Tregs) reactive to self-HSPs and is mediated by an increase of IL-10 production [36]. Similar to our data showing the effect of human HSP70 on both DC maturation and T cell responses [26], microbial HSP70

was demonstrated to delay the DC maturation process and independently reduced proliferative response of T cells upon PHA stimulation [37, 38]. This immunosuppressive activity of microbial HSP70 was later tested in an animal allograft rejection experiment and showed that the skin or tumour allograft rejection can be delayed by preincubation with microbial HSP70. It was also shown that the microbial HSP70 mediated immunosuppressive and tolerising effect was abrogated upon Tregs depletion [39]. Moreover subcutaneous injection of 30 μg of microbial HSP70 was shown to increase representation of Tregs in excised draining lymph nodes. In addition, the lymph node derived cells were shown to be less responsive to PHA stimulation and secreted approximately 25% more IL-10 and approximately 40% less TNFα [39]. These very interesting data correlate very well with our own observation, thus it would be of great interest to compare the immunosuppressive activity of microbial HSP70 with either human or endogenous murine HSP70. Such experiments would show if microbial HSP70 shares the same immunosuppressive capacity with endogenously expressed HSP70 or if it is a result of some other mechanism.

HSP70 upregulation, active secretion, and abundant cell membrane expression are common to tumour cells [40, 41]. Moreover, HSP70 expression is specific to tumour cells and has not been observed in normal cells. In many cases it was associated with poor survival possibly also resulting from poor responses to chemotherapy [42, 43]. Therefore HSP70 can be associated with the immune evasive mechanism of tumour cells. In fact it has recently been shown that HSP70 is indeed being utilised by tumour cells to promote immunosuppression. Tumour cells were found to produce exosomes, which were loaded with membrane bound HSP70. These if incubated with myeloid-derived suppressor cells (MDSCs) induced Stat3 phosphorylation and led to the development of immunosuppressive activity in MDSCs [44]. Knock-down of HSP70 in tumour cells was shown to abrogate the effect of exosomes on MDSCs and the same abolition was obtained when the exosomes were preincubated with anti-HSP70 neutralising antibodies [44]. The role of HSP70 in mediating immunosuppressive activity of MDSCs is very interesting, given that MDSCs are one of the major suppressors of antitumour activity that also reduces $CD8^+$ T cell antigen recognition of tumour cells [45].

HSP70 upregulation was found in many immune mediated diseases. High expression of HSP70 was observed in human patients as well as animal and tissue models of graft versus host disease (GvHD), a major complication of haematopoietic stem cell transplantation. The underlying cause of the disease is an alloreaction of the engrafted immune cells against the host. Increased expression of HSP70 in rat spleen and lymph nodes corresponded with progressive GvHD [46]. Upregulation of HSP70 expression has been demonstrated in a skin explant model of GvHD and correlated with an increased grade of graft versus host reactivity in the biopsy [47]. Increased HSP70 expression has also been reported in the field of solid organ transplantation where rejection of rat cardiac allografts was accompanied by an upregulation of HSP70 by cardiomyocytes [48]. A strong upregulation of HSP70 has also been observed in the epithelial layers and mononuclear cells of colon biopsies from patients with inflammatory bowel diseases [49]. Furthermore HSP70 expression has been the subject of rheumatoid arthritis (RA) research and high expression of HSP70 has been reported in RA patients' synovial fluids [50]. All of these diseases were shown to be associated with HSP70 upregulation in the target rather that effector cells as a consequence rather than a cause of immune reactivity.

These data, in conjunction with the recently revealed immunosuppressive function of HSP70, can argue that upregulation of this protein in various clinical conditions is associated with the protective anti-inflammatory regulation that cells utilise upon a cytotoxic reaction of the immune system. However, this mechanism of cellular response, although feasible, will have to be further examined using a robust experimental approach.

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Review Article

Impact of Exercise and Metabolic Disorders on Heat Shock Proteins and Vascular Inflammation

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Heat shock proteins (Hsp) play critical roles in the body's self-defense under a variety of stresses, including heat shock, oxidative stress, radiation, and wounds, through the regulation of folding and functions of relevant cellular proteins. Exercise increases the levels of Hsp through elevated temperature, hormones, calcium fluxes, reactive oxygen species (ROS), or mechanical deformation of tissues. Isotonic contractions and endurance- type activities tend to increase Hsp60 and Hsp70. Eccentric muscle contractions lead to phosphorylation and translocation of Hsp25/27. Exercise-induced transient increases of Hsp inhibit the generation of inflammatory mediators and vascular inflammation. Metabolic disorders (hyperglycemia and dyslipidemia) are associated with type 1 diabetes (an autoimmune disease), type 2 diabetes (the common type of diabetes usually associated with obesity), and atherosclerotic cardiovascular disease. Metabolic disorders activate HSF/Hsp pathway, which was associated with oxidative stress, increased generation of inflammatory mediators, vascular inflammation, and cell injury. Knock down of heat shock factor-1 (HSF1) reduced the activation of key inflammatory mediators in vascular cells. Accumulating lines of evidence suggest that the activation of HSF/Hsp induced by exercise or metabolic disorders may play a dual role in inflammation. The benefits of exercise on inflammation and metabolism depend on the type, intensity, and duration of physical activity.

1. Introduction

The stress response is a self-protective mechanism against environmental stresses which is mediated via a group of evolutionally conserved proteins, heat shock proteins (Hsp). Hsp regulate the conformation and functions of a large number of cellular proteins in order to protect the body from stress [1]. The expression of Hsp is mainly modulated by a common transcription factor, heat shock factor-1 (HSF1). The activity, translocation, and expression of HSF1 respond to environmental stresses, such as heat shock, wounds, oxidative stress, and radiation [2]. Exercise is associated with transient elevations of Hsp expression, body temperature, hormones, and oxidative stress, which may reduce inflammatory mediators [3]. Metabolic disorders in common chronic diseases (diabetes, metabolic syndrome, and atherosclerotic cardiovascular disease) are associated with a prolonged stress response as a consequence of oxidative stress, altered hormone levels, vascular inflammation, and cell injury [4]. Type 1 diabetes is a common autoimmune disease characterized by pancreatic β-cell destruction and insulin deficiency which can lead to poor circulation and vascular disease [5]. This paper summarized up-to-date knowledge on the relationship between stress responses, oxidative stress, and vascular inflammation under exercise or metabolic disorders. Selected literature searched using PubMed over a period from 1981 to 2012 is provided.

2. Heat Shock Proteins

Hsp has evolved to perform multiple roles within cells, organs, and organisms [1]. These ubiquitous proteins, which are found both inside and outside the cell [7], have a generalized function of interacting with other proteins, hence their

designation as molecular chaperones [8]. These interactions may influence the structure of the client protein(s) so that it may be maintained in a conformation appropriate for functional folding, targeted for degradation, or altered as part of a signaling pathway. Most Hsp have a multitude of activities based upon their cellular location (including extracellular), the client proteins they interact with [9], and their phosphorylation status which may modulate their aggregation [10], their localization [11], or their activation of enzymatic pathways [12]. As a consequence, Hsp not only protect cells and organisms against proteotoxic stresses, but these proteins are also critical in normal functioning of several cellular processes [13]. Amongst those signaling pathways which involve Hsp are several which are implicated in regulation of immune and inflammatory systems [14, 15]. Although there is some controversy regarding their exact role [16], Hsp may activate the immune response [17] but also dampen the inflammatory pathways [14].

Hsp have normally been classified according to their molecular mass with small Hsp, such as αA- and αB-crystallin, Hsp20, 22, 25/27, and other Hsp60, the Hsp70 and 90 families and Hsp110, and their cochaperones (Table 1), often working in concert to maintain cell structure and function [6]. A new nomenclature has more recently been introduced for Hsp [18]; however, for the purposes of this paper, we will refer to the more common mass-based nomenclature (see Table 1).

Table 1: Heat shock protein nomenclature. Comparison of the old molecular-weight-based names with the new nomenclature as outlined in Kampinga et al. [18].

Weight-based nomenclature	New name
Hsp20	HSPB6
Hsp22	HSPB8
Hsp25/27	HSPB1
αA-crystallin	HSPB4
αB-crystallin	HSPB5
Hsc70 (cognate isoform)	HSPA8
Hsp70, Hsp72 (inducible isoform)	HSPA1A
Hsp90	HSPC1
Hsp110	HSPH2

3. Regulation of the Transcription of Hsp

The regulation of the transcription of Hsp is mainly through heat shock factors (HSF). HSF represents a family of transcription factors induced by both stressful and nonstressful stimuli. The fundamental structure of HSF has been well conserved from yeast to humans [2, 19]. Four isoforms of HSF have been reported. HSF1, 2, and 4 are present in humans. HSF1 is ubiquitously expressed in mammalian tissues and relatively abundant in heart, ovary, brain, and placenta [20]. HSF2 is expressed in very low levels in postnatal tissue [21], and HSF4 is mainly expressed in brain and lung [22]. Under basal conditions, HSF1 exists as a monomer. Under stress, HSF1 is converted to a trimer which is required for the binding to the responsive element (heat shock element) of HSF1 in the Hsp promoter. Phosphorylation of specific HSF1 residues is also required for activation [23, 24], and the multiple pathways potentially involved in these phosphorylations [25–28] probably provide tissue and stress specificity. A variety of stresses beside heat shock may activate or upregulate HSF1 [19, 29, 30]. Indeed, activation of HSF1 was detected in diet-induced atherosclerotic lesions in rabbits and humans [31, 32].

Given the importance of the heat shock response, it is not surprising that there are multiple redundant pathways by which the response may be activated [33]. Following exercise, it is likely that these pathways converge with the HSF1 through the translocation of the transcription factor from cytoplasm to nucleus [34, 35]. With exercise, likely candidates are the adrenergic stimuli associated with exercise operating through α- and β-adrenergic receptors [36–38] as well as elevated temperature and its attendant changes [39, 40] (see Figure 1).

4. Exercise and Hsp

Locke et al. [3] were the first to demonstrate that vigorous physical activity is associated with the induction of Hsp70 in rodents. Subsequently, increased expression of Hsp in humans following exercise was confirmed [41, 42]. As noted above, exercise is associated with many stressors, including elevated temperature, metabolic disturbances, altered calcium fluxes, increased production of reactive oxygen species (ROS), changed hormonal environment, and mechanical activation or deformation of tissues [13]. Exercise has also been described as inducing a mild inflammatory state [43]. The magnitude of the exercise stress, including whether it is acute or chronic, plays a major role in inducing the stress response. Generally, the more vigorous the exercise was, the greater the response was [40, 44–46]. Further, isotonic nondamaging contractions, such as these associated with endurance type activities, tend to lead to increases in Hsp60 and 70 with more limited responses in the small Hsp [3, 47]. In contrast, eccentric (often damaging) muscle contractions, also lead to increases, phosphorylation, and translocation of Hsp 25/27 and αB-crystallin [10, 48, 49]. These exercise-induced changes may be associated with protection of the mitochondria [50, 51], the sarcoplasmic reticulum [52], cytoskeletal protection [49], maintenance of enzymatic activity [53], and insulin sensitivity and glucose transport [54, 55]. With repetitive exercise (exercise training), an exercise-induced increase of Hsp70 is maintained whereas the initial response of other Hsp to exercise is diminished as training progresses [39].

Exercise involves the activation of specific muscles for movement but also requires the support of the neural, cardiovascular, and respiratory systems. The primary focus of investigators to date has been on skeletal and cardiac muscles. Such studies have suggested that in the sedentary state Hsp are expressed in a tissue-specific fashion [56]. Exercise is associated with changes in Hsp expression which are also specific to the Hsp in question [47, 49]. For example,

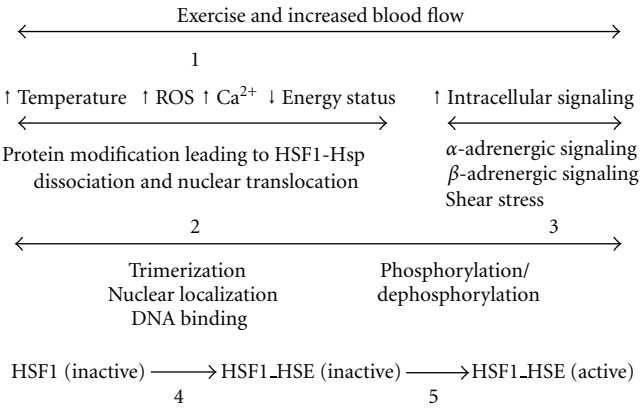


Figure 1: Schematic representation of activation of HSF1 with exercise and accompanying increases in vascular stress. Exercise initiates a number of factors, including elevations in temperature, reactive oxygen species (ROS), intracellular calcium (Ca^{2+}), and decreased energy status [1], which may result in intracellular protein modification leading to dissociation of the heat shock transcription factor (HSF1) and heat shock proteins Hsp in the cytoplasm [2]. In addition, exercise activates adrenergic and shear stress intracellular signaling pathways [3]. Consequently HSF1 trimerizes and binds to heat shock elements (HSE) of nuclear DNA [4], whereupon specific phosphorylation/dephosphorylation events lead to a heat shock response [5]. Adapted from Noble, Melling, and Milne [6].

Hsp70 almost always increases with exercise, whereas the cognate Hsc70 is not normally altered [57–60]. In a similar fashion, some tissues, such as myocardium, may demonstrate a more general response whereas skeletal muscle responds with fiber-specific changes [61, 62]. It is likely that differences in temperature reached during exercise [40] and the specific patters of muscle fiber activation [45] are responsible for some of these tissue-specific observations.

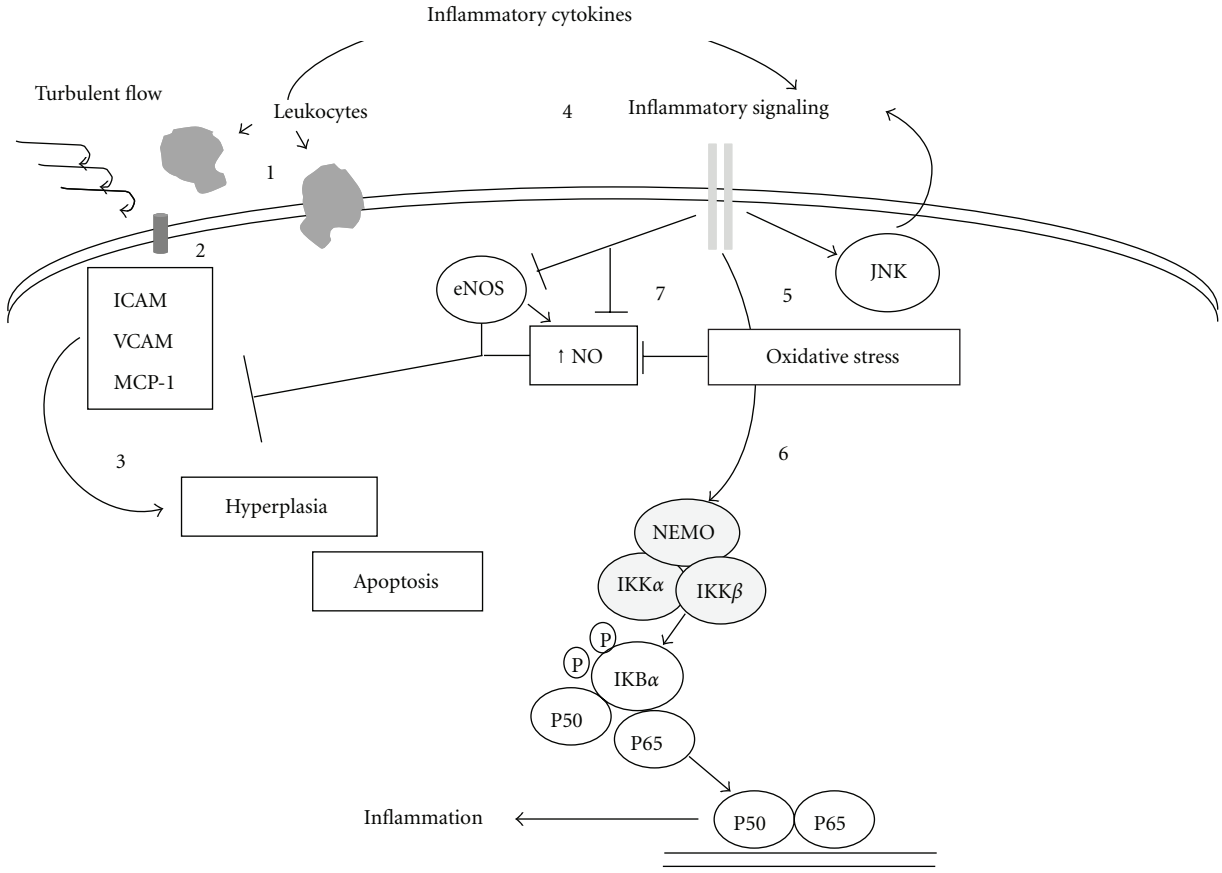
5. Exercise and Vascular Inflammation

Physical activity, or exercise, is known to improve overall health and protect against, delay the progress of, or ameliorate many common chronic diseases [63, 64], in particular those associated with whole body inflammation, including cardiovascular disease [65]. Although those individuals with the greatest cardiorespiratory fitness appear to benefit most [66], simply engaging in regular physical activity seems to be protective [67]. One of the primary targets that may benefit from increased physical activity is the vasculature [68–71]. Amongst the benefits of exercise on the vasculature are increased vasodilation and improved vascular compliance [72] which are likely a result of shear stress and cell stretch on both the endothelium and underlying smooth muscle [73, 74]. Exercise may protect the vasculature through a number of mechanisms [63, 68, 75] including reduced inflammation [76–79]. Short-term exercise reduces the levels of TNF-α, IL-6, plasminogen activator inhibitor-1 (PAI-1) [80], and cell adhesion molecules [81], protects against media-intimal hyperplasia [82, 83] and smooth muscle cell hypertrophy [83], and strengthens the endothelial barrier [84]. The anti-inflammatory role of exercise [43, 65, 78] is complicated; however, as intense unaccustomed exercise may be associated with increased cortisol [85], C-reactive protein [86], and modest increases in other proinflammatory cytokines [87].

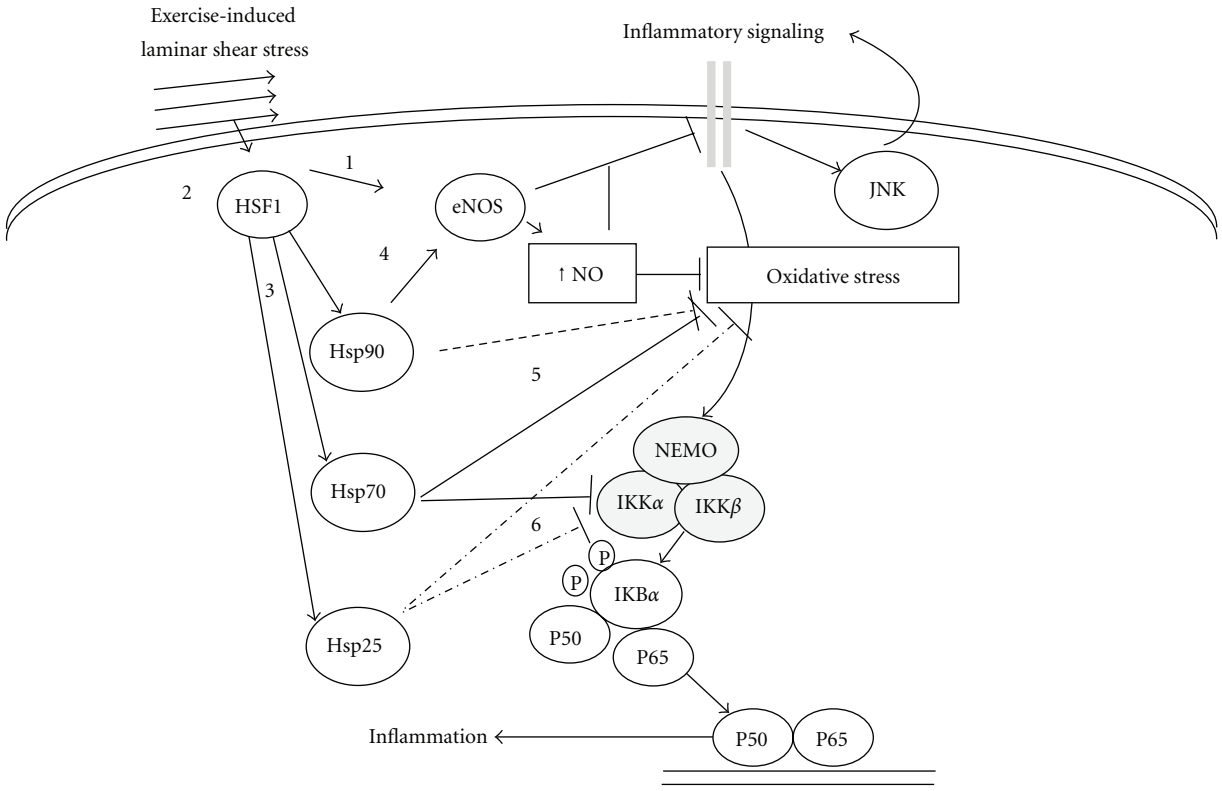
Interestingly, heat shock exhibits beneficial effects on the vasculature which are similar to exercise, with reduced inflammation [88], reduced endothelial interaction with leukocytes [89], enhanced smooth muscle cell survival [90], and inhibition of myointimal hyperplasia and smooth muscle cell hypertrophy [91–95]. Although both heat shock and exercise are complex stressors likely leading to many changes in the integrated physiology of an organism, they both have some common characteristics including activation of stress hormones, ROS, and elevated temperatures leading to the activation of the heat shock response in a variety of tissues including the vasculature. Exercise increases ROS production, and ROS may play a signaling role to initiate the stress response [96]. Also, there is evidence that elevated temperature is critical for the activation of the heat shock response in exercising mammals [39, 40, 97, 98]. These similarities suggest that the protection conferred by exercise against myocardial ischemia-reperfusion injury [99] could be partially a consequence of the vascular expression of Hsp [100, 101]. Indeed, exercise leads to a rapid transcription of Hsp70 mRNA in the vasculature of rodents [62] which eventually results in protein accumulation [102, 103].

6. Vascular Function of Hsp

As throughout the rest of the body, Hsp likely play specific roles within the vasculature. The response of the vasculature to shear stress is complicated. Laminar flow, such as that associated with exercise, induces positive vascular remodeling, whereas turbulent or low flow, such as that associated with vascular inflammation and atherosclerosis, leads to adhesion of blood borne molecules and inflammation [104] (see Figure 2). The increased laminar flow associated with exercise causes endothelial cell remodeling which includes the activation of a number of signaling pathways and either activation or enhanced expression of Hsp [73, 105–107]. Hsp25/27, which is phosphorylated in association with shear stress [105], is involved in cytoskeletal organization [108]. Hsp20 is associated with αB-crystallin in cardiac tissue [109],

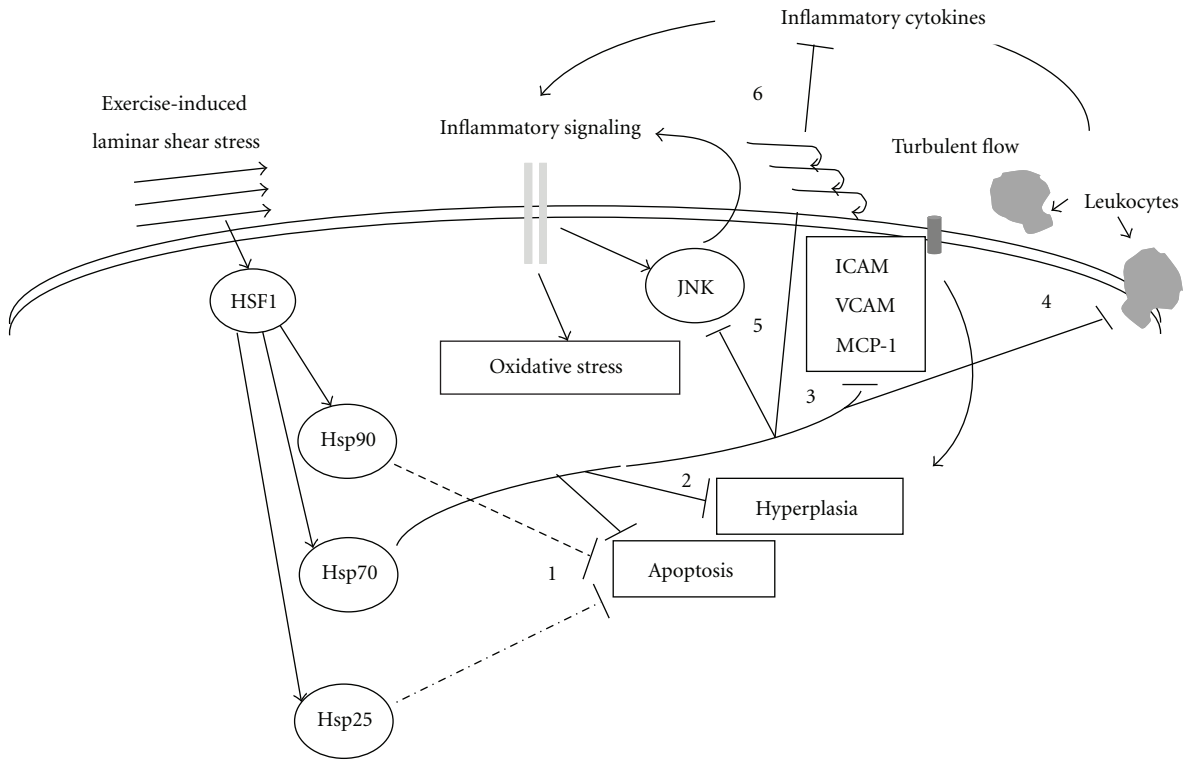


(a)



(b)

Figure 2: Continued.



(c)

Figure 2: Scheme for relationships between exercise-associated hemodynamic changes, inflammatory response, and Hsp. (a) Low or turbulent flow is associated with leukocyte extravasation [1] and expression of adhesion molecules [2], resulting in intimal hyperplasia, cell apoptosis [3] and inflammatory signaling [4]. The associated inflammatory signaling leads to increased oxidative stress, induction of inflammatory pathways such as c-Jun NH_2-terminal kinase (JNK) [5] and NF-κB [6], and suppression of endothelial nitric oxide (eNOS) and oxidation of nitric oxide (NO) [7]. (b) In contrast, an exercise induced increase in laminar shear stress activates eNOS [1] and HSF1 [2]. HSF1 activation leads to increased heat shock proteins 25, 70, and 90 (Hsp25, Hsp70, and Hsp90) [3] which may inhibit many of these inflammatory processes indirectly via activation of eNOS signaling (Hsp90) [4] and directly through suppression of oxidative stress (Hsps 25, 70, and 90) [5] and inflammatory signaling including via the NF-κB pathway (Hsps 25 and 70) [6]. (c) Hsp may also directly reduce apoptosis (Hsps 70 and 90) [1] and hyperplasia (Hsp 70) [2]. Hsp70 has further been implicated in decreased expression of adhesion molecules [3] leading to a reduction of leukocyte extravasation [4] and expression of inflammatory cytokines [6]. Hsp70 also suppresses JNK signaling [5] further inhibiting inflammatory signaling and cytokine release. See text for a more complete description. → — represents activating role; |— represents inhibitory role; - - - -: Hsp90 effects; ——: Hsp70 effects; -·-·-·-·-·: Hsp25 effects.

and both are involved in flow-mediated smooth muscle relaxation [110–112]. Indeed, Hsp25/27 and Hsp20/αB-crystallin may reciprocally assist in controlling venous tone [113]. Hsp70 may modulate vascular contractility through thick filament regulation [114], and Hsp90 is intricately involved in activation of endothelial nitric oxide synthase (eNOS) and the subsequent release of nitric oxide (NO) and vascular relaxation [115].

7. Activation of the Vasculature

Normal endothelium provides an effective barrier to foreign materials and does not interact with circulating factors. With a variety of chronic diseases, including atherosclerosis, metabolic syndrome, and diabetes, there is a subtle change in the endothelium which leads to their "activation" (see Figure 2(a)). Initially, increased membrane permeability leads to the accumulation and modification of proteins, lipids, and lipoproteins on endothelium [116]. The endothelium then becomes "sticky," exhibiting proinflammatory markers such as monocyte chemotactic protein-1 (MCP-1), vascular and intracellular cell adhesion molecules (VCAM-1 and ICAM-1, resp.,) and greater nitrotyrosine content [117, 118]. This leads to the recruitment of blood borne cells which infiltrate the intima resulting in macrophages evolving to foam cells leading to further inflammation and release of pro-coagulant factors, smooth muscle cell death and migration, and the eventual formation of an atherosclerotic plaque [116, 119]. During the course of this progressive dysfunction, NO availability plays a key role, as it is responsible for limiting many of the above processes. However, elevated oxidative stress associated with vascular inflammation leads to diminished NO availability [120]. Oxidation of the eNOS cofactor, tetrahydrobiopterin, uncouples eNOS such that superoxide rather than NO is formed [121]. This leads to NO scavenging to peroxynitrites

and ultimately reduced activation of eNOS and an overall reduction in eNOS content [120, 122].

Although there are a variety of pathways by which inflammation can influence this vascular dysfunction, the nuclear factor kappa light chain enhancer of activated B cells (NF-κB) pathway plays a critical role in this process [123–125] (see Figure 2(a)). NF-κB has both anti- and proinflammatory roles; however, with progression of vascular damage, it primarily activates inflammatory pathways [123, 124, 126]. Members of the NF-κB family, including p50, p52, p65, relB, and C-Rel, form homo- or heterodimers which are found in the cytoplasm in an inactive state bound to the inhibitor IκB. Various stressors can release the IκB from NF-κB through a pathway which involves phosphorylation of IκB by the IKK complex (IKKα, IKKβ, and IKKγ). The phosphorylation of IκB leads to its degradation by the ubiquitin proteasome pathway. The degradation of IκB allows translocation of the NF-κB dimer to the nucleus, where depending on the NF-κB composition, recruited cofactors, and the sequence of targeted genes, variable responses may be observed [124, 127]. NF-κB may also be activated via an IKKα-specific, noncanonical pathway [127]. Knockdown of HSF1 reduced Hsp27 expression and increased angiotensin II-induced NF-κB activation in vascular smooth muscle cells [88]. This suggests that HSF1/Hsp 27 may mediate stress-activated vascular inflammation.

8. Anti-Inflammatory Actions of Hsp

It should be noted that Hsp, particularly extracellular Hsp, may play a key role in activating and exacerbating inflammation including vascular inflammation [17, 128–131] (see the following); however, given the anti-inflammatory phenotype associated with exercise and heat shock, it is likely that the predominate role in the progression of vascular disease is protective under these circumstances.

Both heat shock and exercise increase the vascular content or alter the phosphorylation status of various Hsp and both of these conditions are associated with anti-inflammatory states [14, 132]. Although exact mechanistic activities are often difficult to identify, activation of HSF1, which is the primary transcription factor involved in Hsp induction, may directly reduce general inflammation in vascular tissue [133], but most effects are probably through HSF1-induced increases in expression of Hsp [134] (see Figure 2(b)).

Hsp25/27 and Hsp70 can directly stimulate anti-inflammatory cytokines [135, 136], while Hsp70 can inhibit release of a variety of inflammatory cytokines including TNFα, HMGB1, and IL6 and IL1β [137–139]. This Hsp modulation of cytokine profile also reduces the presence of cell adhesion molecules and thereby leucocyte infiltration of the vascular wall [140, 141]. Hsp may reduce oxidative stress by a variety of mechanisms including facilitation of antioxidant pathways [10, 142, 143]. Of course the reduction in oxidative stress helps maintain NO bioavailability and reduces peroxynitrite formation [120]. In addition, increased Hsp90 in the vasculature has a direct positive effect on eNOS activation [115, 144–146], thereby maintaining vascular function (see Figures 2(b) and 2(c)).

9. Regulatory Role of Hsp on Apoptosis

As the inflammatory process progresses, a progressive cycle of intima expansion occurs with the death, proliferation, and migration of smooth muscle cells [147]. Accumulating lines of evidence suggest that Hsp intervene at multiple locations to inhibit cell death pathways including inhibition of death receptor signaling. Hsp25/27, 70, and 90 are involved in suppression of the mitochondria-dependent apoptosis, by directly limiting cytochrome c release [148, 149] and activation of various caspases [150, 151], by inhibiting caspase-independent pathways [152], and by inhibition of stress [153] and cell death receptor pathways [154, 155]. Hsp directly impacts on this pathway in several ways (Figures 2(b) and 2(c)). Hsp70 and Hsp25/27 can directly interact with IKKα, stabilizing it and preventing the inflammatory activation of the NF-κB pathway [156–158]. These effects appear to be dose and time dependent [159]. Secondly, the stabilization of the cytoskeleton and antiproliferative effects of Hsp25/27 processes negatively influenced by LDL [11] may inhibit inflammation-induced vascular damage [160]. Lastly, although the effects of Hsp on vascular health have been separated by the individual Hsp involved, there is evidence that effective vascular protection requires interaction of multiple types of Hsp [161].

10. Metabolic Disorders and the Stress Response

Metabolic disorders, including hyperglycemia, hypercholesterolemia, hypertriglyceridemia, modified low density lipoproteins (LDL), and insulin resistance, are often associated with diabetes, metabolic syndrome, vascular inflammation, and atherosclerotic cardiovascular disease. Hyperglycemia interrupts the colocalization of Hsp90 and eNOS in endothelial cells, which may affect the production of NO and endothelium-dependent vascular relaxation [162]. Circulating levels of Hsp60 are correlated with triglycerides and small dense LDL in patients with untreated periodontitis [4]. Restriction stress increases the production of Hsp70, MCP-1, PAI-1, and monocyte adhesion but decreases adiponectin in mice [163]. The levels of Hsp27 antigen and antibody in serum of diabetic patients are associated with cardiovascular complications and insulin resistance [164]. Oxidized LDL (oxLDL) has been considered as a circulating marker for coronary artery disease [165]. Glycation increases lipid peroxidation of LDL [166]. The levels of glycated LDL (glyLDL) and oxLDL are increased in diabetic patients [167]. GlyLDL treatment increases the abundance of HSF1 and Hsp70 in endothelial cells [128]. GlyLDL or oxLDL increases the binding of HSF1 to the PAI-1 promoter and PAI-1 expression in endothelial cells [128, 168]. PAI-1 is not only a physiological inhibitor of tissue and urokinase plasminogen activator but also a marker for inflammation. Reduced fibrinolytic activity is associated with coronary artery disease

and diabetic vascular complications [169]. Elevated levels of PAI-1 were detected in acute and chronic inflammatory conditions [170, 171]. Increased levels of circulatory PAI-1 have been considered as a marker of inflammation. However, the precise role of PAI-1 in inflammation remains to be determined. Antioxidants inhibit oxLDL or glyLDL-induced increases of HSF1, PAI-1, and ROS in endothelial cells, which suggests that oxidative stress may play a regulatory role in metabolic stress-induced activation of the stress response and vascular inflammation [168]. In certain stress conditions, such as massive bleeding and wounds, HSF1-mediated PAI-1 production may be protective for the body through its prothrombotic and antifibrinolytic effects. However, chronic elevation of PAI-1 production induced by metabolic disorders may lead to thrombotic tendency and ischemic events. GlyLDL or oxLDL impairs activities of mitochondrial respiratory chain enzymes in vascular endothelial cells [172, 173]. OxLDL induced oxidative stress, activation of HSF1 [168], and apoptosis and the imbalance between caspase-3 and Bcl-2 in endothelial cells [174]. The role of HSF1/Hsp in metabolic disorders-induced vascular inflammation and injury remains to be further investigated but as noted above appears to be both pro- and anti-inflammatory.

11. Inflammatory Imbalance in Type 1 Diabetes and Effects of Exercise

The major underlying mechanism for insulin deficiency in type 1 diabetes is β-cell destruction induced by an autoimmune response. Imbalance between autoreactive Th1 lymphocytes and protective Th2 lymphocytes is found in type 1 diabetes, which leads to both proinflammatory cytokines (IL-2, IL-12, TNF-β, and IFN-γ) and anti-inflammatory cytokines (IL-4, IL-6, IL-10, and IL-13) [175]. Interactions between proinflammatory cytokines (TNF-β and IFN-γ) and the receptors on membrane of β-cells may activate the caspase cascade and result in apoptosis. TNF-β and IFN-γ may also activate macrophages, which leads to the release of TNF-α, IL-1β, NO, and superoxide, which may increase oxidative stress and downregulation of Bcl-2, which activate NF-κB and β-cell apoptosis leading to insulin deficiency [176]. Active macrophages may increase iNOS activity. Elevated NO generation in β-cells may cause oxidative stress, insulin resistance, and β-cell damage [177]. Oxidative stress may reduce insulin secretion from β-cells through stimulating the expression of uncoupling protein 2 (UCP2). UCP2 may inhibit electron transport in mitochondria and increase ROS production. Prolonged hyperglycemia may increase UCP2 in β-cells, which may contribute to insulin deficiency in both type 1 and type 2 diabetes [178]. Relatively less literature is available on the impact of exercise on the clinical outcome or inflammatory mediators in type 1 diabetic animals or humans. A recent study demonstrated resistance exercise before aerobic exercise improved glycemic stability throughout exercise and reduced postexercise hypoglycemia in type 1 diabetic patients [179]. Exercise induced less increase of Hsp70 in insulin-deficient diabetic rats than in control rats [180]. The impact of exercise on inflammatory mediators and the relationship with glucose metabolism in type 1 diabetes remain to be more fully investigated.

12. Conclusion

Both exercise and metabolic stress activate HSF1/Hsp pathway in the body. Transient stress responses induced by regular and moderate exercise tend to downregulate vascular inflammation and protect vessels from injury. Chronic stress responses induced by metabolic disorders upregulate inflammatory mediators, which leads to vascular inflammation, apoptosis, and injury. The HSF1/Hsp-mediated stress response to exercise and metabolic disorders play, distinguishable and possibly opposite roles in vascular inflammation, which may be related to the involvement of different types of Hsp, body temperature, or shear stress of blood flow. The consequences of stress responses induced by exercise and metabolic disorders, particularly of autoimmune diseases such as type 1 diabetes, on vascular inflammation require further investigation.

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Review Article

Heat Shock Proteins: Pathogenic Role in Atherosclerosis and Potential Therapeutic Implications

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Heat shock proteins (HSPs) are a highly conserved group of proteins that are constitutively expressed and function as molecular chaperones, aiding in protein folding and preventing the accumulation of misfolded proteins. In the arterial wall, HSPs have a protective role under normal physiologic conditions. In disease states, however, HSPs expressed on the vascular endothelial cell surface can act as targets for detrimental autoimmunity due to their highly conserved sequences. Developing therapeutic strategies for atherosclerosis based on HSPs is challenged by the need to balance such physiologic and pathologic roles of these proteins. This paper summarizes the role of HSPs in normal vascular wall processes as well as in the development and progression of atherosclerosis. The potential implications of HSPs in clinical therapies for atherosclerosis are also discussed.

1. Introduction

Heat shock proteins (HSPs) were first discovered as being expressed in response to increased temperature, as the name suggests [1]. This family of proteins is highly conserved, displaying high sequence homology between prokaryotes and eukaryotes and between different species [2]. The highly conserved nature of HSPs is a reflection of their essential role in protective mechanisms from stress conditions. At the intracellular level, HSPs act as molecular chaperones and assist in the folding of misfolded proteins, thereby preventing their aggregation. At the extracellular level, HSPs can elicit immunogenic responses. These basic functions of HSPs are evident in the human arterial wall, where HSPs have been shown to be important mediators of protective pathways as well as targets for autoimmunity leading to atherosclerosis [3, 4].

Although the diverse roles of HSPs in normal arterial physiology as well as in atherosclerosis have been discussed in prior reviews, the body of literature at both the basic science and clinical levels has expanded exponentially in this field in recent years [3–5]. As such, the purpose of this paper is to provide an updated overview of our understanding of the role of HSPs in atherosclerosis. In addition, an updated review of the potential clinical implications of HSPs in atherosclerosis-directed therapy is provided as well.

2. Methods

We performed our literature search using MEDLINE, with no limits regarding date of publication. The search terms used were "heat shock proteins" and "atherosclerosis". Limits included articles in English only.

3. Results

3.1. Role of HSPs in Normal Physiologic Processes of the Arterial Wall. The arterial wall is undoubtedly a dynamic structure that continually responds to stresses in its environment [6]. HSPs, which are classified according to their molecular weight, have been implicated in a variety of physiologic processes in the normal arterial wall that are aimed at protecting these structures from such stresses (Table 1). The principal function of HSPs is in protein folding and unfolding. Also, by modulating misfolded proteins, HSPs prevent their aggregation within the cell. Specific subtypes of HSPs, however, exhibit different secondary functions or mechanisms of function.

Table 1: Functions of heat shock proteins.

Heat shock protein molecular weight (kilodaltons)	Function	Pathologic associations
60	Protein folding Protein unfolding Polypeptide assembly Protein translocation across membranes	Atherosclerosis Rheumatoid arthritis Systemic sclerosis Schizophrenia Diabetes mellitus
10	Cofactor for HSP 60	Cardiovascular disease
27	Competes for uptake with lipids Estrogen receptor-β-associated protein	Atherosclerosis
70	Protein folding Protein unfolding Degradation of misfolded or denatured proteins Assembly of new proteins Translocation of proteins across membranes	Atherosclerosis Leprosy Tuberculosis
90	Molecular chaperone involved in protein folding and activation	Atherosclerosis Systemic lupus erythematosus

HSP60, for instance, has been shown to have roles in polypeptide assembly and protein translocation across membranes, in addition to protein folding [7]. Its diverse roles are reflected in the fact that it is found in several intracellular compartments, including the nucleus, cytoplasm, endoplasmic reticulum, and mitochondria [8]. HSP10 acts as a cofactor for HSP60 [9]. HSP27 is a protein associated with estrogen receptor-β, a receptor that is expressed in vascular smooth muscle and endothelium [10]. This HSP subtype has been shown to be protective against atherosclerosis by competing with the uptake of lipids [11]. HSP70 is a two-domain structure consisting of a 45 kilodalton and 25 kilodalton unit, which has several significant roles at the intracellular level, including protein folding, translocation across membranes, and degradation of proteins. HSP70 has also been shown to have anti-inflammatory effects by reducing the activation of the proinflammatory gene transcription factor, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-$\kappa\beta$) [12]. Finally, HSP90 is a molecular chaperone involved in the folding and activation of several proteins integral to transcriptional regulation and signal transduction [13].

3.2. The Link between Autoimmunity to HSPs and the Development of Atherosclerosis

3.2.1. HSP60. HSP60 is the most relevant and well-studied HSP subtype with regards to autoimmunity and development of atherosclerosis. Indeed, earlier studies formulated the hypothesis that T-cell-mediated and humoral immune responses to HSP60 in endothelial regions subject to hemodynamic stress were the initiating event in atherosclerosis [14]. Autoimmunity to HSP60 exists in normal healthy individuals. This stems from its high level of sequence homology with bacterial HSPs, reflecting its conservation through evolution.

3.2.2. HSP65. The HSP60 family also includes HSP65, which is the mycobacterial homologue of mammalian HSP60 [15]. Indeed, more than 95% sequence homology exists between HSP60 from various bacteria including mycobacterial HSP65, and 50%–55% homology exists between human HSP60 and mycobacterial HSP65 with upwards of 70% homology in highly conserved regions [16].

3.2.3. Stress-Related Expression of HSP60 on Vascular Endothelial Cell Surfaces. Under normal conditions, HSP60 is not expressed on the vascular endothelial cell surface. However, under stressed conditions including the traditional risk factors for atherosclerosis, mitochondrial HSP60 is translocated to the cytoplasm and then to the cell surface (Figure 1) [17]. Preexisting immunity to HSP60 then leads to its targeting, with resulting inflammatory cascades and progression of atherosclerosis. In addition to the risk factors for atherosclerosis, the other stressors that can induce expression of HSP60 on the endothelial cell surface include infections, mechanical stress, and temperature change. It is important to mention that in addition to inducing the expression of HSP60 on the vascular endothelial cell surface, the various stressors also induce the expression of adhesion molecules on the cell surface, including VCAM-1, ELAM-1, and ICAM-1 [18]. HSP60 itself induces E-selectin, VCAM-1, ICAM-1, and IL-6 production within the endothelial cell [19]. The upregulated expression of these molecules is an important contributor to the HSP60-directed autoimmune pathogenesis of atherosclerosis.

3.2.4. Immune Cell Types in Atherosclerotic Lesions. With regards to immune cell subtypes, CD4+ T cells are present in the highest concentration in the earliest phases of atherosclerosis. Furthermore, the strongest T-cell reactions against HSP60 are found in intralesional T cells, which display

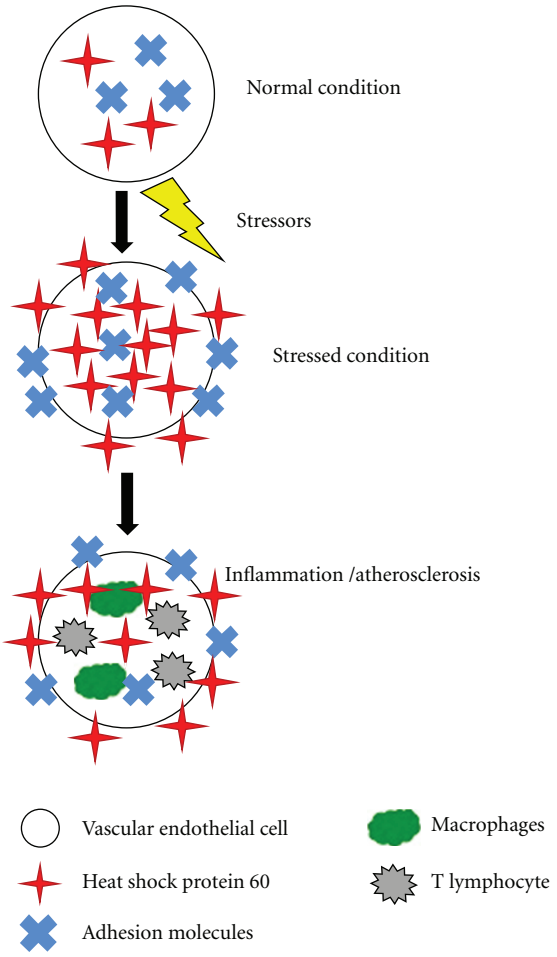

Figure 1: Concept of autoimmunity towards heat shock protein 60 and the development of atherosclerosis. Under normal conditions, heat shock protein 60 is located intracellularly and is not expressed on the vascular endothelial cell surface. Under stressed conditions, heat shock protein 60 and various adhesion molecules are upregulated and expressed on the cell surface. This leads to inflammation and the development of atherosclerosis.

an oligoclonally restricted receptor phenotype, as compared to extralesional peripheral T cells which have weaker reactions to HSP60 and display polyclonal phenotypes [20]. A study of young clinically healthy males found that T-cell reactivity against HSP60 was an independent risk factor for early intima-media thickening (Table 2) [21].

The role of B cells in the development of atherosclerosis is less clearly understood. Several studies have demonstrated progression of atherosclerotic disease with B-cell depletion, whereas other studies demonstrated reduction in the disease with B-cell depletion [22–24]. These differing results may be due to the presence of both atherogenesis-promoting as well as atherogenesis-inhibiting antibodies/mediators. Similar to the different roles of these antibodies, other inflammatory mediators have also been shown to either promote or inhibit atherosclerosis. IL-1β, IL-8, IL-12, IL-18, MCP-1, leukotriene P4, and IFN-γ have been demonstrated as proatherogenic mediators whereas IL-4, IL-10, PDGF-β, and TGF-β have been shown to be antiatherogenic [25].

Table 2: Logistic regression analysis for the impact of various risk factors on high vascular intima-media thickness in a study of 141 young (17- or 18-year old) white males (see [21]).

Risk factor	Odds ratio (95% CI)	*P* value
Cigarette smoking	3.58 (1.34–9.54)	0.0108
High-density lipoprotein level	0.56 (0.36–0.89)	0.0144
Alcohol consumption	0.51 (0.30–0.87)	0.0133
Diastolic blood pressure	1.61 (1.03–2.52)	0.0374
Maximum expiratory flow at 50% vital capacity	0.52 (0.33–0.82)	0.0047
HSP60 stimulation index	2.18 (1.32–3.60)	0.0023
HSP60 antibody titer	1.52 (1.00–2.31)	0.0514

Odds ratios were calculated based on a 1 standard deviation unit change in the given variable.

3.2.5. Soluble HSP60. In addition to being expressed on the endothelial cell surface, HSP60 can be shed into the circulation in a soluble form under stressed conditions. A study of 826 human patients found that levels of soluble HSP60 were significantly elevated in patients with carotid atherosclerosis [26]. The authors postulated that the release of HSP60 from cells may be mediated by infectious agents. More specifically, chlamydiae are known to exhibit both nonlytic and lytic infective phases, and during the latter, the human host cell releases both its own HSP60 as well as chlamydial HSP60. This postulate is supported by evidence that both HSP60 subtypes exist in high concentrations in atherosclerotic lesions, and that soluble HSP60 levels correlate with anti-Chlamydial antibody titers [27]. More recent studies have also shown the association between elevated levels of soluble HSP subtypes and various cardiovascular diseases (Table 3) [15, 28–32].

3.2.6. Serum Antibodies to HSP60 and HSP65. Similar to soluble HSP60, prior studies have also demonstrated elevated serum antibody levels to HSP65, which is the mycobacterial homolog of human HSP60 [33]. A followup study also showed that anti-HSP65 antibodies remained consistently elevated over several years in humans with progressive atherosclerosis [34]. Furthermore, levels of anti-HSP65 antibodies correlated strongly with antibody titers to *Chlamydia pneumoniae* and *Helicobacter pylori*, suggesting an infectious role [35]. Coronary events were observed more frequently in patients that had high HSP60 IgA levels coupled with high titers of antibodies to *Chlamydia pneumoniae* and high C-reactive protein levels [36]. However, a polymerase chain reaction study of 40 atherosclerotic human patients and 20 nonatherosclerotic human controls found similar detection rates of *Chlamydia pneumoniae*, *Mycoplasma pneumoniae*, *Helicobacter pylori*, herpes simplex virus, and cytomegalovirus in the aortic wall, which does not support the infectious etiology hypothesis for atherosclerosis [37].

3.2.7. Establishing Causality in HSP60/65 Antibody-Mediated Atherosclerosis. Although soluble HSP60 and antibodies to HSP60/65 have been shown to be elevated in human patients

Table 3: Soluble heat shock proteins and their association with cardiovascular diseases.

Soluble heat shock protein subtype	Number of patients	Cardiovascular disease	Study finding	Reference
HSP70	24	Acute myocardial infarction	Soluble HSP70 is released into the circulation after an acute myocardial infarction	[28]
HSP60	684	Carotid atherosclerosis	Levels of soluble HSP60 are associated with early carotid atherosclerosis	[29]
HSP70	52 cases 20 controls	Acute myocardial infarction	Levels of soluble HSP70 are associated with progression of heart failure after acute myocardial infarction	[30]
HSP60, HSP72	88 cases 44 controls	Idiopathic left ventricular dysfunction	Levels of soluble HSP60 and HSP72 correlate with severity of cardiac and microvascular dysfunction in patients with idiopathic left ventricular dysfunction	[15]
HSP70	167	Congestive heart failure	Levels of soluble HSP70 are associated with severity of heart failure in patients with congestive heart failure	[31]
HSP60	1003 cases 1003 controls	Coronary artery disease	Levels of soluble HSP60 correlate with the presence of coronary artery disease	[32]

with atherosclerosis, it is unclear whether this simply represents an association or whether a causal relationship exists. Administration of a murine monoclonal antibody (II-13) to amino acid residues 288 to 366 of HSP60 induced atherosclerosis in apolipoprotein E-deficient mice [38]. II-13 injection resulted in endothelial cell damage, leukocyte attachment, and accumulation of macrophages and smooth muscle cells in lesions. The same study demonstrated that isolating anti-HSP60 antibodies from humans with coronary atherosclerosis and injecting them into apolipoprotein E-deficient mice caused significant increases in aortic atherosclerotic lesions [38]. Passive transfer of T cells from mice immunized with mycobacterial HSP65 to nonimmunized mice led to the development of atherosclerosis in the nonimmunized cohort [39].

A study of 120 normocholesterolemic rabbits found that those immunized with recombinant mycobacterial HSP65 had increased atherosclerosis [40]. In the rabbits that were fed a cholesterol-rich diet in addition to being immunized with HSP65, the atherosclerotic lesions were even more severe. A followup investigation by the same group demonstrated that the early atherosclerotic lesions induced by HSP65 could be inhibited by T-cell depletion using an anti-CD3 monoclonal antibody [41].

3.2.8. HSP10. HSP10 is an important cofactor for HSP60 [9]. The significant interplay between these HSP subtypes is further evidenced by the fact that their genes are localized in a head-to-head manner on chromosome 2, separated by a bidirectional promoter [42]. Similar to HSP60, the overexpression of HSP10 is met with an overexpression of BcL-2 and Bcl-xL [43]. These molecules protect vascular endothelial cells from TNF-mediated apoptosis in addition to inhibiting activation of NF-$\kappa\beta$ and thereby inhibiting the upregulation of proinflammatory genes [44]. The antiapoptotic roles of HSP10 are evidenced by the fact that transfecting doxorubicin-treated cardiomyocytes with HSP10 and HSP60 by an adenoviral vector suppresses apoptosis and resulting cardiomyopathy [43]. A study of antibodies to HSP10 of *Chlamydia pneumoniae* in patients with coronary artery disease failed to demonstrate significant differences in levels versus controls; however, the importance of HSP10 to the development of atherosclerosis may indeed lie in its genetic and physiologic link to HSP60 [45].

3.2.9. HSP27. Emerging data has implicated HSP27 in the pathogenesis of atherosclerosis. A study of human atherosclerotic plaques revealed an increase in expression of HSP27 in normal-appearing vessel adjacent to the plaque, with decreased levels in the plaque itself [46]. HSP27 phosphorylation was decreased in both plaque and adjacent vessel compared to reference vessel. And finally, when the investigators examined HSP27 levels in plasma, they found that in patients with acute coronary syndrome, levels of HSP27 were increased and found to correlate with levels of HSP70, C-reactive protein, and CD40L [46].

Another study similarly found that HSP27 release was significantly decreased in atherosclerotic plaques [47]. Circulating levels of soluble HSP27 were also significantly decreased in patients with carotid stenosis compared with healthy controls [47]. A study of 22 heart transplant recipients found that those with cardiac allograft vasculopathy had significantly reduced levels of phosphorylated HSP27 in biopsy samples as compared to those recipients without vasculopathy [48]. The decreased expression of HSP27 within plaques may be related to its degradation by enhanced proteolytic pathways which are known to be important contributors to vascular remodeling [49].

HSP27 may also offer protection from atherosclerosis due to its role in plaque stability. A proteomic analysis of stable versus unstable human carotid artery atherosclerotic plaques found reduced levels of HSP27 in unstable lesions [50]. Moreover, at the molecular level, phosphorylated HSP27 is known to be a regulator of actin filament dynamics [51]. Furthermore, HSP27 may modulate the effects of plasmin and other extracellular mediators of apoptosis in vascular smooth muscle cells, a process which has been shown to lead to plaque instability through the weakening of the fibrous cap of the atheroma with potential plaque rupture and resultant atherothrombosis [52].

In addition, HSP27 has been demonstrated *in vitro* to be released into the extracellular space in response to various stimuli, including estrogen or acetylated low-density lipoprotein, where it binds the scavenger receptor A to prevent low-density lipoprotein uptake and foam cell formation [53]. HSP27, which is an estrogen receptor-β associated protein, also modulates estrogen signaling and may have additional atheroprotective functions via this mechanism [54]. Increased estrogen receptor-β expression has indeed been noted in both males as well as pre- and postmenopausal females with atherosclerosis [55, 56].

In an apolipoprotein E-deficient animal model, overexpression of human HSP27 resulted in a 35% reduction in aortic atherosclerosis in female, but not male, mice [53]. Serum levels of HSP27 were over tenfold higher in females as compared to males, again using the model of HSP27-overexpressing apolipoprotein-E deficient mice. Circulating HSP27 levels demonstrated a strong inverse correlation with atherosclerotic lesion area in both female and male mice [53].

3.2.10. HSP70. In early atherosclerosis, dendritic cells exclusively overexpress HSP70 as well as HLA-DR and CD1d, the latter being a unique molecule used in lipid antigen presentation [57]. These HSP70-expressing dendritic cells also frequently interact with T cells within the arterial wall and therefore may be responsible for presenting lipid antigens to them. Unlike the early stages, several immune cell types, including macrophages, smooth muscle cells, monocytes, and dendritic cells, have been shown to overexpress HSP70 in advanced atherosclerosis [57]. Furthermore, a gene expression profiling analysis revealed that two HSP70 family members were expressed within aortic atherosclerotic lesions but not within nonlesional tissue [58].

Another study found that oxidized low-density lipoprotein stimulated the expression of HSP70 and that supernatants from oxidized low-density lipoprotein-treated macrophages could induce both IL-1β and IL-12 secretion in naïve macrophages [59]. Furthermore, this latter effect on cytokine production was inhibited by inhibiting HSP70 transcription or secretion. Extracellular HSP70 could therefore be an important inducer of cytokine expression and inflammation.

HSP70 may also have anti-inflammatory roles. In one immunization study, a peptide sequence of myobacterial HSP70 was found to induce the production of IL-10 by peptide-specific T cells, a phenomenon that was also seen with T cells responsive to the whole HSP70 protein [60]. IL-10 is known to be a potent anti-inflammatory cytokine, and indeed, its production was found in the prior study to prevent arthritis. Another study found that HSP70 attenuated NF-$\kappa\beta$ activation and its associated proinflammatory gene upregulation [61]. The potentially protective roles of HSP70 were further supported by a study of 421 blood samples from human subjects which found that high levels of HSP70 were associated with low risk of coronary artery disease [62]. Another study also found low plasma levels of HSP70 in patients with atherosclerosis, with activated neutrophils being a potential source for proteases involved in HSP70 degradation [63].

HSP70 may also be implicated in the calcification of blood vessels. HSP70 was found to enhance bone morphogenetic protein-4-induced proliferation in endothelial cells and to enhance bone morphogenetic protein-induced calcium deposition in vascular cells [64]. The same study found that HSP70 mediated the IL-6 procalcific effect on vascular cells. Levels of HSP70, bone morphogenetic protein-4, and IL-6 were all elevated within the aortic wall as well as the serum in a mouse model of atherosclerosis [64]. Antibodies to HSP70 diminished this procalcific effect.

3.2.11. HSP90. A study of human carotid atherosclerosis demonstrated overexpression of HSP90 in both plaque and serum as compared to healthy controls [65]. Moreover, plaque-derived and circulating T cells from patients with atherosclerosis proliferated in response to HSP90 whereas cells from controls did not. Finally, HSP90-specific T cells expressed both proinflammatory and anti-inflammatory cytokines, implying a dichotomous role [65].

Another study of human atherosclerotic plaques found that the expression of HSP90 was associated with plaque instability in advanced lesions [66]. Inhibitors of HSP90 also reduced atherosclerosis-related inflammation in their analysis. Another investigation by the same group found that HSP90 inhibitors interfere with oxidative stress by reducing pro-oxidative factors in experimental atherosclerosis [67].

The potential anti-inflammatory therapeutic benefits of HSP90 inhibitors have been demonstrated in other diseases as well. In a mouse model of systemic lupus erythematosus, HSP90 was found to have a potential role in regulating T-cell differentiation and activation, and its inhibition was associated with reduced inflammation [68]. In a murine sepsis model, the administration of HSP90 inhibitors resulted in reduced systemic and pulmonary inflammatory markers compared to controls as well as improved lung function and survival [69].

3.3. Clinical Implications

3.3.1. Screening, Diagnosis, and Prognosis. There are several clinical implications related to HSPs and their role in atherosclerosis. One potential clinical application would be to exploit the presence of HSP antibodies for screening at-risk patients to detect significant atherosclerosis. A study of 750 human subjects demonstrated the correlation between HSP65 antibody titers and advanced carotid atherosclerotic lesions [29]. Another study similarly showed that anti-HSP65 antibody titers correlated strongly with severity of coronary atherosclerosis [70]. In both of these studies, these findings persisted after adjusting for potential confounders such as patient age and smoking history. In addition to identifying atherosclerosis, there may be a role in identifying patients who have suffered from myocardial infarction. HSP70, for instance, was found to be rapidly released in significant quantities following an acute myocardial infarction in 24 patients, highlighting its potential as a marker for myocardial damage [28].

Screening patients based on titers could be a useful strategy for the detection of significant atherosclerosis, although

Table 4: Summary of studies on potential heat shock protein-related treatments for various diseases.

Study	Disease	Subjects	Study summary and major findings
Maron et al. [74]	Atherosclerosis	Mice	Nasal vaccination with HSP65 resulted in a significant decrease in the size of atherosclerotic plaques, a reduced number of T cells, and an increased IL-10 expression
Harats et al. [75]	Atherosclerosis	Mice	Oral tolerance induced with HSP65 led to a reduction in atherosclerosis
Jun et al. [77]	Atherosclerosis	Rabbits	Vaccine targeting HSP65 and cholesterol ester transfer protein reduced low-density lipoprotein levels and atherosclerotic burden
Ishii et al. [80]	Multiple myeloma	Human	The addition of an HSP90 inhibitor enhanced the antitumor activity of a proteasome inhibitor both *in vitro* and *in vivo*
Kaiser et al. [81]	Acute leukemia	Human	HSP70 inhibitor displayed antileukemic effects both alone and in combination with other antineoplastic agents

the sensitivity and specificity of such a test are unknown, as are the cost implications. Such blood tests may have more utility in directing diagnostic workup for coronary artery disease, particularly in patients with marginal indications for further testing. In these intermediate risk patients, antibody titers could be used as further risk stratification, with those patients with high antibody levels undergoing further workup and those with undetectable or low levels continuing to undergo clinical surveillance.

HSPs may also have a role in prognosis [71]. In a study of 750 patients, HSP65 antibody titers were found to predict 5-year mortality [29]. Another investigation found that among 79 individuals with angiographic evidence of coronary artery disease, anti-HSP65 titers were higher among those with future cardiovascular events than in those without [72]. In addition, in a study of 588 consecutive emergency admissions of patients with acute chest pain of suspected cardiac origin, those with high anti-HSP60 titers had a worse one-year prognosis [73].

3.3.2. Treatment. In addition to its potential screening, diagnostic, and prognostic roles, there may be potential utility for HSPs in the treatment of atherosclerosis (Table 4). There has been growing interest in the role of HSP vaccination in this effort. Mice lacking the receptor for low-density lipoprotein were nasally vaccinated with HSP65 in one study and subject to cholesterol-rich diets [74]. Vaccinated mice had significant decreases in atherosclerotic plaque size, a reduced number of T cells, and an increased IL-10 expression, the latter of which is an antiatherogenic mediator. Another study also found that inducing oral tolerance by feeding low-density lipoprotein receptor-deficient mice with HSP65 led to IL-4 (antiatherogenic) production and a reduction in atherosclerosis [75]. Both of these studies suggest that inducing shifts from a Th1 to Th2 phenotype could be associated with protection from atherosclerosis.

There are clinical concerns with such vaccines given the high sequence homology between human and bacterial HSPs. Inducing tolerance may result in increased susceptibility to serious infections. Despite these concerns, there have been phase I and II trials in the realm of cancer that have demonstrated the safety of similar vaccines. In metastatic melanoma, for instance, vaccination with autologous tumor-derived HSP peptide complexes resulted in only mild toxicity in some patients, limited mostly to local erythema and induration at the injection site [76]. The safety of HSP vaccines directed at atherosclerosis remains to be elucidated, however, as does the longer term safety profiles of these vaccines in general.

There have been several recent studies published regarding potential HSP-related therapies for atherosclerosis. One study examined the effect of a vaccine that targets HSP65 and cholesterol ester transfer protein simultaneously and found more protective IL-10, less adverse IFN-γ, less serum, low-density lipoprotein, and a significant reduction in aortic atherosclerotic plaque burden in vaccine-treated rabbits [77]. An expert opinion piece highlighted the potential of combining regulatory T-cell-targeted therapies using dominant HSP peptides with current biological therapies for autoimmune and inflammatory conditions such as atherosclerosis [78]. Another study in a diabetic rat model concluded a potential therapeutic role of glutamine-induced HSP70 expression, a finding that was observed in both serum and aortic wall [79].

There have also been several recent studies regarding the potential of HSP-related therapies in diseases other than atherosclerosis (Table 4). One such study demonstrated that KW-2478, which is a novel HSP90 inhibitor, enhanced the antitumor effects of bortezomib (a proteasome inhibitor) both *in vitro* and *in vivo* in multiple myeloma [80]. Another study in acute leukemia also demonstrated significant antitumor effects with an HSP70 inhibitor both alone and in combination with other antineoplastic agents [81]. Additionally, an analysis of flavaglines, which are a family of natural products with known neuroprotective properties, found that they also have cardioprotective effects in the setting of doxorubicin therapy, and that this effect is mediated by HSP27 [82]. Finally, remote ischemic preconditioning was found to reduce spinal cord damage in a rat model likely through HSP70 overexpression [83].

4. Conclusions

A growing body of evidence in both animal models and human subjects has implicated autoimmunity towards HSPs as a potential pathogenic mechanism for the development of atherosclerosis. Ongoing and future studies that further elucidate the mechanisms whereby HSPs, infection, and immune response pathways interact and lead to the common pathway of atherosclerosis will be essential to developing more specific and potentially safer novel therapies for this devastating disease process. A better understanding of the functions of HSPs in other pathologies such as cancer may also be useful in advancing our knowledge of the role of this important family of molecules in atherosclerosis and their potential therapeutic utility.

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Review Article

CD91-Dependent Modulation of Immune Responses by Heat Shock Proteins: A Role in Autoimmunity

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Heat shock proteins (HSPs) have been known for decades for their ability to protect cells under stressful conditions. In the 1980s a new role was ascribed for several HSPs given their ability to elicit specific immune responses in the setting of cancer and infectious disease. These immune responses have primarily been harnessed for the immunotherapy of cancer in the clinical setting. However, because of the ability of HSPs to prime diverse immune responses, they have also been used for modulation of immune responses during autoimmunity. The apparent dichotomy of immune responses elicited by HSPs is discussed here on a molecular and cellular level. The potential clinical application of HSP-mediated immune responses for therapy of autoimmune diseases is reviewed.

1. Introduction: HSPs in Immunity

Expression of HSPs is generally upregulated in cells in response to a variety of stressful conditions including nonphysiological temperature, nutrient deprivation, and hypoxia [1]. It is the inherent chaperoning function of HSPs that allows them to provide their cytoprotective function in assisting correct protein/polypeptide folding and preventing further protein denaturation. It has become evident over the past two decades that the chaperoning function of HSPs also plays a key role in several processes during the development of immune responses [2]. Within the cell, several HSPs act as chaperones of peptides that are ultimately presented by MHC I and MHC II molecules. Thus, the HSPs in the cytosol and in the endoplasmic reticulum form a relay line for the transport of peptides from their formation by the proteasome to the MHC I heavy chain (HC). This is discussed in the next subheading. As the HSPs are some of the most abundant proteins in cells, their liberation into the extracellular environment has been shown to be a key indicator of loss of cellular integrity and they are rapidly recognized by the cellular sentinels of the immune system. Such recognition allows for cross-priming of the potential antigens that the HSPs chaperone. The efficiency of this pathway predicted a cell surface receptor on the cross-presenting cells and that receptor has now been shown to be CD91. These events are discussed in the next two subheadings. The isolation of HSPs (and the associated peptides) from tumor cells or cells infected with pathogens therefore provides a single entity that primes immune responses specific for the chaperoned peptides and thus for the cell that harbored these antigens. This application has been tested in a vast number of rodent models of cancer and infectious disease and is being tested in the clinical setting. We discuss this in the third subheading. A search for optimal immunizing doses of HSPs led to the fortuitous dampening of the immune response at higher doses of HSPs. This phenomenon has been applied to the therapy of autoimmunity and is the focus of the last subheading. This chapter is largely restricted to the HSPs gp96, hsp70, hsp90, and calreticulin although others such as hsp110, grp170 have been shown to elicit similar immune responses [3, 4].

2. HSPs Form a Relay Line in MHC I Antigen Processing and Presentation

The classical and current view of antigen processing and presentation by MHC I can be summed up as follows: production of peptides occurs within the cytosol by the

large multi-subunit catalytic body called the proteasome. The proteasome ingests polypeptides and trims them down to small peptides usually with the correct C-termini, but extended N-termini, for MHC I binding. The transporter associated with antigen processing (TAP) pumps peptides into the ER in an ATP-dependent manner. Additional N-terminal trimming proteases such as the ER-associated aminopeptidase (ERAP) are present in the ER for final processing [5]. For a period these peptides were envisioned to diffuse to TAP, and once in the ER, they diffuse and get loaded onto MHC HC with the assistance of the peptide loading complex. We now know that diffusion plays a minor role if any at all in antigen presentation; instead, peptide trafficking is possible only because peptides are actually chaperoned by HSPs. This was first proposed in 1994 [6] and the evidence for the involvement of HSPs in peptide processing and presentation is now abundantly clear and comes in both direct and indirect forms. The evidence is as follows: (i) disruption of peptide binding by HSPs abrogates MHC I peptide presentation [7–9], (ii) the extremely poor efficiency of diffusion of peptides within the cell cannot account for the calculated efficiency of antigen presentation [10, 11], unless other more efficient methods of peptide trafficking, such as the HSP chaperoning effect, are integrated into the pathway, (iii) free peptides have not been found in cells even after a careful search for them [12]. The peptides are readily seen once they are released from HSPs by protein denaturation or treatment with ATP or acid [13], (iv) given the hydrophobic residues of amino acids in the hydrophilic environment, solubility issues that this poses [14] must be resolved, and peptide association with HSPs does so, (v) isolation of peptides from highly purified HSP preparations reveals MHC binding peptides and their precursors (intermediates of the processing events) [15–20]; (vi) peptides chaperoned by hsp90 in the cytosol are less processed (longer) than those chaperoned by the ER HSP gp96 revealing a continuum of processing events by proteases in different compartments [15], and (vii) shuttling of MHC binding peptide precursors between HSPs and MHC I HC has been observed in the ER [17]. These lines of experimental evidence suggest and provoke the idea that the relatively recent evolutionary development of the MHC I antigen processing and presentation pathway, a key component of adaptive immunity, has taken advantage of the ancient property of HSP chaperoning.

3. HSP-Peptide Complexes in Antigen Cross-Presentation

The initial event in priming T-cell responses to cancer or infectious disease involves the transfer of antigens from the cells that harbor them to antigen presenting cells. This pathway is called cross-presentation and allows for the presentation of antigens in the form of peptides to T cells in the context of MHC molecules. Cross-presentation of antigens by APCs also directs that the antigen is presented in the context of costimulation, which is a combination of cytokines and a series of APC-T cell interactions through receptors and their corresponding ligands. The specific costimulation received by the naïve T cell dictates the type of T cell response that is primed. HSPs have been shown to play a critical role both in cross-presentation of antigens and in provision of a dynamic set of signals for costimulation.

Calculations on the amount of antigen that is available for cross-presentation in two independent studies have shown that it is insufficient if the antigens were transferred as a whole protein [21, 22]. This triggered an investigation into the role of HSP-antigen complexes as a necessary alternative to antigen transfer during cross-presentation. Antigens chaperoned by HSPs are cross-presented approximately 50,000 times more efficiently than naked protein and/or peptide alone [22]. This increase of efficiency is in large part due to the presence of the HSP receptor CD91 that is present on APCs [23, 24]. Although the role of HSPs in cross-presentation has been demonstrated *in vivo*, it has been modeled *in vitro* in several antigenic systems in mice and humans over a period of many years (Table 1). These studies have shown that the initial interaction of the APC with the HSP is mediated through the cell surface receptor CD91 and potentially others. The evidence, or lack thereof, for these other suggested HSP receptors is discussed elsewhere [25]. Following binding, the HSP with the chaperoned peptide is internalized into endosomal vesicles. Through an as yet unidentified mechanism, peptides are delivered to the cytosol for trimming by the proteasome where they enter the MHC I processing and presentation pathway. Other mechanisms include internalization of HSP-peptide complexes into MHC I containing vesicles where there can be direct peptide transfer between these molecules [26–30]. However with this mechanism only fully processed peptides chaperoned by HSPs would be presented by MHC I. The requirement for MHC I antigen processing machinery such as the proteasome and TAP appears to be dependent on the antigenic system being tested [7, 24, 31]. Other mechanisms leading to presentation of peptides chaperoned by HSPs may also be dependent on the HSP chaperoning the antigen, as endogenous and exogenous hsp90 have been shown to be directly involved in transendosomal membrane transport [32, 33]. Since the peptides bound to HSPs do not appear to be limited by length or amino acid sequence, HSP chaperoned peptides can also be presented by MHC II of the APC to stimulate $CD4^+$ T cells [34–37].

By extension of this very efficient mechanism of cross-presentation of HSP-chaperoned antigens, immunization with HSP-antigen complexes primes antigen-specific T-cell responses while comparable amounts of antigen alone does not. This has been demonstrated with gp96 [19, 59–63], hsp90 [15, 62], hsp70 [13, 19, 62], calreticulin [64, 65], hsp110 [3, 4], and grp170 [3, 4]. In these immunization regimens, the HSP-peptide complexes can be purified intact from the antigen bearing cell. Thus, purification of HSPs from tumor cells will yield complexes that represent the entire antigenic fingerprint of that tumor and will prime T-cell responses specific for that tumor. The same applies to cells infected with bacteria or viruses and cells expressing minor histocompatibility antigens or model antigens. The peptides bound to HSPs are not restricted to the MHC

Table 1: A summary of reported antigen cross-presenting systems described for HSP-chaperoned peptides and the corresponding receptor.

HSP	Antigen presenting cell	Uptake mechanism	Reference and year	Notes
rhsp70 (bovine), rhsp70 (human)	Bovine cultured monocytes, B-LCL lines L721.45 and L721.174, and human monocytes	Macropinocytosis	[38] 2010, [39] 2007	CD4 stimulation
rgp96, gp96 and hsp70, rhsp70 (human), hsp70 (human), rhsp70	BMDC, macrophage cell line (p388d1) and DC line (D2SC1), RAW264.7 and RAW309Cr.1, human monocytes, elicited PEC	Receptor (unidentified) mediated endocytosis	[40] 2011, [26] 1999, [41] 2008, [42] 2002, [31] 2000	Differences in lipid raft involvement with N or C terminus of HSP70, also MHC II presentation
gp96, hsp90, hsp70, CRT, (*E. coli* and Mtb) hsp70, gp96 (frog), rhsp70 (human)	B-LCL, human DC, RAW264.7 and peritoneal macrophages, BMDCs, BM-macrophages, CD11c+ cells, frog macrophage-like cells, human PBMC	CD91	[35] 2004, [23] 2000, [24] 2001, [43] 2004, [44] 2004, [45] 2008, [46] 2011	MHC I and II presentation
rhsp70 (human) and rhsp60 (human)	Human monocytes, epidermal LC	CD91	[28] 2002	Internalization with MHC I and II
gp96	Splenic APC, B220+, CD11c+, CD11b+, BMDC	CD91 and partial Lox-1	[37] 2010	MHC II presentation
rhsp70 (human)	Monocytes, MDDC	CD91 and partial CD36/scavenger receptor	[47] 2010	MHC II presentation and CD4 memory
rhsp70 (*M. avium* paratuberculosis)	Bovine macrophage cell line BoMac, bovine DC, macrophages, and monocytes	CD91 and other	[48] 2005	
gp96 (porcine), rgp96 (canine, NTD)	Elicited peritoneal macrophages, MEF-1, PEA-13, DC2.4		[49] 2002, [50] 2010	MHC I and II presentation
hsp90 (human, purified), rhsp72 (human)	RMA-S/A*2402, BMDC	Receptor dependent	[30] 2007	
rhsp90 (human)	BMDC, CHO, HeLa and RAW264.7	SREC-1 and Lox-1	[51] 2010	
rCRT	DC2.4, elicited peritoneal macrophage, MEF-1, PEA-13	SREC-1	[52] 2005	
gp96 (porcine) and rCRT	CHO, elicited peritoneal macrophages, BMDC	SREC-1	[53] 2004	
rhsp70	Human myeloid DC, monocytes, macrophages, CD19+ cells	Lox-1	[54] 2002	
gp96 (porcine) and rCRT	Elicited peritoneal macrophages, RAW264.7, HEK	SRA	[55] 2003	
rgp96 and rCRT	Fibroblasts, BMDC and BM macrophages	SRA and other	[56] 2008	
rgrp170 and rhsp110	RAW264.7, DC1.2, CHO, BMDC	SREC-1 and SRA	[57] 2007	
gp96	D2SC/1, D1, BMDC, splenic DC, macrophages, and B cells	Receptor dependent but not MHC class II or DEC205	[27] 2000	Internalization with MHC I and II
gp96 (porcine)	Elicited peritoneal macrophages, RAW264.7, CHO, COS7, BRL	Unidentified receptor mediated and macropinocytosis	[58] 1999	
gp96 (porcine)	Elicited peritoneal macrophages, RAW264.7, BMDC	Unidentified receptor mediated and macropinocytosis	[29] 2002	

haplotype of the originating cells [66]. Various methodologies are also currently available to artificially bind peptides to HSPs noncovalently [67] or covalently through fusion constructs to form immunogenic complexes [68]. In most cases, the immune response measured after priming is of the Th1 phenotype and characterized by $CD8^+$ cytotoxic T lymphocytes. In a few situations, immunization with HSPs has led to priming of Th2/antibody or Th17 responses [69–72].

4. CD91 Is Pivotal in Regulating HSP-Mediated Costimulation

HSP-chaperoned peptides can be cross-presented by professional APCs; however, presentation and recognition of antigen alone by T cells are not sufficient to prime adaptive immunity. In order to prime T and/or B cells, help is needed from an expanding family of costimulatory molecules on APCs. The cytokine milieu provides additional signals for activation and expansion of these effector cells. The immunogenic HSPs were the first endogenous molecules proven to be particularly adept in stimulating APCs to provide costimulation [73]. Studies have shown that the signals provided by the HSPs to the APCs do not occur through the traditional pattern recognition receptors which include the TLRs. Rather, recent studies have shown that the immunogenic HSPs utilize CD91 to transmit signals to the APC [71]. Primary APCs were shown to be activated by HSPs in a CD91-dependent manner suggesting that CD91 was acting as a signaling receptor for the immunogenic HSPs. The β-chain of CD91, which has two NPXY sequences that are consensus motifs for phosphorylation and signal transduction, was subsequently mutated. Upon tyrosine to phenylalanine mutation, CD91 failed to transmit intracellular signals in response to HSP stimulation, abrogating the costimulation provided by the APC. The signaling pathway(s) initiated by CD91 upon HSP stimulation involves the activation of NF-κB and p38 MAPK although other molecules are yet to be identified. Downstream of intracellular signaling, a number of cytokines are released by HSP-stimulated APCs including TNF-α, IL-1β, IL-6, IL-12, and GM-CSF [71]. Other studies have shown that, in addition to cytokine production by HSP-stimulated APCS, the APCs upregulate expression of costimulatory molecules and maturation markers including CD80, CD86, CD40, and MHC II [74]. The complete array of costimulatory molecules and cytokines is dependent on the type of APC (macrophage or DC subsets) that is stimulated and the HSP (hsp70, hsp90, calreticulin or gp96) that is used for stimulation (Table 2).

CD91 thus has a role in signal 1 (cross-presentation) and 2 (costimulation) that is provided by the APC to T cells in response to extracellular HSP. Similar to HSP-mediated cross-presentation of peptides, other receptors besides CD91 have been suggested to be signaling HSP receptors. However there is abundant published literature that the suggested TLR2/4 receptors were implicated because of the use of endotoxin-contaminated HSP preparations, especially from recombinant sources. A discussion of HSP receptors has been published elsewhere [25]. The flexibility in the pattern of costimulation triggered by various HSPs in a variety of experimental settings has implications in several fields of immunology and we focus here on a discussion on a role in autoimmune diseases.

5. Extracellular HSPs and the Etiology of Autoimmunity

As discussed above, APCs stimulated with various HSPs secrete proinflammatory cytokines such as IL-1β, IL-6, and TNF-α among others (Table 2). In addition, HSPs chaperone self-peptides that can be cross-presented as efficiently as the antigenic peptides. The former of these events has the potential to trigger chronic inflammation during the continuous presence of HSPs in the extracellular environment. The two events concurrently can prime autoreactive T cells if such T cells are not thymically deleted. In at least one autoimmune disease, this concept is strongly supported. The etiology of rheumatoid arthritis remains largely unresolved; however, several factors that *contribute* to the initiation and/or progression of the disease can be pinpointed. The observation of elevated levels of hsp70 in synovial fluids from inflamed joints of RA patients is one of these factors. Hsp70 is found both within the fibroblasts at the joint and in the fluid itself [99, 100]. The significant increase in extracellular hsp70 in arthritic joints is profoundly correlative because nonarthritic joints in the same individual patients have no elevation in hsp70. As mentioned above, hsp70 can interact with its cell surface receptor CD91, and potentially other receptors, on cells to induce the release of proinflammatory cytokines such as IL-1β, IL-6, and TNF-α. The observation of elevated hsp70 levels in synovial fluids from inflamed joints implicates hsp70 as an initiator of inflammation and/or a perpetrator of these events. In this disease hsp70 will chaperone self-peptides that can be cross-presented by local APCs [101]. Such cross-presentation of self-antigens appears to be sufficient to break tolerance and for priming self-antigen-specific T cells which could contribute to cellular destruction observed in arthritic joints.

6. HSP Immunotherapy for Autoimmune Diseases

Immunization of mice with HSPs typically elicits Th1 responses characterized by CTL specific for antigenic peptides chaperoning the HSP. Optimal immunizing doses range from 1 to 10 μg at the intradermal or subcutaneous route. Upward titrations of this dose revealed a surprising and apparently paradoxical result. Doses of HSPs that were 10 times higher than the immunizing dose administered to mice were shown to prime an immunosuppressive phenotype characterized by expansion of $CD4^+$ Tregs [60, 102–104]. The immunosuppressive response could also be transferred to naïve mice by transfer of the expanded $CD4^+$ Treg cell population [103]. The phenotype is observed when there is a prior ongoing CTL response, and in at least one system, Tregs from high dose gp96-immunized mice significantly

Table 2: A summary of reports showing activation of APCs by HSPs.

HSP	Species	Antigen presenting cell	Receptor	Innate immune responses					Reference and year
				Cytokine	Chemokine	NO	Maturation	NF-κB	
hsp60, hsp70	Bacteria, human, mouse	Peritoneal macrophage	ND	IL-12					[75], 1996
hsp60	Human	Macrophage	CD14	TNF-α, IL-12, IL-15		Yes			[76], 1999
hsp60	Chlamydia and human	Human EC, SMC, and macrophage	ND	IL-6				Activation	[77], 1999
gp96, hsp70, hsp90	Mouse	DC	ND	TNF-α, IL-12, IL-1β			↑MHC II, B7-2	Activation	[74], 2000
hsp70	Human	Monocyte	CD14	TNF-α, IL-1β, IL-6				Activation	[78], 2000
gp96	Mouse	human DC	ND	TNF-α, IL-12			↑MHCII, CD86		[79], 2000
hsp60	Mouse	Macrophage	TLR4	TNF-α		Yes			[80], 2000
gp96	Mouse	CD11c$^+$ cell	ND				↑MHC II, B7-1, B7-2, migration		[81], 2000
hsp70	Mouse	Mouse splenocyte	ND	TNF-α, IL-1β, IL-6					[82], 2000
hsp70	Mouse	Mouse BMDC	ND	TNF-α, IL-12, IL-1β, IL-6					[83], 2000
rhsp70	Human	DC	ND				↑CD40, CD86, CD83		[84], 2001
Mtb hsp70	Mycobacteria	THP1, KG1 cells, and DC	CD40		RANTES				[85], 2001
hsp70, gp96	Human	DC	ND				↑HLA-DR, CD40, CD86, CD83, and DC-LAMP		[86], 2001
hsp70	Human	DC	Toll/IL-1R	IL-12					[87], 2002
gp96	Human	BMDC	TLR2/4	IL-12			↑CD86	Activation	[88], 2002
gp96 and hsp70	Human and mouse	Macrophage, immature DC	ND			Yes			[89], 2002
rhsp70	Mycobacteria	Human DC	CD40	IL-12, TNF-α	RANTES	Yes	↑CD83, CCR7, CD86, CD80, and MHC II		[90], 2002
hsp60	Human	DC	TLR4	TNF-α, IL-12, IL-1β					[91], 2003
rhsp70	Human	DC	CD40	IL-12p40					[92], 2003
hsp72	Rat	Splenocyte and macrophage	ND	TNF-α, IL-1β, IL-6		Yes			[93], 2003

Table 2: Continued.

HSP	Species	Antigen presenting cell	Receptor	Innate immune responses					Reference and year
				Cytokine	Chemokine	NO	Maturation	NF-κB	
hsp70L1	Human	DC	CD91, TLR2/4	TNF-α, IL-12p70, IL-1β	IP-10, MIP-1α, MIP-1β, RANTES				[94], 2004
hsp60	Human	Mouse B-cell	TLR4	IL-10 and IL-6			↑MHC II, CD69, CD40, and B7-2		[95], 2005
HSPB8	Human	DC	TLR4	TNF-α, IL-6, IL-12, IL-10			↑CD80, CD83, CD86 and MHC II		[96], 2006
hsp70	Mouse	Tumor cells and DC	TLR4		CXCL10		↑CD80, CD86, CD40, MHC II	Activation	[97], 2009
gp96	Human	Macrophage	TLR2	TNF-α, IL-8					[98], 2009
gp96, hsp70 Calreticulin	Mouse	RAW264.7, or PEC	CD91	TNF-α, IL-1β, IL-6, etc.	CXCL10 (IP-10), CXCL11 (IP-9)			Activation	[71], 2012

ND: not determined; NO: nitric oxide; PEC: peritoneal exudate cells; BMDC: bone-marrow-derived dendritic cells; DC: dendritic cells; EC: endothelial cell; SMC: smooth muscle cell.

suppressed the IFN-γ production by autologous $CD4^+$ and $CD8^+$ T cells [105].

The application of this "high dose" phenomenon has been tested in mouse models of autoimmunity including diabetes and experimental autoimmune encephalomyelitis [103]. In both models, administration of "high doses" of HSPs reduced the severity of disease or prevented its development outright. In the diabetic model, high doses of HSP were administered to NOD mice that were older than 4 months at which point β-islet-specific pathogenic T cells were already present in the pancreas. In the EAE model, high dose of HSP was administered after immunizing mice with the MOG peptide which primes pathogenic $CD8^+$ T cells. The HSPs used in these studies did not chaperone any antigenic peptides related to the disease model and led to the conclusion that the ability to prime Treg cells was inherent to the HSP molecule itself. The less preferred explanation would be a common self-peptide associated with gp96 regardless of the source of HSP in a global disease setting. Our current understanding of the diversity of responses of APCs stimulated with HSPs (Table 2) sheds some light on the mechanism. It strongly suggests that the disparity of responses in immunization with high and low doses of HSP results from targeting different sets of APCs, possibly through CD91 and additional cell surface receptors, leading to a new repertoire of cytokines and/or costimulatory molecules. Higher doses of HSPs may target different subsets of APCs or stimulate multiple receptors in the same APCs (as the immunogenic dose) to elicit distinct costimulatory profiles [105]. The alternative costimulation could include TGF-β, PD-L1, and other Treg skewing molecules and would be predicted to be dominant over other signals. This area is under investigation and definition of these mechanisms will offer novel targets for inhibition in autoimmune diseases.

These data suggest a delicate balance between regulatory and effector T cells mediated by HSPs and is supported by the recent demonstration of significant enhancement of gp96-primed CTL activity after anti-CD25 treatment [106]. By blocking Treg generation, gp96 was able to mediate stronger peptide-specific CTL responses in BALB/c mice and synergistically enhanced gp96 tumor vaccine-induced antitumor immunity.

7. Conclusions

Over the past 3 decades the various roles of HSPs in the immune systems have been explored and characterized. It appears that the evolutionarily ancient chaperone functions of HSPs in binding peptides and proteins have been commandeered by the relatively recent development of the adaptive immune system. However recent studies suggest that, parallel to evolution of innate responses, multicellular organisms are alerted to aberrant cellular damage by utilization of pre-existing receptors (CD91) to detect the presence of abundant intracellular molecules (HSPs). We draw many similarities between the innate immune responses elicited by PAMPs through PRRs and those by HSPs through CD91 in terms of costimulation for T-cell priming. Indeed the HSP-CD91 network has been well documented not only in mammals but also in amphibians. While CD91 is a well-studied receptor for HSPs (Table 1), there may be other molecules that may serve as receptors, offering a diversity of responses that may be elicited by each HSP. Again, the diversity of PRRs for recognition of various PAMPs is well noted in innate immunity. The immune responses primed by extracellular HSPs are dictated by the costimulation that is elicited and is as diverse as the APC the HSP will encounter. The immune responses range from antitumor and pathogen immunity to suppressive responses, with the latter being applied to the therapy of autoimmune diseases. With greater understanding of the immunobiology of these proteins, we anticipate that vaccine design will be enhanced.

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Review Article

Role of Hsp90 in Systemic Lupus Erythematosus and Its Clinical Relevance

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Heat shock proteins (HSP) are a family of ubiquitous and phylogenically highly conserved proteins which play an essential role as molecular chaperones in protein folding and transport. Heat Shock Protein 90 (Hsp90) is not mandatory for the biogenesis of most proteins, rather it participate in structural maturation and conformational regulation of a number of signaling molecules and transcription factors. Hsp90 has been shown to play an important role in antigen presentation, activation of lymphocytes, macrophages, maturation of dendritic cells, and in the enhanceosome mediated induction of inflammation. Systemic lupus erythematosus (SLE) is a chronic autoimmune inflammatory disease with complex immunological and clinical manifestations. Dysregulated expression of Type I interferon α, activation of B cells and production of autoantibodies are hallmarks of SLE. The enhanced levels of Hsp90 were detected in the serum of SLE patients. The elevated level of Hsp90 in SLE has also been correlated with increased levels of IL-6 and presence of autoantibodies to Hsp90. This suggests that Hsp90 may contribute to the inflammation and disease progression and that targeting of Hsp 90 expression may be a potential treatment of SLE. The pharmacologic inhibition of Hsp90 was successfully applied in mouse models of autoimmune encephalomyelitis and SLE—like autoimmune diseases. Thus targeting Hsp90 may be an effective treatment for SLE, especially if combined with other targeted therapeutic approaches.

1. Introduction

Heat shock proteins (HSPs) are a family of ubiquitous and phylogenically highly conserved proteins and play an essential role as molecular chaperones in protein folding and transport within the cell [1]. These proteins are named according to their molecular weight, which ranges from 17 kDa small HSP families to more than 100 kDa, and they are classified into six families, namely, the HSP100, HSP90, HSP70, HSP60, and HSP40. The classification of HSPs is based on their related functions and sizes (molecular masses). Using the nomenclature adopted after the Cold Spring Harbor Meeting of 1996 [2], family names are written in capitals, for example, HSP70. Major classes of HSPs include the small HSPs, HSP40, 60, 70, 90, and 110. Interestingly, HSP60, 70, and 90 families are the major HSPs implicated in autoimmune diseases, antigen presentation, and innate immunity, Hsp90 is one of the most abundant proteins in the eukaryotic cell. It constitutes up to 1-2% of the cellular protein under physiological conditions, and its expression is several-fold enhanced in response to stress. Under physiological conditions, HSPs exert housekeeping functions and act as molecular chaperones [3, 4] that assist in the proper folding of nascent polypeptide chain preventing their aggregation and misfolding, or as chaperonins that directly mediate protein folding. Moreover, HSPs also play an important role in preventing protein aggregation, degrading unstable and misfolded proteins, and transporting proteins between cellular compartments. Their basal levels facilitate normal protein folding and guard the proteome from the dangers of misfolding and aggregation [5]. Heat shock proteins are expressed both constitutively and under stressful conditions. In addition to the heat shock, a variety of stressful situations, including environmental (ultraviolet radiation or heavy metals), pathological (infections or malignancies), and

physiological (growth factors or cell differentiation) stimuli, induce a marked increase in HSP synthesis, a phenomenon known as the stress response [6]. Traditionally, HSPs are regarded as intracellular molecules; however, upon necrotic, but not apoptotic, cell death, HSPs are released into the extracellular compartments [7]. In addition, HSPs can be released extracellularly in response to a number of stressful conditions [8, 9]. The mechanism and the physiological significance of the HSP release independent of necrotic cell death are not clear. However, HSPs are present in circulation of normal individuals [10], and their circulating levels are decreased in aging [11], and increased in a number of pathological conditions [12]. Their increased expression in tissues that are subjected to various proteotoxic stressors (including heat, heavy metals, hypoxia, and acidosis) is an adaptive response that enhances cell survival. Both functions are needed under diseases state. The aim of this paper is to investigate the structure, localization, and regulation of highly conserved HSP90 and its role in SLE by looking for HSP90 containing immune complexes in kidney biopsies of lupus patients. It also describes the role of HSP90 and its family members in etiology of systemic lupus erythematosus and its potential use in designing appropriate therapeutic approaches.

2. Structure, Localization, and Regulation of HSP90

In vertebrates, two distinct genes encode inducible and constitutively expressed isoforms of the protein (HSP90-α and HSP90-β), but the functional differences between these isoforms are not well understood [13]. Homologues of HSP90 are also found in the endoplasmic reticulum as glucose related protein 94 (GRP94) and in the mitochondria as TNF receptor associated protein 1 (TRAP1). While HSP90 resides primarily in the cytoplasm, one of the HSP90 variant (HSP90N) with a unique hydrophobic N-terminal domain has been found to be a membrane-associated protein. However, its precise cellular function is not clear [14]. The cytoplasmic HSP90 exists predominantly as a homodimer and each homodimer is made up of monomer units which consist of three main domains which are involved in important functional interactions with other cellular targets. The N-terminal domain contains an unusual adenine-nucleotide binding pocket known as the Bergerat fold [15]. The hydrolysis of ATP to ADP in the Bergerat fold has an essential role in the chaperoning activity of the HSP90 dimer. In eukaryotes, a flexible, highly charged linker sequence connects the N-terminal domain to the "middle region" of HSP90. Most molecular chaperones share common functional domains: an adenine nucleotide-binding domain that binds and hydrolyzes ATP and a peptide-binding domain that binds exposed hydrophobic residues of substrate proteins. Binding of ATP triggers a critical conformational change leading to the release of the bound substrate protein [16]. As folding of most newly synthesized proteins in the cell involves interactions with one or more chaperones, protein-binding sites of HSPs by necessity have a broad specificity, and their binding to other cellular proteins is facilitated by hydrophobic interactions [4].

Under conditions of stress, such as heat shock, inducible HSPs are highly upregulated by heat shock factors (HSF), which are generated as part of the heat shock response (HSR), to maintain cellular homeostasis and to develop cell survival functions. The heat-shock factors (HSFs) bind to the heat-shock element (HSE) in the promoters of the genes encoding hsps. Four heat shock factors (HSFs) have been identified and well characterized and their roles clearly elucidated. The functional role of HSF1 and HSF3 has been linked to regulating Hsps in response to thermal stress whereas HSF2 and HSF4 are involved in Hsp regulation in unstressed cells and have been linked to a wide variety of biological processes such as immune activation and cellular differentiation [17]. The stresses results in HSF1 oligomerization and nuclear translocalization, followed by enhanced DNA binding on the Hsp gene promoters. It was shown recently that HSF1 is negatively regulated by Hsp90, thus suggesting a negative-feedback loop for the regulation of Hsp90 genes following a heat-shock response [18]. During heat shock response, HSF1 is known to undergo post-translational modification by various processes including phosphorylation, acetylation, and sumoylation [17]. The HSF2 has also been shown to be bound to the HSE promoter elements of other heat-shock genes, including Hsp90 and Hsp27, as well as the protooncogene c-Fos [19]. These data suggest that HSF2 is important for constitutive as well as stress-inducible expression of HSE-containing genes.

3. Role of HSP90 and Its Homologues in Autoimmune Diseases

Infection is a stressful process for both the pathogen and the host and therefore inevitably results in increased production of molecular chaperones by the pathogen as well as by the host. The conservation of HSPs through prokaryotes and eukaryotes, together with the increased production of host and microbial HSPs at the site of infection, suggests that cross-reactivity between host and pathogen HSPs might be responsible for a variety of autoreactive disorders that are associated with high frequency recognition of HSPs [20]. In this context, the possible involvement of mycobacterial HSP70 in the autoantibody production in systemic lupus erythematosus (SLE) has been indicated in one study [21].

Autoimmune diseases remain among the most poorly understood and recognized categories of illnesses in the world. Many autoimmune diseases are much more common in women than in men, and estrogens exacerbate systemic lupus erythematosus in murine models of the disease by altering the B-cell repertoire in the absence of inflammation [22]. In addition to serving as molecular chaperones, HSPs have been implicated in autoimmune diseases, antigen presentation, and tumor immunity. Considerable work has also suggested that HSPs, such as Hsp60, Hsp70, Hsp90 and gp96, may be potent activators of the innate immune system capable of inducing the production of proinflammatory cytokines by the monocyte-macrophage system and the

activation and maturation of dendritic cells *via* the TLR2- and 4-signal transduction pathways [23].

Chaperones function as stimulators of the innate immune system; HSPs have been also shown to play a role in generating antigen-specific T-cell responses [24]. The proposed mechanism is that peptides, complexed with the HSPs including HSP70, Gp96, and calreticulin are delivered to antigen-presenting cells (APCs) by receptor-mediated internalization of the HSPs, making them available for processing and presentation on major histocompatibility complex (MHC) molecules. Specific receptor-mediated mechanisms exist for the capture and internalization of HSPs, suggesting that cross-presentation of HSP-derived antigenic determinants is a legitimate mechanism for cross-priming by professional APCs. Moreover, HSPs can be upregulated in different pathologic conditions and serve as specific targets of the adaptive immune response. Regardless of the participation of HSPs in the pathogenesis of autoimmunity via antigenic cross-reactivity, HSPs are capable of eliciting immune responses [25]. Autoantibodies and cells reactive to HSP have been detected in patients with rheumatoid arthritis, [26], SLE [26] inflammatory bowel disease [27] and multiple sclerosis [28]. The role of this autoimmune response to HSPs in various diseases has not been yet identified. In one case, autoantibodies to HSP90 have been correlated with elevated levels of IL-6 in SLE [12].

Misdirected immune responses target self-antigens and induce severe inflammatory responses, which is a typical sign of autoimmune diseases and sometimes cause death. In addition to numerous components in autoimmune responses, heat shock proteins (HSPs) have been also implicated in autoimmune and inflammatory diseases [29, 30]. Because protein folding is easily impaired by various cytotoxic stresses, such as heat shock, cytotoxic chemicals, hypoxia, and inflammation, prokaryotic to higher eukaryotic organisms have evolved unique mechanism to respond to these stresses. The transcription of HSPs and their subsequent protein expression are stimulated by cytotoxic stresses, and they immediately restore protein folding and cellular homeostasis to counter toxic stresses [5]. Thus, HSPs could be postulated to act as an intracellular protein homeostasis maintenance factors. However, HSPs have also been reported to be observed in the extracellular fluid [11], and act as pro- and anti-inflammatory factors especially in autoimmune and inflammatory diseases in diverse manners. In inflammatory lesions, chaperones are upregulated by inflammatory stress and are released into the extracellular fluid. Subsequently, extracellular HSPs specifically induce proinflammatory cytokines and enhance the antigenicity of autoantigens through modulations of antigen presentation [31, 32]. However, the extracellular HSPs can also stimulate anti-inflammatory regulatory T cell responses, thereby inducing the negative feedback control of inflammation [29, 30]. Indeed, immunization with HSP peptides prevents disease development in autoimmune model animals, such as adjuvant arthritis and collagen-induced arthritis [30]. Presently, besides HSPs, another class of stress proteins is also known to exist in eukaryotic cells known as endoplasmic reticulum (ER) stress proteins, which are specialized factors involved in protein quality control in the secretory pathways [33, 34] are induced by ER stress, which is very different from cytosolic stress [35, 36]. However, similar to HSPs, ER stress proteins are also induced by inflammatory stress [37], suggesting that ER stress proteins might also participate in autoimmune and inflammatory responses.

4. Role of HSP90 in Systemic Lupus Erythematosus (SLE)

Systemic lupus erythematosus (SLE) is a chronic autoimmune inflammatory disease with complex immunological and clinical manifestations. Reduced immune tolerance and abnormal activation of T and B cells lead to autoantibody production mainly against protein-nucleic acid complexes, such as chromatin, and small ribonucleoprotein particles [38]. These autoantibodies complexed with their cognate self-antigens deposit within capillaries of various organs and subsequently mediate systemic disorders [12]. The commonly affected organs include the skin, heart, kidneys, lungs, joints, and central nervous system. SLE is more common in women than in men (>8 : 1). Studies using animal models suggest a role of estrogens in the disease development. The induction of SLE depends on hereditary factors and environmental agents, and inherited genes, infections, ultraviolet light, and some medications are all involved [39].

Autoreactivity to HSP is often associated with autoimmune pathology. The investigations have demonstrated the presence of autoantibodies to the HSP90 in a significant proportion of patients with systemic lupus. Anti-HSP90 autoantibodies of the IgG class were detected in approximately 50% [2] and 26% [3], and of IgM class in 35% [40] of patients with SLE. The presence of high concentration of hsp90 autoantibodies was found to correlate with renal disease and low C3 levels [40]. Although HSP90 is an intracytoplasmic protein, surface expression of HSP90 on peripheral blood mononuclear cells was found in approximately 25% of the patients with systemic lupus erythematosus (SLE) during active disease [41]. It has been shown that increased expression of HSP90 is due to the enhanced transcription of the HSP90-alpha gene [5]. While, these results indicate an association of anti-HSP90 autoreactivity with SLE, however, no direct involvement of HSP90 and anti-HSP90 antibodies in the pathogenesis of the disease has been proven. Although the etiology of SLE is still unclear, deposition of antigen-antibody complexes plays a role in the tissue damage of blood vessels and kidney [42]. Autoantibodies of different specificities are found in the sera of patients with SLE. Antibodies to double-strand DNA are found in 52% of the patients with SLE [40], anti-Sm antibodies in 25% [39], and antibodies to components of RNP particles in 25% and 10%, respectively [40]. Anti-HSP90 antibodies were present in 30–50% of the patients with SLE and those patients were more likely to have renal disease and a low C3 level [43]. HSP90 has also been found to be elevated in some subsets of systemic lupus erythematosus (SLE) patients, but its role in the disease is still unknown [39]. Elevated serum levels of HSP90 have been correlated to elevated levels of IL-6 [12] and IL-1.

Furthermore, the glomeruli of some SLE patients have been found to have deposits of HSP90 [40]. In addition, HSP90 and its endoplasmic reticulum homologue, glycoprotein 96 (gp96), have also been linked to autoimmunity [44]. In this study the presence of anti-HSP90 autoreactivity was found in 6/10 sera from lupus patients with active disease [40]. The presence of high concentration of HSP90 autoantibodies in the sera of SLE patients might be the most probable cause for the deposition of HSP90 in the kidneys. The increased expression of HSP90, in lupus patients [45], could play an important role as an auto-antigen in the pathogenesis and development of SLE.

HSP90 is abundant in normal tissues and the relative increase found is difficult to explain the deposits. With these considerations, the results of the present study are in support of the hypothesis that anti-HSP90 autoreactivity is involved specifically in the renal pathology associated with SLE. IgG autoantibodies to HSP90 from SLE patients are found to be less idiotypically regulated within the normal IgG repertoire than natural anti-HSP90 antibodies. These results were supported by the demonstration of an antiidiotypic inhibition of natural anti-HSP90 autoantibodies by IgG antibodies which were more pronounced in normal than in SLE IgG. Thus, the complete IgG repertoire present therein seems to be beneficial in experimental SLE and primary antiphospholipid syndrome [46]. Further, pathogenic anti-HSP90 autoreactivity seems specific SLE, since self-HSP90 participates in the formation of the kidney deposits only in SLE glomerulonephritis [47]. It appears to be a functional expression of the naturally occurring anti-HSP90 IgG autoreactivity due, at least in part, to an altered idiotypic IgG repertoire in sera of SLE patients. The hallmark of SLE is the dysregulation of the immune system, resulting in polyclonal activation of T and B cells, leading to multiorgan inflammation damages from immune complex deposition and infiltrations with inflammatory cells [48].

Although lupus-like diseases can be caused by defects in many pathways of the immune system, studies suggest that one of the mechanisms for SLE is the defect in peripheral B- or T-cell tolerance as a result of chronic stimulation and activation of APCs. For example, chronic activation of Langerhans cells by ectopic CD40 ligand expression in the murine epidermis led to not only chronic skin inflammations but also systemic autoimmunity, including immune complex-mediated glomerular nephritis [44]. Activation by genomic DNAs released from dead cells by means of TLR-9 has been implicated in the generation of autoantibodies against Ig [49]. Peripheral monocytes from patients with active, but not quiescent, SLEs were found to differentiate into active DCs more readily, due to higher serum levels of type I IFN [50]. However, whether chronic activations of DCs alone may provoke autoimmunity [51] is still not clear. Chronic activation of B cells, which can be mediated either by activation of TLRs or inflammasomes, certainly contributes to SLE; the association between SNPs in transcription factor IRF-5 (rs 2004640) and predisposition to SLE has also been reported [51].

5. Role of HSP90 as Therapeutic Agent for SLE Treatment

Heat shock proteins have been implicated as endogenous activators for dendritic cells (DCs). Chronic expression of heat shock protein gp96 on cell surfaces induces significant DC activations and systemic-lupus-erythematosus- (SLE)-like phenotypes in mice [52]. However, its potential as a therapeutic target against SLE remains to be evaluated. The investigations on chemical approach to determine the role of SLE-like phenotypes could be compromised by controlling surface translocation of gp96. From chemical library screening a compound has been identified that binds and suppresses surface presentation of gp96 by facilitating its oligomerization and retrospective transport to endoplasmic reticulum. [52]. In vivo administration of this compound reduced maturation of DCs, populations of antigen presenting cells, and activated B and T cells. The chemical treatment also alleviated the SLE-associated symptoms such as glomerulonephritis, proteinuria, and accumulation of antinuclear and DNA antibodies in the SLE model mice resulting from chronic surface exposure of gp96. These results suggest that surface translocation of gp96 can be chemically controlled and gp96 is a potential therapeutic target to treat autoimmune disease like SLE. It has also been envisaged that T-cell regulation has long been pursued as a potential therapy for lupus. There are a number of altered or distinct T-cell subtypes in lupus. The DNT cells are known to be upregulated in lupus mice and it is believed that this contributes to the disease state when they infiltrate target organs to produce proinflammatory cytokines and activate B-cell antibody production [51]. Han et al. [52] found that an inhibitor for the endoplasmic reticulum homologue of HSP90, gp96, reduced both the CD44 memory T cells and activated CD44 T cells in the spleen and lymph nodes. The effects of HSP90 inhibition on T-cell populations might be explained by the fact that stimulation of the T-cell receptor leading to T-cell activation requires HSP90 to stabilize lymphocyte-specific protein tyrosine kinase (Lck) in order to initiate activation. Furthermore, it has been shown that HSP90 is an essential regulator for gene expression of linker for activation of T cells (LAT) and that following inhibition of HSP90, both LAT messenger RNA and total LAT protein were decreased. Taken together, HSP90 plays a role in activation of T cells which may be the mechanism behind the changes in T-cell subtypes, found in mice treated with an HSP90 inhibitor. Consequently, targeting HSP90 may be an effective treatment for SLE, especially if combined with other targeted therapeutic approaches [51].

HSP90 has also been reported to play important roles in antigen presentation, activation of lymphocytes and macrophages, and activation and maturation of dendritic cells, indicating a potential treatment target for inflammatory diseases, including autoimmune diseases [7]. This includes patients with systemic lupus erythematosus (SLE) who have elevated levels of Hsp90. Interestingly, elevated levels of circulating IL-6 have also been reported in SLE. Moreover, spontaneous production of IgG by normal and SLE-derived B lymphocytes in culture can be enhanced by

the addition of exogenous IL-6 and inhibited by antibody to IL-6. These findings therefore suggest that IL-6 might play a role in the pathogenesis of autoimmune diseases. Moreover, infusion of an antibody to IL-6 can relieve disease symptoms in lupus-prone NZB/NZW mice. Thus, elevated levels of IL-6 in SLE patients induce increased levels of HSP90 protein which in turn results in the production of autoantibodies to this protein. Interestingly, pharmacologic inhibition of Hsp90 has recently been successfully applied in mouse models of autoimmune encephalomyelitis [53], rheumatoid arthritis [54], and systemic lupus erythematosus-like autoimmune diseases [52]. More importantly, in response to stress, HSP90 not only changes its level of expression but may also undergo subcellular redistributions and its trafficking to extracellular milieu, either actively or passively under pathologic stress, is a critical signal for the activation of DCs, which in turn contributes to the initiation and fate determination of adaptive immunity against pathogens as well as self antigens.

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Review Article

Aberrant Activation of Heat Shock Protein 60/65 Reactive T Cells in Patients with Behcet's Disease

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Behcet's disease (BD) is a multisystemic inflammatory disease and is characterized by recurrent attacks on eyes, brain, skin, and gut. There is evidence that skewed T-cell responses contributed to its pathophysiology in patients with BD. We found that heat shock proteins (HSPs) reactive T cells were prevalent in patients with BD. Here, we summarize current findings on HSP reactive T cells and their contribution to the pathogenesis in patients with BD.

1. Introduction

Behcet's disease (BD) is a systemic inflammatory disease, characterized by recurrent signs and symptoms of oral aphthosis, genital ulcers, skin lesions, and uveitis. It is well known that BD is prevalent along the Silk Route, but BD patients are occasionally found in other regions of the world.

The etiology of BD is largely unknown and skewed T-cell responses are associated with development and maintenance of BD [1]. Excessive cytokine productions by T helper type 1 (Th1) cells were reported using immunohistochemistry [2, 3] and intracellular cytokine staining [4, 5]. Th1 dominance was observed in BD uveitis [6] and stomatitis as well [7]. We reported excessive Th1 cell infiltration in BD skin and intestinal lesions [8–10].

Immune responses against microbes and microbial antigens were thought to play an important role in the pathogenesis of BD. Regional differences of the disease distribution [11] suggested association of disease development with locally prevalent microbes. Oral health was often impaired in patients with BD and was correlated well with BD disease severity [12]. *Streptococcus sanguinis* is a commensal oral bacterium and often forms dental plaque. *S. sanguinis* was found frequently in oral flora in patients with BD and the strain showed uncommon serotype (KTH1) compared with the standard ATCC strains [13]. T cells and peripheral blood mononuclear cells (PBMCs) from patients with BD responded to KTH1 antigens and produced interferon γ (IFNγ) and interleukin (IL)-12 [14]. Skin tests of streptococcal antigens caused various systemic reactions, such as fever, ocular attack, and genital and oral ulcer in patients with BD [15]. Accumulation of indirect evidence suggested participation of bacteria (or associated antigens) in the pathogenesis of BD.

Pathergy reaction is a cutaneous phenomenon where a minor injury, such as a needle prick, causes major skin lesions, such as ulcerations, panniculitis, and pyoderma, and positive pathergy reaction is included in the diagnostic criteria for BD proposed by the International Study Group [16]. Massive neutrophil infiltration and subsequent T-cell infiltration were frequently observed pathologically in the lesion caused by the reaction, even without any exogenous microbes [2]. The underlying mechanisms of the reaction remain largely unknown. On the other hand, it was suggested that skin florae and some skin self-antigens played a role because bacterial sterilization of skin reduced the reaction [17].

Heat shock proteins (HSPs) function as an intracellular chaperonin for other proteins, and significant sequence homology is found between mammalian HSP and microbial HSP (Table 1). For example, mycobacterial and streptococcal HSP65 have more than 90% homology, and mycobacterial HSP65 and human HSP60 have 42% homology [18]. HSP60/65 were thought to be a major cause of the

Table 1: Comparison of the amino acid sequences of human, Chinese hamster (C) HSP60, *Escherichia coli* (E), and *Mycobacterium leprae* (M) HSP65 [18].

```
hHSP60 MLRLPTVFRQMRPVSRVLAPHLTRAYAKDVKFGADARALMLQGVDLLADAVAVTMGPKGRTVIIEQSWGS 70
CHSP60 -------L--------A---------------------------------------------------- 70
EHSP65                        MA-------N---VK--R--NV-----K--L-----N-VLDK-F-A 46
MHSP65                         M--TIAYDEE--RGLER-LNS-----K--L-----N-VL-KK--A 45

       PKVTKDGVTVAKSIDLKDKYKNIGAKLVQDVANNTNEEAGDGTTTATVLARSIAKEGFEKISKGANPVEI 140
       ------------A--------------------------------------- --------------- 140
       -TI-----S--RE-E-E--FE-M--QM-KE--SKA-DA------------QA-IT--LKAVAA-M--MDL 116
       -TI-N---SI--E-E-E-P-EK---E--KE--KK-DDV------------QALV---LRNVAA----LGL 115

       RRGVMLAVDAVIAELKKQSKPVTTPEEIAQVATISANGDKEIGNIISDAMKKVGRKGVITVKDGKTLNDE 210
       ---------------------------------------D----------------------------- 210
       K--IDK--T-AVE---AL-V-CSDSKA----G----TS-ETV-KL-AE--D---KE-----E--TG-Q-- 186
       K--IEK---K-TET-L-DA-E-E-K-Q--AT-A---E--QS--DL-AE--D---NE-----EESN-FGLQ 185

       LEIIEGMKFDRGYISPYFINTSKGQKCEFQDAYVLLSEKKISSIQSIVPALEIANAHRKPLVIIAEDVDG 280
       ------------------------------------------V-------------------------- 280
       -DVV---Q-----L------KPETGAV-LESPFI--AD----N-REML-V--AVAKAG---L------E- 256
       --LT---R--K----G--VTDAER-EAVLEEP-I--VSS-V-TVKDLL-L--KVIQAG-S-L------E- 255

       EALSTLVLNRLKVGLQVVAVKAPGFGDNRKNQLKDMAIATGGAVFGEEGLTLNLEDVQPHDLGKVGEVIV 350
       ------------------------------------------------N-------A----------- 350
       ---A-A-V-TIRGIVK-A---------R--AM-Q-I-TL---T-IS--NIGME--KATLE---QAKR-VI 326
       -------V-KIRGTFKS----------R--AM-Q----L--AQ-IS--NVG-T--NTDLSL---ARK-VM 325

       TKDDAMLLKGKGDKAQIEKRIQEIIEQLDVTTSEYEKEKLNERLAKLSDGVAVLKVGGTSDVEVNEKKDR 420
       ------------E-----------T---EI---------------------------------------- 420
       N--TTTIID-V-EE-A-QG-VAQ-RQ-IEEA--D-DR---Q--V---AG----I---AATE--MK---A- 396
       ---ETTIVE-A--TDA-AG-VAQ-RTEIEMSD-D-DR---Q------AG----I-A-AATE--LK-R-H- 395

       VTDALNATRAAVEEGIVLGGGCALLRCIPALDSLTPANEDQKIGIEIIKRTLKIPAMTIAKNAGVEGSLI 490
       ----------------------------------K-S-------------A------------------- 490
       -E---H---------V-A---V--I-VASK-AD-RGQ----NV--KVAL-AMEA-LRQ-VL-C-E-P-VV 466
       IE--VRNAK--------A---VT--QAA----K-KLTGE-EAT-AN-V-VA-EA-LKQ--F-S-M-PGVV 465

       VEKIMQSSSEVGYDAMAGDFVNMVEKGIIDPTKVVRTALLDAAGVASLLTTAEVVVTEIPKEEKDPGMGA 560
       ----L-----I-----L-----------------------------------A---------------- 560
       ANTVKGGDGNY--N-ATEEYG--IDM--L-----T-S--QY--S--G-MI-T-CM--DL--NDAADLGA- 536
       A--VRNL-VGH-LN-AT-EYEDLLKA-VA--V--T-S--QN--SI-G-FL-T-A--ADK-EKTAA-ASDP 535

       MGGMGGGMGGGMF                                                          630
       -------------                                                          630
       G-MG-M-GM---M                                                          606
       T-----MDF                                                              605
```

Residues identical to the human HSP are indicated by dash. Significant sequence homology is found between mammalian and microbial HSP. *E. coli*-derived GloEL fragment and human HSP60 have 45.2% homology.

autoimmunity in patients with BD because of the molecular mimicry between human and microbial HSP. In this paper, we summarize current understanding of T-cell responses against HSP in patients with BD.

2. HSP Expressions in BD Lesions

Lehner et al. found that monoclonal antibodies against HSP65 reacted with six *S. sanguinis* strains and *Streptococcus pyogenes* [19]. They revealed that both IgG and IgA antibodies against HSP65 and *S. sanguinis* were significantly increased in BD patients compared to normal controls. They showed molecular mimicry between HSP and streptococcal antigens and suggested HSP-antigen-specific autoimmunity in the pathogenesis of BD.

After the initial report, researchers tried to identify the expression of HSP and to analyze immune cell functions on biopsy specimens in patients with BD. Several researchers

Table 2: Conserved peptide sequences of mycobacterial HSP65 and human HSP60 [20].

Mycobacterial HSP65	(111–125)	N	P	L	G	L	K	R	G	I	E	K	A	V	E	K				
Human HSP60	(136–150)	N	P	V	E	I	R	R	G	V	M	L	A	V	D	A				
Mycobacterial HSP65	(154–172)	Q	S	I	G	D	L	I	A	F	A	M	D	K	V	G	N	E	G	V
Human HSP60	(179–197)	K	E	I	G	N	I	I	S	D	A	M	K	K	V	G	R	K	G	V
Mycobacterial HSP65	(219–233)	L	L	V	S	S	K	V	S	T	V	K	D	L	L	P				
Human HSP60	(244–258)	L	L	S	E	K	K	I	S	S	I	Q	S	I	V	P				
Mycobacterial HSP65	(311–326)	D	L	S	L	L	G	K	A	R	K	V	V	V	T	K	D			
Human HSP60	(336–351)	Q	P	H	D	L	G	K	V	G	E	V	I	V	T	K	D			

The four peptide sequences were well conserved between human HSP60 and mycobacterial HSP65. Peptide 336–351 derived from human HSP60 effectively stimulated the pathogenic T cells in patients with BD [10, 20–22].

observed massive expressions of HSP60 in BD skin [23] and oral ulcer lesions [24, 25]. HSP60 was expressed more diffusely [25] and intensely [23, 25] in BD lesions than those in other types of inflammation, such as oral lichen planus and recurrent aphthous stomatitis. Not only infiltrating cells but also vascular endothelial and epithelial cells expressed HSP60 in the BD skin lesions [25].

We reported excessive Th1 cell function and aberrant HSP expression in patients with BD [8–10]. HSP60 mRNA expression was found in PBMC and in intestinal tissues of BD but not in those of normal controls [8]. We found that infiltrating mononuclear cells, including CD4+ T cells, CD8+ T cells, and CD68+ macrophages, expressed HSP60, IFNγ, and IL-12 in the intestinal lesions in patients with BD [8, 9]. C-C type chemokine receptor (CCR)5 and macrophage inflammatory protein (MIP)1β, a Th1-related chemokine receptor and its ligand, were detected in the intestinal lesions in patients with BD, and we suggested that CCR5/MIP1β interaction played a role in the migration of activated Th1 cells [8]. It is possible that HSP60 acted as a crucial signal to trigger excessive Th1 cell accumulation and subsequent Th1-cell-mediated inflammatory responses in the intestinal lesions in patients with BD where activated T cells and macrophages promoted the destructive processes.

3. T-Cell Responses against HSP Peptides in Patients with BD

Lehner et al. analyzed the frequency of short-term cell lines stimulated with four selected peptides (111–125, 154–172, 219–233, 311–326) (Figure 1, Table 2) derived from *Mycobacterium tuberculosis* HSP65 in the ocular, arthritic, and mucocutaneous types of BD. T cells from ocular-type BD patients responded excessively to the stimulation with the four HSP peptides, especially peptides 111–125 and 311–326 [20].

Excessive T- and B-cell responses to the four peptides and human counterparts were observed in patients with BD who lived in Europe, Far Eastern Asia, and the Middle East [10, 20–22].

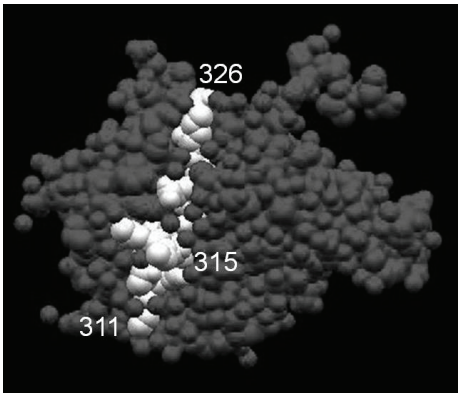

Figure 1: Crystal structure of HSP. GroEL fragment apical domain, a bacterial homologue of human HSP 60, comprising residues 191–345 derived from *Escherichia coli* in the Protein Data Bank Japan (PDBj) is shown. Residues 311–326 which corresponded to human HSP60 331–356 are colored with white.

In our study, CD4+ T cells, but not CD8+ T cells, yielded proliferative responses to the peptide 336–351 derived from human HSP60 in Japanese BD patients [26]. We evaluated T-cell receptor Vβ gene usage of T cells which responded to the peptide 336–351 in the patients [22]. To this end, we first conducted amplification by PCR of TCR Vβ gene of T cells which had been stimulated with the peptide *in vitro* and then visualized several bands which represented each TCR clonotype by PCR-single-strand conformation polymorphism (SSCP) based technique. T-cell receptor Vβ gene oligoclonality was found in T cells which had been freshly isolated from peripheral blood and the exactly same PCR products were remarkably increased after stimulation with the peptide 336–351 in patients with BD. These data suggested that HSP-specific T cells in patients with BD showed antigen-driven expansion by HSP stimulation and that the HSP peptide reactive T cells increased in BD peripheral blood. A longitudinal study of the TCR clonotypes in 6 BD patients showed that the oligoclonal expansion of particular T-cell clonotypes correlated with the severity of uveitis [22].

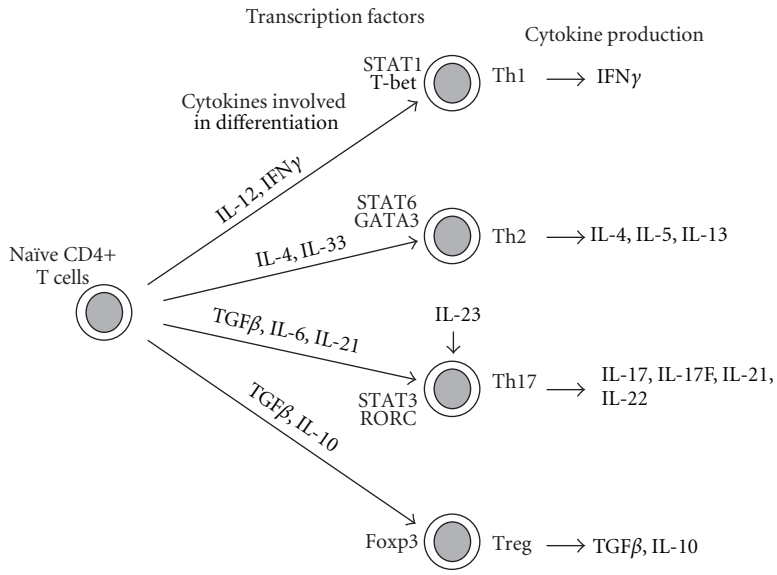

Figure 2: Current view of CD4+ T-cell subsets in humans [27]. Naïve CD4+ T-cells differentiate into several T-cell subsets in the presence of appropriate cytokines. In response to the cytokines, the corresponding signaling molecules and transcription factors are expressed to regulate lineage commitments. Th17 cells require IL-23 for their expansion.

4. $\gamma\delta$ T-Cell Activation and HSP60/65

HSP60/65 presented by antigen presenting cells (APCs) stimulated not only $\alpha\beta$ T cells but also $\gamma\delta$ T cells which played important roles in the oral mucosal immunity as the first defense against microorganisms. It was thought that Vγ9δ2+ T cells, a major subset of $\gamma\delta$ T cells of peripheral blood lymphocytes (PBL), recognized the antigens produced by bacteria [26]. Vδ1+ $\gamma\delta$ T cells responded to the stress inducible major histocompatibility complex class I related chain A (MICA) mainly expressed on damaged intestinal epithelial cells [28].

Vδ2+ $\gamma\delta$ T cells increased in peripheral blood, while Vδ1+ $\gamma\delta$ T cells increased in bronchoalveolar lavage fluid and cerebrospinal fluid in patients with BD [29]. Infiltrating cells expressed HSP, and $\gamma\delta$ T-cell numbers were increased in oral ulcer in the patients [23, 25].

We found that CD45RA+ Vγ9δ2+ $\gamma\delta$ T cells increased in BD PBL irrespective of disease activity. Vγ9δ2+ $\gamma\delta$ T cells in the active phase of BD expressed IL-2 receptor β chain and HLA-DR, suggesting that the cells were activated *in vivo* [30]. The CD45RA+ $\gamma\delta$ T cells produced tumor necrosis factor (TNF)α and contained perforin granules. Moreover, Vγ9δ1+ $\gamma\delta$ T cells preferentially responded to *S. sanguinis*-derived KTH1 antigen without HLA restriction [31]. It is possible that $\gamma\delta$ T cells respond to HSP both in peripheral blood and in affected lesions and enhance the inflammation in patients with BD.

5. Th17 Cells and HSP

Recently, the classical Th1/Th2 paradigm was challenged by the discovery of various subsets of T helper cells [27] (Figure 2). Th17 cells produce a number of proinflammatory cytokines, including IL-17, IL-17F, IL-21, and IL-22. IL-6, IL-21, and transforming growth factor (TGF)β were reported to play a role in the differentiation of Th17 cells which proliferated in the presence of IL-23. Regulatory T (Treg) cells control T-cell immune responses and also need TGFβ for their differentiation [27] (Figure 2).

TGFβ activates Smad pathway via TGFβ receptor I/II complex and activated Smad protein leads to forkhead box P3 (Foxp3) expression which is a master gene of Treg cells [32] (Figure 3). TGFβ also activates p38 mitogen-activated protein kinase (MAPK) which regulates Th17 cell differentiation [32, 33]. In the presence of TGFβ, IL-6/STAT3 signaling pathway plays a critical role in the reduction of Foxp3 expression and in the induction of retinoic acid receptor-related orphan receptor C (RORC) expression which is a master gene of Th17 cells [34] (Figure 3). Also it is reported that STAT3 and MAPK are involved in the immune tolerance induction and Treg cell differentiation [35, 36]. Signaling molecules such as STAT3 and MAPK may transduce positive and negative signals depending upon the surrounding microenvironment. To address these issues, further studies are needed. In addition, specific antigen such as HSP directly and indirectly regulated the balance between Th17 and Treg cells [37–40].

In patients with BD, monocytes and T cells overproduced IL-6 in the presence of HSP [41]. Overexpression of RORC mRNA [42, 43], underexpression of Foxp3 [44, 45], and high frequencies of Th17 cells [42–44, 46] were reported in patients with BD. We recently reported that TGFβ/Smad signaling pathway of T cells was overactivated in patients with BD [47]. We also reported the possibility that CD4+ T cells in patients with BD showed higher sensitivity to IL-23 and produced more IFNγ and IL-17, as compared with normal controls [43]. Recent genome-wide association studies identified IL-12 receptor β2 (IL-12Rβ2)/IL-23 receptor and IL-10 genes as BD susceptibility genes [48, 49].

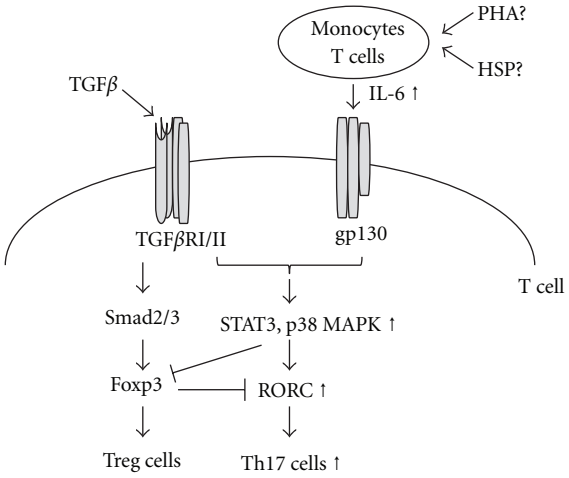

Figure 3: A schematic representation of skewed Th17/Treg cell differentiation in patients with BD, a hypothesis. TGFβ activates Smad pathway via TGFβ receptor I/II complex, and activated Smad protein leads to forkhead box P3 (Foxp3) expression which is a master gene of Treg cells [27]. TGFβ also activates p38 mitogen-activated protein kinase (MAPK) which regulates Th17 cell differentiation [32, 33]. In the presence of TGFβ, IL-6/STAT3 signaling pathway plays a critical role in the reduction of Foxp3 expression and in the induction of retinoic acid receptor-related orphan receptor C (RORC) expression which is a master gene of Th17 cells [34]. In patients with BD, monocytes and T cells overproduce IL-6 in the presence of HSP [41]. Overexpression of RORC [42, 43], underexpression of Foxp3 [44, 45], and higher frequencies of Th17 cells [42–44, 46] are reported in patients with BD. PHA: phytohemagglutinin, TGFβRI/II: TGFβ receptor types I and II, STAT3: signal transducer and activator of transcription 3.

Based on the above findings, we proposed that HSP induced imbalance of Th17 cells to Treg cells in patients with BD (Figure 3). Further study on HSP and Th17 cell differentiation is necessary to understand the pathogenesis of BD.

6. Toll-Like Receptors (TLRs) and HSP60/65

TLRs play a key role in recognition of microbes in the innate immune system. Activation of dendritic cells by TLR ligands is a crucial event in the initiation of both innate and adaptive immune responses. Several classes of TLR ligands are identified which interact with distinct members of the TLR family. TLR2/CD14 complex and TLR4/CD14 complex were suggested to be important for APC to recognize HSP60 [50], and TLR2/6 heterodimer was reported to promote IL-6 production of mononuclear cells [51]. We need to study the expression of the heterodimer because IL-6 concentrations were elevated in patients with BD [41].

We found that TLR2 and TLR4 mRNA were expressed on ileocaecal ulcer lesions of BD, but less on unaffected sites of BD and on Crohn's disease lesions. We found that IL-12 producing TLR2+ macrophages are located neighboring to CD3+ T cells. HSP60 was expressed on the same region of the intestinal lesions [9]. We suggested that TLR/HSP60 interactions induced destructive Th1-type responses at the intestinal lesion in patients with BD.

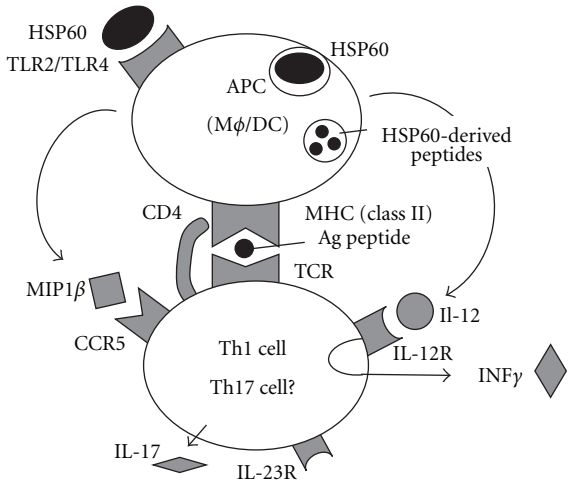

Figure 4: HSP60 regulates Th1 cell differentiation through multiple pathways, a hypothesis. The interactions between TCR- and HSP-derived peptides on MHC [22] and between IL-12 and its receptor [8–10] may induce aberrant T-cell differentiation in patients with BD (Figure 4). The interaction between MIP1β and C-C-type chemokine receptor (CCR)5 was suggested to induce migration of the pathogenic T cells [8]. TLR2/6 heterodimer is involved in IL-6 production [51] and thus we need to study the expression of the heterodimer in patients with BD.

7. Conclusions

We reviewed here HSPs reactive T-cells and their contribution to the pathogenesis of BD. It is possible that HSPs regulate T-cell differentiation through several interactions. The interactions between TCR- and HSP-derived peptides on MHC [22] and between IL-12 and its receptor [8–10] may induce aberrant T-cell differentiation in patients with BD (Figure 4). The interaction between MIP1β and CCR5 was suggested to induce migration of the pathogenic T cells [8]. TLR2/6 heterodimer is involved in IL-6 production [51] and thus we need to study the expression of the heterodimer in patients with BD. It is important to clarify whether HSP bring about generation of pathogenic Th17 cells in patients with BD because Th1 and Th17 cells share a common structure (IL-12Rβ1) on their characteristic receptor complexes, namely, IL-12R and IL-23R [52].

Acknowledgments

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Review Article

Heat Shock Proteins, Autoimmunity, and Cancer Treatment

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Heat shock proteins (HSPs) have been linked to the therapy of both cancer and inflammatory diseases, approaches that utilize contrasting immune properties of these proteins. It would appear that HSP family members Hsp60 and Hsp70, whether from external sources or induced locally during inflammation, can be processed by antigen-presenting cells and that HSP-derived epitopes then activate regulatory T cells and suppress inflammatory diseases. These effects also extend to the HSP-rich environments of cancer cells where elevated HSP concentrations may participate in the immunosuppressive tumor milieu. However, HSPs can also be important mediators of tumor immunity. Due to their molecular chaperone properties, some HSPs can bind tumor-specific peptides and deliver them deep into the antigen-processing pathways of antigen-presenting cells (APCs). In this context, HSP-based vaccines can activate tumor-specific immunity, trigger the proliferation and CTL capabilities of cancer-specific CD8+ T cells, and inhibit tumor growth. Further advances in HSP-based anticancer immunotherapy appear to involve improving the properties of the molecular chaperone vaccines by enhancing their antigen-binding properties and combating the immunosuppressive tumor milieu to permit programming of active CTL capable of penetrating the tumor milieu and specifically targeting tumor cells.

1. Introduction

The primary function of the immune response is to distinguish between molecules, usually proteins, that are construed as either components of self or nonself molecules likely derived from invading organisms. Through the mechanisms of central and peripheral tolerance, the immune response is deterred from attacking cells recognized as self [1, 2]. The case of tumor immunity is however more ambiguous. Tumor cells arise from normal cells that the immune cells are educated to tolerate. However, it has been shown that tumors can express specific antigens not displayed by the corresponding normal tissues and that these epitopes can be recognized by CD8+ T lymphocytes [3, 4]. These polypeptides may be derived from embryonic antigens reawakened during malignant progression or from mutated proteins that arise due to development of a mutator phenotype and loss of DNA repair mechanisms that characterize tumorigenesis. Despite the potential for specific immunity and the existence of tumor antigens, it is evident that cancers arise, grow, progress, and lead to the death of greater than 30% of the human population. A depressing variety of mechanisms have been found which may account for the ability of tumors to dismiss the attentions of the immune response. These include a "loss-of-self" mechanism in which major histocompatibility class I antigens cease to be expressed on the tumor cell surface, thus masking the presence of the tumor proteome and evading CD8+ killing [5]. Additional mechanisms include the expression by cancer cells of Fas ligand that can recognize the presence of proapoptotic Fas on the tumor cell surface and trigger programmed cell death of CTL [6]. In addition, the nonmalignant cell populations that migrate into the tumor microenvironment appear to play a key role in deterring immunity [7–10] (Figure 1). It is known that although cytotoxic CD8+ cells progress to and arrest at the periphery of many tumors, crossing the tumor capillary wall comprises a barrier to entry of such cells; indeed, ability of CTL to penetrate tumors is a favorable prognostic feature [11–13]. As mentioned, however, tumors also attract a range of normal cells in

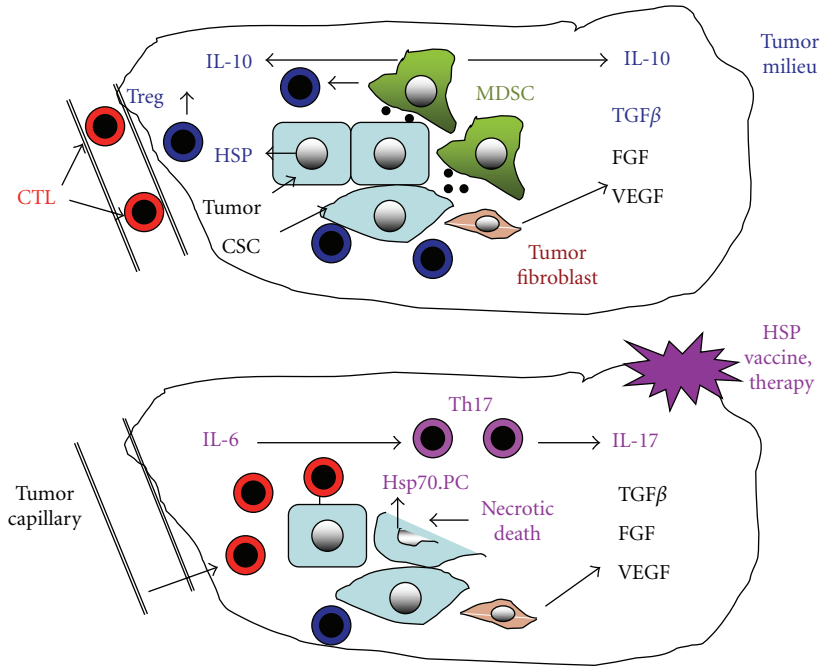

Figure 1: The cell biology of the tumor milieu: the role of heat shock proteins. The upper part of the figure depicts tumor cells (pale blue), including cancer cells with a cuboid epithelial shape and more spindle-shaped cancer stem cells (CSCs), suggesting the EMT (epithelial-mesenchymal transition) characteristics ascribed to CSC. The tumor is represented as a heterogeneous cell colony containing myeloid suppressor cells (MDSCs; green), Treg (dark blue), and tumor-associated fibroblast (TAF; orange). Dominant cytokines in the tumor microenvironment include IL-10 and TGFβ. The growth factors FGF and VEGF are secreted by TAF. To the left of the figure is depicted a tumor capillary containing CD4+ T cells (red) that have stalled at the capillary wall. Tumor cells are depicted as secreting Hsp70-containing exosomes (black circles) that recruit MDSCs as well as free Hsp70 that may also trigger immunosuppressive responses. The lower section suggests the potential effects of therapy using molecular chaperone vaccines, in which IL-6 is now at high levels and the cytokine profile is proinflammatory, cognate CTL has crossed the capillary wall, penetrated the tumor interstitial spaces, and recognized MHC class I associated with tumor antigens. Such tumor cells can then be killed in an antigen-specific manner. In addition, Hsp70-peptide complexes (Hsp70.PC) are secreted from necrotic tumor cells and can trigger anticancer CTL after entering APC and cross-presentation to CD4+ T cells in afferent lymph nodes.

a process that resembles a normal wound-healing response. Invading cells include regulatory T lymphocytes (Treg), primary players in peripheral tolerance and in the defense against autoimmunity [14, 15]. Treg can be distinguished by a surface phenotype of CD4+CD25+, as well as the expression of the forkhead box transcription factor Foxp3 that regulates many Treg functions. Treg exhibit multiple immunosuppressive mechanisms including the secretion of cytokines such as transforming growth factor beta (TGFβ) and interleukin-10 (IL-10), the killing of CTL, and inhibition of immune cells through a cell contact mechanism [15]. Treg are known to congregate in tumors and may thus mediate immunosuppression [14]. It may be significant that cancer stem cells (CSCs) that account for tumor initiation, metastasis, and resistance to many forms of therapy can attract Treg and lead to the expression of immunosuppressive IL-10 [16, 17]. Another class of immunoregulatory cells associated with tumors includes myeloid-derived suppressor cells (MDSCs). MDSCs are a heterogeneous population of early myeloid precursors, immature granulocytes, macrophages, and DC that can suppress CD4+ and CD8+ T cells, NK and NKT cells and promote development of Treg by multiple mechanisms [10]. In addition, many tumors contain tumor-associated macrophages (TAMs) that are also suppressors of tumor immunity through production of IL-10, stimulation of Treg, and synthesis of the coinhibitory factor CTLA-4 [9]. Also attracted to the tumor are mesenchymal stem cells (MSCs) that can give rise to a tumor-associated fibroblast (TAF) population that supplies growth factors such as FGF, TGFβ, and VEGF required for growth and angiogenesis (Figure 1). The tumor milieu can contain a small fraction of cells of mesenchymal origin identified by surface *fibroblast activation protein-a* (FAP cells) that suppress antitumor immune responses [18].

2. Immune Properties of Heat Shock Proteins

Heat shock proteins (HSPs) are stress proteins whose synthesis is triggered by proteotoxic stresses such as heat shock [19, 20]. The dominant functions of the HSPs are the holding and folding of other intracellular proteins [21]. The HSPs are thus classified among the molecular chaperones, a group of polypeptides that mediate intracellular protein quality control under both housekeeping and stressed situations. As HSPs are often required to interact with "client" proteins in a stoichiometric rather than catalytic manner (holding), they are synthesized in

high intracellular concentrations, particularly during stress [21, 22]. Thus, HSPs are induced in prodigious quantities, and their expression dominates ongoing transcription and translation in heat-shocked cells [23]. Early studies showed that some bacterial HSPs of the Hsp60 and Hsp70 families, due in part to their high concentrations, were dominant antigens in the host responses to pathogens [24]. It was therefore widely expected that human host HSP paralogs might in turn promote autoimmunity by a peptidomimetic mechanism due to the close similarity between domains in the HSP paralogs in humans and bacteria [25]. Structural domains conserved between the well-conserved pathogenic and host HSPs were thus thought to be a potential source for autoimmune/inflammatory diseases. However, it has turned out that when the host Hsp60 or Hsp70 is elevated in cells, as might be observed during inflammation or immunotherapy using purified HSPs, they can be processed by professional or nonprofessional antigen-presenting cells and thus trigger Treg cells, mediate immunosuppression, and ameliorate the inflammatory effects of pathogenic proteins [26, 27]. Thus, HSPs from intracellular or extracellular sources, after processing in cells and presentation to T lymphocytes, tend to be immunomodulatory and can ameliorate symptoms of inflammatory diseases (Figure 2). It is now well established that HSP levels become amplified in a broad spectrum of cancers, are required for tumor progression, and are targets for cancer therapy [28, 29]. Increases in tumor HSP levels have been ascribed to either the high concentrations of mutated and misfolded oncoproteins that drive oncogenesis or to induction of the heat shock proteins by corruption of the signaling pathways leading to HSP expression during malignancy [30, 31]. The elevated cohort of HSPs may thus be required to chaperone the abundant and denatured tumor proteins or processed peptides derived from such proteins and as such offers a target for therapy. For immunologists, inspection of such a situation in the cancer cell might suggest an opportunity for immune attack on the tumor cells [32]. Hsp70 has been observed in the extracellular milieu and has been detected in plasma from mice and humans [33]. In addition, the chaperone is released from necrotic cells with compromised plasma membranes as well as from intact unstressed cells under basal conditions, using a defined secretion mechanism [34, 35]. The situation in tumors, with cells rich in HSP expression some of which exist in a highly stressed microenvironment, therefore might suggest that HSP polypeptides could be released either from disintegrating cells in areas of necrosis or in the normal metabolism of viable cells. However, *in vivo* studies showed that depletion of intracellular Hsp70 enhances tumor growth due in part to a decrease in immune killing in the tumor [36]. These effects of Hsp70 secretion were ascribed to the fact that Hsp70 can be released from the tumor cells packaged in exosomes, lipid-bounded particles that can attract immunosuppressive myeloid-derived suppressor cells (MDSCs) [36] (Figure 1). These cells have been found in the tumor milieu and mediate immunosuppression by secreting immunosuppressive interleukin-10, decreasing CD4+ and CD8+ T-cell viability, and attracting Treg [10]. In this context, extracellular HSPs can be seen to be contributing to

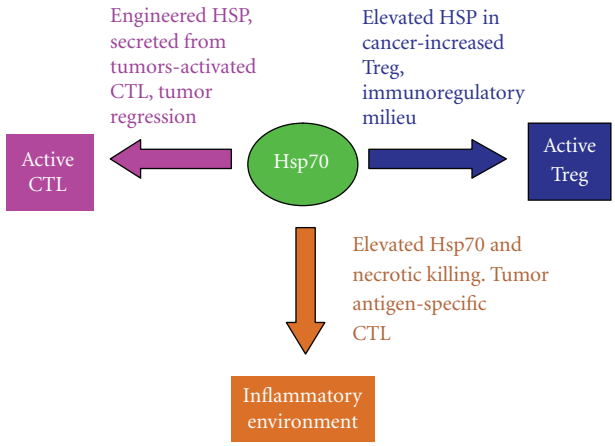

Figure 2: Contrasting immunological influences of HSPs under differing contexts. We show that HSPs in cancer cells can inhibit or promote tumor immunity, depending on the tissue context. Tumor HSP levels become elevated during progression (blue line). This can lead to immune-suppressive effects of intracellular Hsp60 and Hsp70 as well as Hsp27, Hsp60, and Hsp70 secreted from tumor cells. However, if tumor cells are engineered to overexpress secretable forms of Grp78 or Gsp170 (purple line), antitumor immune response can be generated that are at least partially due to release of HSP tumor antigen complexes. In addition, necrotic killing of cells along with forced expression of Hsp70 (orange line) can lead to an inflammatory environment that triggers a tumor antigen-specific immune response.

the immunosuppressive microenvironment associated with many tumors, which are often enriched in Treg, MDSC, and TAM, as well as tumor-associated fibroblast that can secrete TGFβ (Figure 1). The role of Hsp70 released in free form from tumor cells as opposed to exosomal Hsp70 is less clear. It is however known that the major inducible Hsp70 member, *hsp70.1/hspA1A,* can induce the formation of immature tolerogenic DC and decrease T-cell proliferation [37]. Thus, Hsp70 epitopes expressed on either tolerogenic DC or tumor cells themselves may lead to an immune-suppressed environment due to interaction with Treg and suppression of CTL by multiple mechanisms [27]. In addition, the tumor cells are particularly enriched in the small HSP Hsp27 (*hspB1* gene product) [29, 31]. Extracellular Hsp27 is frankly immunosuppressive due to inhibition of DC differentiation and production of inhibitory mediators thrombospondin-1 and IL-10 [38, 39]. Likewise with Hsp60, an HSP is known to be secreted from cells [40]. When released into the extracellular milieu, Hsp60 increases levels of CD4+CD25+Foxp3 cells and suppresses CTL [41, 42].

Few studies have addressed the role of humoral immunity in the responses of tumor cells to HSP. However, Hsp70/MAGE-3 fusion vaccines were shown to enhance both cellular and humoral immunity to MAGE-3 expressing melanoma, and B- and plasma-cell infiltration was a strong prognostic factor in the response [43]. Likewise in human esophageal cancer, simultaneous occurrence of Hsp70 expression and B- and plasma-cell infiltration into tumors was an indicator of good prognosis, even exceeding

the benefits of tumor infiltration of CD4+ and CD8+ T cells [44]. It is not clear how HSPs might influence cells of the humoral response. However, Hsp60 has been shown to interact with B cells through a pathway involving TLR signaling and can activate naïve cells, upregulate expression of MHC class II, and protect the cells from apoptosis [45, 46]. The latter effects are accompanied by release of IL-10 suggesting that effects of extracellular Hsp60 on B cells might provoke an immunoregulatory response. Thus, effects of extracellular HSPs on cells of the humoral response in the tumor milieu likely involve a balancing act between potential immunostimulatory and immunosuppressive responses.

Evidence to suggest that HSPs secreted from tumor cells can also be immunostimulatory and lead to antitumor immunity comes from immunotherapy studies carried out with HSPs such as Gp96 and Grp170 engineered for spontaneous secretion [47–50] (Figure 2). In this context, Gp96 and Grp170 appear to trigger tumor immunity due to their ability to chaperone intracellular tumor antigens, enter the canonical protein secretion pathway, be taken up by APC, and trigger a CTL response targeting the cancer cells. Secreted extracellular HSPs released from malignant cells can thus either function as part of the immunosuppressive tumor milieu or can enhance tumor immunity by exporting tumor-associated antigens and triggering a CTL response directed at the tumor (Figure 2). Factors influencing the activating or immunosuppressive effects of extracellular HSPs may include the molecular nature of the HSP (Hsp70, Gp96, Hsp90, Hsp110, or Grp170), whether HSPs are released in soluble form bound to antigenic polypeptides or secreted in exosomes, as well as the relative rate of secretion of the HSP.

3. Molecular Chaperone-Based Anticancer Vaccines

Despite the evidence that Hsp60 and Hsp70 can be immunosuppressive and downregulate inflammatory autoimmune diseases, it seems that HSPs can be used as vaccines to induce antitumor immunity under the appropriate conditions [32, 51]. It was proposed that members of the HSP family such as Hsp70 and gp96 could form the basis of anticancer vaccines due to their assumed ability to bind a sample of the antigenic polypeptides in tumor cells, as mentioned above. Indeed, it was shown that when cells are lysed under controlled conditions, HSP-antigen complexes can be isolated, used as vaccines, and thus induce immunity to cancer [32, 52–57]. This effect was achieved by utilizing the biochemical properties of Hsp70 and Hsp90 family members. In the case of Hsp70, binding to ATP causes loss of affinity for the client polypeptide, while binding to ADP stabilizes peptide binding [22, 58]. HSP polypeptide complexes (HSP-PCs) prepared in this way were shown to induce antigen-specific tumor immunity [59]. For instance, Hsp70 peptide complexes prepared using ADP affinity chromatography retain peptide clients and induce tumor immunity while use of ATP-agarose, although permitting isolation of Hsp70 leads to a preparation devoid of ability to induce anticancer immunity [59]. HSP-based vaccines have been prepared using these general principles from Hsp70, Gp96, Hsp90, Hsp110, and Grp170 and shown to be effective in deterring tumor growth [55]. The methods permit either isolating HSPs coupled to the (largely uncharacterized) tumor antigen repertoire to give a personalized, polyvalent vaccine, or loading one of the small minority of known tumor antigens to produce a highly concentrated vaccine based on one known antigenic protein [51] (Figure 2). Clinical trials to test the efficaciousness of this approach are underway (reviewed recently, [51]).

Immunoregulatory responses may accompany or follow the triggering of tumor immunity by HSP antigen complexes. For instance, in the case of Gp96, lower concentrations of the chaperone-antigen complex lead to immunity, while higher doses cause immune suppression [52, 60]. These findings might be explained by more recent studies using a GP96-HPV vaccine which showed that lower doses of the preparation lead to activation of CD8+ T lymphocytes, while higher concentrations cause strong induction of CD4+CD25+Foxp3 Treg [11]. At least in the case of Gp96, successful vaccination appears to involve a balancing act between immune stimulation and regulation, with the aims of enhancing the immunotherapy arm. It is not known if the use of other chaperones invokes the same dilemma. However, both Hsp60 and Hsp70 have been shown to produce immunomodulatory effects and stimulate anti-inflammatory Treg when used to treat inflammatory diseases such as arthritis [61]. Preparation of tumor vaccines so as to stabilize antigen binding may bias the response towards tumor antigen-specific immunity, and use of the chaperone complexes at relatively low concentrations may minimize the immunomodulatory effects of anticancer vaccines and favor antitumor immunity (Figure 2).

The responses elicited in different immune cells by chaperone vaccines may also depend on the mechanisms by which they interact with the surface of target cells and are taken up into these cells. HSP peptide complexes (HSP-PCs) have been proposed to induce antitumor immunity by stimulating both antigen cross-presentation and by triggering innate immunity [62–65]. Although some investigators have suggested that HSPs can be taken up by nonreceptor route, the majority of studies suggest a receptor-mediated mechanism [62, 66–68]. A considerable amount of effect has been expended on studying how HSP-PCs can trigger antigen cross-presentation in dendritic cells (DCs) [69]. Search for receptors that might mediate this process has not revealed a dedicated HSP receptor. Instead, HSP uptake appears to involve scavenger receptors including LOX-1, SRECI, and CD91 with a broad specificity as regards ligand binding [67, 70]. There is still some controversy concerning the relative role of the individual HSP receptors, although the absence of CD91 from the DC surface casts some doubt on its significance at least in DC [62]. Both LOX-1 and SRECI have been shown to bind avidly to Hsp70 and Hsp90 in DC and mediate antigen cross-presentation [62, 64, 71]. Indeed, non-APC such as Chinese Hamster Ovary cells can be endowed with cross-presenting properties when stably expressing SRECI [64]. Hsp90 can be endocytosed by scavenger receptors (SRs) into endosomes and transported all the way to intracellular proteasome—the site of processing

of internalized antigens [64, 72, 73]. Hsp90 appears to assist in transporting the antigens complexed to it across the endosomal membrane and insertion into the proteasome. Thus, molecular chaperones appear to be able to penetrate deeply into the intracellular antigen processing pathways in DC and may in this way trigger cross-presentation of associated antigens to CD8+ and trigger CTL [72, 73]. Some doubt exists as to the peptide-binding capacity and role in antigen cross-presentation of the ER chaperone Gp96 [65, 74]. However, there is strong evidence for a role for the capacity of other chaperones such as Hsp90 to bind and mediate cross-presentation of antigenic peptides by DC [51, 64, 69].

In addition to activating cross-presentation to CD8+ cells, HSPs may be able to interact with other immune cells. For instance, Hsp70 can activate the class II pathway in DC leading to CD4+ cell activation [71]. Extracellular antigens are usually sorted and distributed between the class I and class II pathways in DC, for presentation to CD4+ and CD8+ T lymphocytes by a number of mechanisms (reviewed in [69]). However, this mechanism may not apply to HSP-chaperoned antigens and, for instance, SRECI may be able to permit Hsp90-bound chaperones to enter both the class I and class II pathways (Murshid and Calderwood, in preparation). For DC to interact productively with CD8+ T cells, a second signal, in addition to activation of the T-cell receptor, is required [75]. Such a signal could be provided by the CD40 receptor on the surface of DC cells that can bind to the CD154/CD40-L counterreceptors on the CD4+ surface. Indeed for strong activation of DC and activation of naïve T cells, individual DC interacts with the T-cell receptors of both CD4+ cells and CD8+ T through surface MHC class II and class I. Interaction with the CD4+ cell "licenses" the DC for full CTL programming, permitting survival and proliferation [75, 76]. Licensing includes a range of alterations, not all of them understood but involving the induced expression of costimulatory molecules such as CD80/B7.1 and CD86/B7.2 that bind to counterreceptors such as CD28 constitutively expressed on the CD8+ cell surface and, in concert with T-cell receptor ligation, trigger a productive interaction [77]. The HSP scavenger receptor system may permit presentation of antigens to—and activation of both CD4+ and CD8+ T cells—DC licensing and a fully activated CD8+ T cell capable of killing tumor cell targets.

In the immune response to pathogens, a similar activation of DC can be produced by the innate immune response. In this case, an abundant class of *pathogen-associated molecular patterns* (PAMPs), designated as "danger signals," are released from microorganisms and can interact with receptors on APC designated as *pattern recognition receptors* (PRRs). The PAMPs trigger powerful signal transduction responses that emanate from PRR such as Toll-like receptors (TLRs) and result in triggering transcription of cytokines such as tumor necrosis factor alpha (TNFa), interleukin-6, and interleukin-12 as well as costimulatory molecules such as CD28 [78, 79]. This second signal resembles the stimulus provided by the licensing effects of CD4+ cells discussed above and permits CD8+ cell programming and lysis of specific cell targets. It was suggested that in stressed tissues, endogenous danger signals might be released from cells and trigger effects similar to the innate response to PAMPs. Such compounds may underlie the enhanced immunogenicity of cells that die from necrosis, rather than apoptosis: necrotic cells would be expected to release their contents, including endogenous danger signals rather than the cryptic pathways of apoptotic death in which cell contents are retained until engulfment by scavengers [80]. A number of compounds released from stressed or dying, notably uric acid crystals appear to fit the billing of endogenous danger signals or DAMPs and lead to sterile inflammation [81]. A large number of studies have suggested that Hsp70 in particular can stimulate the PRR Toll-like receptor 4 (TLR4) *in vivo* [82]. This receptor was characterized as the PRR for lipopolysaccharides (LPSs), PAMPs derived from the cell coat of Gram-negative bacteria. LPSs from a range of organisms, but most commonly *E. coli,* are endemic on the surfaces of laboratory glassware, contaminate many laboratory reagents, and associate avidly with HSPs [83]. This property made some of the earlier studies using recombinant Hsp60 and Hsp70 in *in vitro* studies of HSP-TLR interactions somewhat controversial [84, 85]. However, *in vivo* studies show almost overwhelming evidence of a role for Hsp70 and other HSPs in triggering TLR4 (recently reviewed in [86]). In terms of the responses of tumor-bearing animals to HSP-based vaccines, TLR signaling appears to be essential. An Hsp70 vaccine derived from MC38 cells expressing the tumor antigen MUC1 was shown to trigger DC maturation and expression of costimulatory molecules and trigger CTL that could kill target tumor cells in an antigen- (MUC1-) specific manner [87]. These effects were abrogated in mice bearing mutations that lead to inhibition of TLR signaling. For instance, in mice deficient in the signaling intermediate Myd88, an adaptor molecule downstream of TLR4 that is essential for activation of proinflammatory NFκB signaling and innate immune transcription, ability of the Hsp70 vaccine to trigger T-cell activation, and CTL activity was reduced [87]. Knockout of TLR2 and TLR4 almost completely abrogated the ability of the vaccine to activate either CD4+ or CD8+ cells and prevented induction of CTL [71]. Free Hsp70 may also induce other components of innate immunity. For instance, natural killer (NK) cells can be activated by *ex vivo* treatment with Hsp70 [88]. In addition, NK cells appear to target a population of tumor cells that express Hsp70 on the cell surface, and exteriorized Hsp70 appears to act as a receptor for killing by NK in tumor cells [88, 89]. NK cells may also form part of the tumor response to chaperone-based vaccines. For instance, in mice responding to an Hsp70-Mage3 fusion vaccine, NK cells as well as CD4+ and CD8+ T cells were required for antitumor activity [90].

4. Autoimmunity, Heat Shock Proteins, and Cancer Therapy

Much available evidence, particularly from study of autoimmune responses, suggests that some HSPs play an anti-inflammatory, immunosuppressive role *in vivo* and could

contribute to the immunoregulatory properties of the tumor milieu by, among other effects, recruiting MDSC and Treg [27]. However, these immunosuppressive properties may be reversed under certain circumstances, and an Hsp70-based vaccine prepared from a fusion of tumor cells and DC (Hsp70.PC-F) is able to overturn tolerance to tumor antigens *in vivo* and mediate CTL killing of tumor cells [87, 91, 92]. In these studies, Hsp70 was prepared in a complex with Hsp90 and a broad repertoire of tumor-associated antigens. Another approach has attempted to recruit autoimmunity in the cause of tumor immunotherapy. Initially it was shown that if proliferating melanocytes were subjected to necrotic killing in the presence of elevated levels of intracellular Hsp70, an immune response could be generated capable of killing distantly located, transplanted B16 melanoma cells [93]. The rationale behind the treatment was that melanoma cells can reexpress antigens that are also present in proliferating melanocytes and that necrotic killing should in principle be proinflammatory [80]. A broad consensus agrees that while necrotic death is immunostimulatory, engulfment of apoptotic cells is suppressive to the immune response [80]. The role of Hsp70 in this system was not fully defined although it could involve chaperoning tumor antigens for delivery to APC and immunostimulation as shown previously [62]. In an inflammatory environment, Hsp70 peptide complexes can reverse the tolerance that develops to tumor antigens and trigger a CTL response that inhibits tumor growth as shown previously [87]. Another feature of this approach is that an autoimmune response, targeting melanocytes although predicted, was not in fact observed, a feature that was ascribed to a delayed Treg response triggered by the therapy that presumably dampened autoimmunity [93]. Some of the molecular determinants that may underlie these effects were discovered in a subsequent study. In a similar approach, normal prostatic tissue was destroyed by viral lysis, and this led to regression of distant transplanted prostate cancer. These effects required a combination of necrotic killing of and Hsp70 overexpression in the normal prostate tissue. In the absence of Hsp70, necrotic killing led to induction of the cytokines TGFβ and IL-10 and a Treg response. When cell killing occurred in Hsp70 overexpressing cells, IL-6 was expressed to high level and the combination of IL-6 and TGFβ led to the synthesis of IL-17 [94]. IL-17 is a powerful inflammatory cytokine and is involved in the conversion of CD45+Cd25+Foxp3 Treg cells to inflammatory, ROR, gamma-expressing Th17 cells that are known to play a profound role in the inflammatory response [95]. Thus, the cytokine milieu in tissues exposed to elevated HSP concentrations may be critical in determining the direction of the response (Figures 1 and 2).

5. Discussion

As tumors progress, they develop distinct characteristics due in part to the recruitment of nonmalignant cells to the growing mass (Figure 1). The malignant lesions appear to share some of the characteristics of healing wounds and are known to attract MSC from bone marrow that can develop into TAF and secrete cytokines that permit tumor growth and angiogenesis [96]. In addition, tumor cells rapidly outgrow the local microcirculation and become deficient in nutrient and oxygen supplies leading to necrosis and attraction of tumor-associated macrophages [9]. However, despite these vestiges of inflammation, the tumor milieu tends to be immunosuppressive and is often rich in MDSC and TAM that secrete IL-10 and chemokines [10]. The cytokine milieu, although supporting tumor growth, is thus rich in TGFβ and IL-10, conditions that favor development of Treg. In addition, cancer stem cells are known to attract Treg and be resistant to immunotherapy [17, 97] (Figure 1). The tumor microcirculation also appears to be resistant to penetration by CD8+ T cells [12–14]. The cell biology of the tumor microenvironment thus seems to be an important component in permitting tumor cells to evade immunity, and these properties appear to reflect the cytokine makeup that develops in the tumor interstitium (Figure 2). HSPs may play a number of roles in this process as Hsp70-containing exosomes released from tumor cells can attract MDSC and suppress CTL-mediated immune killing. In addition, release from cells of free Hsp27, Hsp60, and Hsp70 may be immunosuppressive [36–38]. However, use of chaperone-based vaccines in experimental animals can lead to the "Holy Grail" of antigen-specific antitumor immunity and can overturn tolerance to tumor antigens such as MUC1 [51]. These promising findings have not yet translated into clinical advances, and results of clinical trials with HSP vaccines have so far been modest [98]. Improvements in responses to chaperone vaccines could come from increasing the inflammatory nature of the tumor milieu by combination with other modalities. Conditions that increase the levels or activity of IL-6 can increase uptake of CTL into tumors and lead to conversion of regulatory Treg into inflammatory Th17 cells [94, 99, 100]. Combination of chaperone vaccines with ionizing radiation or focused ultrasound could be envisaged [101, 102]. It has also been shown recently that tumors are enriched in coinhibitory molecules that can suppress the proliferation and viability of the CTL that may penetrate tumors even though their T-cell receptors may be engaged with cognate MHCI-antigen complexes on tumors. Indeed, although CTL triggered by activated DC in the lymph node may express costimulatory CD-28 and are primed for immune killing, influence of the tumor microenvironment may switch expression to high-affinity coinhibitors such as CTLA-4 or PD-1 that suppress the signals generated by the T-cell receptor [103–105]. Currently, monoclonal antibodies with the properties of blocking the effects of CTLA-4 and PD-1 are being used clinically to boost antitumor immunity and could potentially be used in combination with chaperone-based vaccines [103, 104]. This may come at considerable costs to the health of patients bearing in mind the severe side effects of preventing coinhibition in terms of autoimmunity. Another addition to treatment that could be contemplated might be adding an innate immune stimulus to boost costimulation and levels of inflammatory cytokines. Although original studies suggested that HSPs might be potent activators of TLR signaling, it remains to be shown that HSP vaccines activate innate immunity in tumors

in vivo. Combination with virally derived oligonucleotides containing CpG motifs (TLR9 agonists) might be suggested [106]. Such agents are known to activate innate immunity in DC and are being tested clinically in cancer treatment [106].

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Review Article

M. paratuberculosis Heat Shock Protein 65 and Human Diseases: Bridging Infection and Autoimmunity

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Mycobacterium avium subspecies *paratuberculosis* (MAP) is the known infectious cause of Johne's disease, an enteric inflammatory disease mostly studied in ruminant animals. MAP has also been implicated in the very similar Crohn's disease of humans as well as sarcoidosis. Recently, MAP has been associated with juvenile sarcoidosis (Blau syndrome), autoimmune diabetes, autoimmune thyroiditis, and multiple sclerosis. While it is intuitive to implicate MAP in granulomatous diseases where the microbe participates in the granuloma, it is more difficult to assign a role for MAP in diseases where autoantibodies are a primary feature. MAP may trigger autoimmune antibodies via its heat shock proteins. Mycobacterial heat shock protein 65 (HSP65) is an immunodominant protein that shares sequential and conformational elements with several human host proteins. This molecular mimicry is the proposed etiopathology by which MAP stimulates autoantibodies associated with autoimmune (type 1) diabetes, autoimmune (Hashimoto's) thyroiditis, and multiple sclerosis. This paper proposes that MAP is a source of mycobacterial HSP65 and acts as a trigger of autoimmune disease.

1. Introduction

The term "molecular mimicry" is accredited to Damian in 1964. He was first to suggest that antigenic elements of microorganisms may resemble antigenic elements of their host. Damian suggested that this similarity served as a defense mechanism of a microorganism from the host's immune system and prevented the development of immune response to the microorganism, thereby protecting it from host defense [1]. Over time the term "molecular mimicry" was given a different meaning, namely, antigenic elements of microorganisms may trigger an autoimmune response that harms the host. The concept of molecular mimicry is based on a structural similarity between a pathogen and self. The similarity could be expressed as shared amino acid sequences or similar conformational structure between a pathogen and self-antigens. Molecular mimicry has become a very popular explanation for the frequent association of infection with autoimmune disease [2].

Under cellular stress such as heat, ethanol, heavy metals, endotoxins, and inflammatory processes, heat shock proteins (HSPs) are rapidly synthesized, increasing their concentration in the intracellular compartment as well as on the cell surface [3–9]. Heat shock proteins are found in virtually all life forms and are closely linked to the immune response. HSP65 of microorganisms is an immunodominant antigen. In human mycobacterial infection, for example, it has been estimated that up to 40% of the T-cell response is directed against this single protein [10, 11]. Although there is great homology between mycobacterial HSP65 among the species, genotyping of the 3′ end of the HSP65 gene has proven to unambiguously distinguish between the *M. avium* complex of mycobacteria as well as the cattle and sheep strains of *M. paratuberculosis* [12]. Immune responses to these proteins have been described in both infectious [13] and autoimmune diseases [14, 15] in human and animal models, suggesting that they could be attractive target molecules for interfering with these processes [16, 17].

This paper proposes that *Mycobacterium avium* ss. *paratuberculosis* (MAP) is a source of mycobacterial HSP65 and acts as a driver of autoimmunity [18].

2. MAP

MAP is a Gram-positive, acid-fast staining small rod-shaped bacterium. As with members of the Mycobacteriaceae genus, it has a unique cell wall structure rich in complex lipids. The thick and chemically distinctive cell wall of mycobacteria is largely responsible for the robust nature of these bacteria, both within the host cell and in the environment. The pathogenic potential of mycobacteria is correlated with their growth rate. Paradoxically, slow-growing mycobacteria are more virulent than fast-growing mycobacteria. With the exception of *Mycobacterium leprae* (the cause of leprosy in humans), which cannot be cultured in vitro, MAP has the slowest growth rate of pathogenic mycobacteria. After isolation from infected animals and grown under optimal conditions colonies of MAP are typically not visible for 3 months or more [19]. MAP causes a chronic granulomatous inflammation of the intestines in ruminant animals called Johne's disease. Mostly studied in dairy cattle, goats, and sheep, MAP also causes a chronic inflammation of the intestines in beef cattle and in a wide variety of other domestic and wild ruminants. MAP-induced enteric inflammation has been found in monogastric animals including dogs and pigs as well as four different types of subhuman primates—macaques, baboons, gibbons, and cotton-top tamarins [20]. A majority of the dairy herds in the United States and Europe have MAP infected animals within the herd [21].

3. MAP and Human Exposure

Infected cows shed up to 1.6×10^7 organisms per 2 grams of manure (0.07 oz)—a dose large enough to infect a calf. A single high-shedding animal can excrete up to 15 *gallons* of such contaminated manure per day—a staggering *25,000 infective doses per day* [22]. MAP is present in pasteurized milk [23, 24], infant formula made from pasteurized milk [25], surface water [26, 27], soil [26], cow manure "lagoons" that leach into surface water, cow manure in both solid and liquid forms that is applied as fertilizer to agricultural land [28, 29], and municipal tap water [29, 30], providing multiple routes of transmission to humans.

4. MAP and Human Granulomatous Disease: Crohn's and Sarcoidosis

In addition to Johne's disease of animals, MAP is the putative cause of the strikingly similar Crohn's disease of humans. Although there has been a century-long debate, the role of MAP in Crohn's is evolving from controversial to compelling [31–33]. The major source of the debate is that conventional methods of detecting bacteria—namely, culture and stain—are largely ineffective in detecting MAP. However, with newer laboratory techniques, primarily PCR, evidence of MAP is readily found in Crohn's tissues [34, 35]. In a study that evaluated the inflammatory bowel disease of attendants of goat herds where caprine Johne's disease is endemic, MAP was detected in the attendants compared to controls; the risk was correlated with the duration of association with the endemic goat herd [36]. The DNA of MAP can be identified within the granulomas of Crohn's biopsies [37] and, with extreme care and patience, MAP can be grown from the gut and blood of Crohn's patients [38–40]. In limited series, antimycobacterial therapy directed at MAP has been shown to have a favorable effect on patients with Crohn's disease [41].

Moreover, MAP has been historically linked is sarcoidosis; a multisystem inflammatory disease in which DNA evidence of MAP has been found (sporadically) in sarcoid granulomas [42]. Juvenile sarcoidosis (Blau syndrome) is an inherited granulomatous disease of children. The DNA of MAP was detected from every sample in a small series of archived tissues [43].

5. Genetics of Mycobacterial Infection and Autoimmunity

A complete discussion of shared genetic association to mycobacterial infection and autoimmune disease is beyond the scope of this paper. However, the role of the SLC11a1 gene is worth discussing in some depth. Natural resistance-associated macrophage protein 1 (NRAMP1) is now referred to as SLC11a1 (solute carrier 11a1). The gene that encodes for this protein is recognized as having a role in the susceptibility of humans and animals to a number of infections, including mycobacterial infections, and is associated with a number of autoimmune diseases as well.

The product of the SLC11a1 gene modulates the cellular environment in response to activation by intracellular pathogens by acidifying the phagosome thus killing the pathogen [44]. As such, it plays a role in host innate immunity [45]. Mutation of SLC11a1 impairs phagosome acidification yielding a permissive environment for the persistence of intracellular bacteria [46].

SLC11a1 polymorphisms are associated with paratuberculosis in cattle [47], goats [48], and sheep [49]. When researchers at the Belgium Pasteur Institute developed a murine model for MAP infection, they created an SLC11A1 defect mouse [50]. Given the pivotal roles that SLC11A1 plays in innate immunity and, as such, is not surprising that the relationship between polymorphisms in SLC11A1 and a number of mycobacterial as well as autoimmune diseases has been explored [51]. Associations have been found with leprosy [52], tuberculosis [53], and the aforementioned sarcoidosis [54]. Additionally, SLC11a1 associations have been found with rheumatoid arthritis [55], visceral leishmaniasis [56], multiple sclerosis [57, 58], inflammatory bowel disease [59–61], and type 1 diabetes mellitus (T1DM) [62, 63].

6. MAP and Type 1 Diabetes, Autoimmune Thyroiditis, and Multiple Sclerosis

While it is not difficult to envision a role for MAP in human disease where there is a granuloma, it is more difficult to

divine a role for MAP in diseases that feature autoantibodies. This divide is bridged by the concept that MAP HSP65 mimics host protein elements. An example is that of MAP as a proposed infectious trigger of autoimmune diabetes. T1DM is an autoimmune disease manifest by progressive T cell-mediated autoimmune destruction of insulin-producing beta cells in the pancreatic islets of Langerhans [64]. In 2005, Dow postulated a causative role for MAP in the T1DM [65]. Sechi et al. in 2007 found the DNA of MAP in the blood of autoimmune (type 1) patients but not nonautoimmune (type 2) diabetics [66–68]. (Sechi also found an association of polymorphisms of the SLC11a1 gene and MAP in T1DM patients [61].) The link connecting MAP and T1DM: MAP HSP65 mimic the host pancreatic glutamic acid decarboxylase (GAD) [69]. Similar mechanisms are proposed for the role of MAP in autoimmune (Hashimoto's) thyroiditis [70, 71] and multiple sclerosis [72].

7. Discussion

Not specific to MAP but to mycobacteria in general, mycobacterial HSP has been found in several additional autoimmune diseases [73]: the mycobacterial HSP65 has been implicated in the pathogenesis of rheumatoid arthritis [74], autoimmune hepatitis [75], primary biliary cirrhosis [76], and scleroderma [77]. HSP65 is also implicated in multiple vasculitis-associated systemic autoimmune diseases such as Kawasaki disease [78], Behcet's disease [79], and Takayasu's arteritis [80].

Although the SLC11a1 gene was featured in our discussion, there are several gene defects that are associated with mycobacterial infection and autoimmune disease. Besides SLC11a1, genes with strong mycobacterial susceptibility/autoimmune associations are the NOD2 gene [81], VDR (vitamin D receptor) gene [82], the LTA (lymphotoxin-alpha) gene [83], and the complement C4 gene [84]. The NOD2 (CARD15) gene has been of interest as different domains of the gene are associated with two aforementioned human diseases: Crohn's and Blau syndrome [43].

The list of diseases in which MAP has been implicated in a causal role is growing. This paper illuminates a parsimonious path linking MAP and a number of autoimmune diseases. The link proposed is mycobacterial HSP65 of MAP.

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Review Article

H11/HspB8 and Its Herpes Simplex Virus Type 2 Homologue ICP10PK Share Functions That Regulate Cell Life/Death Decisions and Human Disease

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Small heat shock proteins (sHsp) also known as HspB are a large family of widely expressed proteins that contain a 90 residues domain known as α-crystallin. Here, we focus on the family member H11/HspB8 and its herpes simplex virus type 2 (HSV-2) homologue ICP10PK, and discuss the possible impact of this relationship on human disease. H11/HspB8 and ICP10PK are atypical protein kinases. They share multi-functional activity that encompasses signaling, unfolded protein response (UPR) and the regulation of life cycle potential. In melanocytes H11/HspB8 causes growth arrest. It is silenced in a high proportion of melanoma prostate cancer, Ewing's sarcoma and hematologic malignancies through aberrant DNA methylation. Its restored expression induces cell death and inhibits tumor growth in xenograft models, identifying H11/HspB8 as a tumor suppressor. This function involves the activation of multiple and distinct death pathways, all of which initiate with H11/HspB8-mediated phosphorylation of transforming growth factor β-activated kinase 1 (TAK1). Both ICP10PK and H11/HspB8 were implicated in inflammatory processes that involve dendritic cells activation through Toll-like receptor-dependent pathways and may contribute to the onset of autoimmunity. The potential evolutionary relationship of H11/HspB8 to ICP10PK, its impact on human disorders and the development of therapeutic strategies are discussed.

1. Introduction

Small Hsp (sHsp) also known as HspB are a large family of widely expressed proteins that contain a 90-residue domain known as α-crystallin [1]. The 10 members of the human HspB family (17.0–28.4 kDa in size) are expressed widely and function as molecular chaperones, which restore protein folding and cellular homeostasis [2]. They have a flexible quaternary structure that allows structural changes, which alter their oligomeric substructure and interaction with target proteins depending on intracellular conditions and posttranslational modifications [3]. Current understanding of the HspB structure and function has recently been reviewed [4, 5]. Here, we focus on the family member H11/HspB8 and its herpes simplex virus type 2 (HSV-2) homologue known as ICP10PK and discuss the possible impact of this relationship on human disease.

2. HSV-2 Encodes Hsp Homologues with Antiapoptotic Activity

To the extent of our knowledge, HSV-2 is the only human viral pathogen that encodes Hsp homologues. One of these is UL14 that has 27% sequence identity with the protein-binding domain of Hsp70, but lacks ATPase activity. Like Hsp70, UL14 undergoes nuclear translocation after heat shock, it appears to aid in protein folding, and it has antiapoptotic activity [6]. The second HSV-2 protein that is an Hsp homologue is ICP10PK. It has 32% sequence identity with H11/HspB8, in the catalytic core and they share a number of unique functional properties.

2.1. ICP10PK Has Intrinsic Kinase Activity. ICP10PK is a serine/threonine kinase that is fused in frame to the large

subunit of the HSV-2 ribonucleotide reductase (R1, also known as ICP10) [7]. Phylogenetic analyses indicate that ICP10PK is a member of a family of atypical PKs (aPKs), also known as pseudokinases [8]. These kinases retain the 3 motifs required for kinase activity but lack one or more residues retained by the classical eukaryotic PKs. They can also function as scaffold proteins or allosteric activators [9, 10]. Pseudokinases do not always display catalytic activity but they are widely located in every branch in the phylogenetic tree, suggesting that they function like the eukaryotic PKs [10]. The ICP10PK functional domains are schematically represented in Figure 1(a). The catalytic core contains the 3 motifs essential for enzymatic activity that are best conserved relative to the eukaryotic PKs, including the Gly-rich loop (catalytic motif I), which is involved in stabilizing nontransferable ATP β-phosphates, and the two conserved charged residues that, respectively, constitute catalytic motifs II (Lys) and III (Glu) [11, 12]. The core is preceded by a transmembrane (TM) helical segment that is required for kinase activity, likely related to the conformational context that results from its anchorage into the plasma membrane [11–13]. It is followed by a degenerate α-crystallin motif [14] that establishes ICP10PK as being evolutionarily related to the HspB family [1].

Significantly, despite its overall similarity to HSV-2, HSV-1 has an R1 protein that contains a poorly conserved additional N-terminal domain, which lacks kinase activity [7, 8]. While the failure to detect kinase activity in the HSV-1 R1 protein has been interpreted to indicate that the kinase activity of ICP10 is due to contamination by casein kinase [15], available data provide incontrovertible evidence that the ICP10 kinase activity is intrinsic. Specifically, (i) kinase activity was lost through replacement of the Lys and/or Glu residues in catalytic motifs II and III or deletion of the TM domain, (ii) ICP10 binds the ^{14}C-labeled ATP analogue FSBA, and binding is specifically competed by another ATP analogue, AMP-PNP, (iii) treatment with epidermal growth factor activates the kinase activity of a chimeric protein consisting of the ligand-binding domain of the epidermal growth factor receptor and the PK domain of ICP10, and (iv) the ICP10 kinase activity favors Mn^{2+} ions, does not require monovalent cations, and is not inhibited by zinc sulfate, all of which are properties distinct from those of the putative contaminant [11, 12]. In fact, the HSV-1 R1 protein (ICP6) lacks kinase activity under conditions that allow for the putative contamination impugned for ICP10 [7].

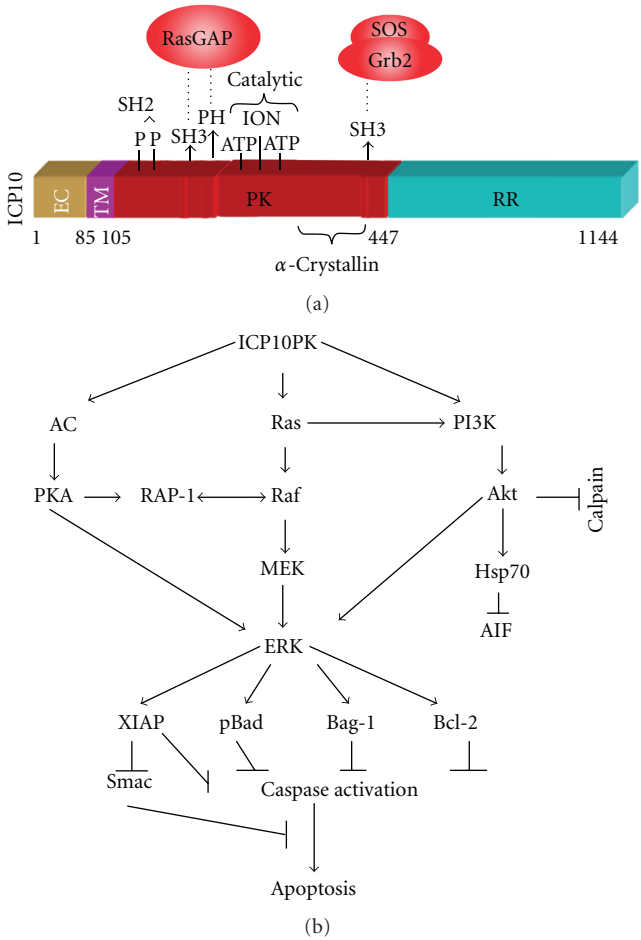

Figure 1: ICP10PK activates survival/proliferative pathways that initiate with Ras activation. (a) Schematic representation of the ICP10PK functional motifs. Shown are the binding sites for the Grb2-Sos complex and Ras-GAP, the PK catalytic motifs the TM and extracellular (EC) domains, and the α-crystallin domain. (b) The activated signaling pathways include the Ras/c-Raf/MEK/ERK, the PI3-K/Akt, and the AC/PKA. The pathways converge on ERK activation and modulate apoptosis regulatory proteins that control caspase activation (Bcl2 family) and caspase activity (iAP family) and their inhibitors (Smac). ICP10PK also inhibits calpain activation and AIF release. Ras activation is through recruitment of the GEF Sos and inhibition of the Ras-GAP through direct phosphorylation [10–13].

2.2. ICP10PK Is Required for HSV-2 Growth. ICP10PK is required for HSV-2 growth and latency reactivation [16–19]. This is related to its ability to inhibit neuronal apoptosis caused by proapoptotic viral genes and it involves the activation of survival pathways, notably Ras/MEK/ERK [20–27] (see Section 3 for further details). By contrast, both HSV-1 and a HSV-2 mutant deleted in ICP10PK (ΔPK) cause neuronal apoptosis [21] and activate the stress JNK/c-Jun pathway, which is required for their optimal replication ([28], Colunga and Aurelian, unpublished). The activated JNK/c-Jun pathway and neuronal apoptosis are involved in HSV-1-mediated adult encephalitis [29], which is the most common viral encephalitis. Encephalitis in adults is virtually never caused by HSV-2, likely related to the ability of ICP10PK to override the JNK/c-Jun-induced apoptosis [21]. The findings that (i) HSV-1 lacks Hsp homologues, (ii) the two viruses have distinct amino-terminal R1 domains, and (iii) HSV-1 activates stress as opposed to survival pathways in order to support its replicative cycle are indicative of a divergent evolution for these two largely similar viruses.

3. ICP10PK Activates Survival Pathways That Inhibit Apoptosis

ICP10PK behaves as a constitutively activated growth factor receptor that activates multiple survival/proliferative pathways (Figure 1(b)). Ras activation is a pivotal function. ICP10PK activates Ras both by recruiting the guanine nucleotide exchange factor (GEF) Sos and inactivating the Ras inhibitor Ras-GAP. To recruit Sos to the membrane and bring it into the proximity of Ras, ICP10PK binds the SRC homology 3 (SH3) domain of the adaptor protein Grb2 in the Grb2-Sos complex. To inactivate Ras-GAP, ICP10PK binds and phosphorylates it. Once it is activated, Ras further activates the downstream Raf/MEK/ERK and PI3-K/Akt pathways. In addition to these two major survival pathways, ICP10PK also upregulates/stabilizes adenylate cyclase (AC) and activates PKA, upregulates the Ras family member Rap-1, activates the B-Raf kinase activity, inhibits calpain activation and blocks the release of apoptosis-inducing factor (AIF), and upregulates/stabilizes Hsp70. Downstream signaling includes (i) increased activation/stability of the transcription factor CREB, (ii) stabilization of the anti-apoptotic protein Bcl-2 and the co-chaperone Bag-1, (iii) inactivation of the proapoptotic protein Bad through phosphorylation, and (iv) MEK-dependent upregulation of the antiapoptotic protein XIAP and downregulation of the apoptogenic protein Smac/DIABLO. In normal diploid cells, these ICP10PK functions are mitogenic (induce cell proliferation). In immortalized cells, they cause neoplastic transformation. However, in postmitotic neurons they inhibit programmed cell death (PCD) caused by different stress conditions, including HSV-1 infection [21–27]. Because cancer cells have constitutively activated Ras and PI3-K pathways, the ICP10PK deleted mutant ΔPK has potent oncolytic activity, which is based on cancer cell lysis through selective virus replication. Significantly, the ΔPK oncolytic activity also includes the activation of multiple programmed cell death (PCD) pathways and is accompanied by the upregulation of H11/HspB8 that functions as a tumor suppressor [30]. The role of the α-crystallin motif in these ICP10PK functions is still unknown.

4. H11/HspB8 Cloning and DNA Methylation

H11/HspB8 was cloned in our laboratory using ICP10PK as a probe and called H11 [31, 32]. It is also known as Hsp22 [33] and the product of the *E2IG1* gene expressed in estrogen-treated breast cancer cells [34]. H11/HspB8 has 32% identity and 59% homology to ICP10PK in the catalytic core [14, 31]. It was classified as an sHsp based on the presence of an α-crystallin sequence, which is believed to provide *sine qua non* evidence of evolutionary relationship, even when characteristic residues in the sequence have been lost [1]. Using FISH and M-FISH we originally reported that H11/HspB8 maps at position 12q24.1-12q24.31 [35] and its molecular location has since been established as 12q24.23. Our original data [35], confirmed by various investigations [4, 5], indicate that H11/HspB8 is ubiquitously expressed in many tissues, being most abundant in human skeletal and smooth muscle, heart, and brain, with smaller expression in prostate, lung, kidney, and skin and virtually no signal detected in ovaries, testes, liver, pancreas, and spleen. While H11/HspB8 has two heat-shock-factor- (HSF-) binding sites, 1,000 bases upstream of the translation initiation site, its expression is not always heat inducible and it appears to differ in tumor as compared to normal cells [36, 37]. In melanocytes, where it is not heat inducible, H11/HspB8 causes cell-cycle arrest through the inhibition of cyclin E/Cdk2 and β-catenin phosphorylation at the transcriptional activity site Ser^{552} [38]. Consequently, H11/HspB8 is silenced in a large proportion of melanoma tissues by aberrant DNA methylation (a CpG island is present at the 5′ UTR, 216 bp upstream of the transcription start site) [36, 38]. H11/HspB8 is similarly silenced in prostate cancer and Ewing's sarcoma cells [36] and in hematologic malignancies [39]. H11/HspB8 is unique among all Hsps in terms of methylation-associated transcriptional repression in human cells. However, the mechanism by which H11/HspB8 acquires a new methylation pattern in some, but not other, cells and the relationship between altered methylation patterns and loss of accessibility to heat shock transcription factors are still unknown.

5. H11/HspB8 Is Membrane Associated and Has Intrinsic PK Activity

Confocal microscopy confirmed that H11/HspB8, like ICP10PK, is associated with the plasma membrane [35]. However, unlike ICP10PK, which has a TM and extracellular domain, H11/HspB8 forms tight complexes with phospholipids located in the intracellular leaflet of biological membranes [40]. H11/HspB8 has myristoylation motifs (Gly-Xaa-Xaa-Xaa-Ser/Thr; residues 62 and 132) that could enhance membrane-binding potential. However, it is unknown whether H11/HspB8 is myristoylated and the functional significance of its membrane-binding potential, whether myristoylation related or not, is unclear. Indeed, ICP10PK is myristoylated [41], but this modification does not seem to affect its membrane-binding potential which is TM-determined and is required for its signaling activity (Sections 2.1 and 3). However, H11/HspB8 retains catalytic motifs I–III and has intrinsic serine/threonine kinase activity, which favors Mn^{2+} ions, like ICP10PK [31, 36]. Kinase activity was lost upon mutation of the invariant Lys (Lys113) in catalytic motif II [31] but not Lys115 that is not located within a conserved catalytic motif. The Lys113 mutant retains all the other potential phosphorylation sites in H11/HspB8, supporting the interpretation that kinase activity is not a contamination artifact. Studies of the isolated H11/HspB8 protein confirmed that it undergoes autophosphorylation [42]. Like ICP10PK, H11/HspB8 has an SH3 module located between the PK catalytic motifs and a potential *N*-glycosylation site (amino acid 138), suggestive of potential structural similarity [14, 36]. Significantly, however, H11/HspB8 has cell-type-specific

functions, which may or may not be kinase dependent (see Section 7).

6. H11/HspB8 and ICP10PK Inhibit UPR

Over 30% of cellular proteins are synthesized in the endoplasmic reticulum (ER). Delayed or impaired protein folding causes misfolded proteins accumulation thereby triggering ER stress that is characterized by distension and loss of homeostasis. To overcome the toxicity associated with the accumulation of misfolded proteins and/or their aggregation, the ER induces a cascade of reactions called the unfolded protein response (UPR). Cellular response to UPR restores normal cell function/survival and includes (i) translational attenuation, (ii) induction of ER-resident proteins that assist in protein folding, notably chaperones, (iii) induction of ER-associated degradation machinery, and (iv) ER enlargement to accommodate the large load of unfolded proteins. Translational attenuation is mediated by the double-stranded RNA-activated protein-kinase-like endoplasmic reticulum kinase (PERK) signaling pathway that reduces the activity of the ribosomal initiating factor (eIF2α) by phosphorylation of its α-subunit. Induction of ER-resident proteins is mediated by activated transcription factor 6 (ATF6) and inositol requiring kinase 1 (IRE1) receptors. ATF6 activates the transcription of molecular chaperones such as BiP, and IRE1 induces the synthesis of a potent X-box-binding protein 1 (XBP1) and consequently activates the transcription of ER-associated degradation proteins such as EDEM (reviewed in [43]). If the UPR response fails to restore cellular homeostasis, such as under conditions of prolonged UPR stress, the cell initiates apoptosis. Prolonged ER-stress-induced apoptosis is an important pathologic element of neurodegenerative diseases, diabetes, renal diseases, and atherosclerosis.

H11/HspB8 has basic chaperone activity. Original studies reported its interaction with HspB1 (HSP27) [33] but this proved to be assay dependent and was not seen in cross-linking and immunoprecipitation assays [31, 36] that in turn revealed its interaction with itself, HspB2, HspB6, β-crystallin, HspB3, and HspB7 [44]. Other proteins shown to interact with H11/HspB8 include Src-associated protein in mitosis 68 kDa (Sam68), which is involved in RNA transportation and processing [45], Ddx20, that has helicase activity [46], and β-crystallin mutants associated with the development of desmin-related cardiomyopathy or myofibrillar myopathy [47]. H11/HspB8 prevents in vivo aggregation of polyglutamine-containing proteins [48] and binds amyloid β-peptides (A 1–42 and A 1–40). However, it only inhibits the death of cardiovascular cells induced by Dutch-type A 1–40 mutants and the role that chaperone activity and/or UPR inhibition plays in protection is still poorly understood [49]. Two missense mutations, K141E and K141N, correlate with development of distal hereditary motor neuropathy type II (dHMN) [50] apparently related to decreased chaperone-like activity [48].

The ability of H11/HspB8 to inhibit UPR is underscored by the finding that it can remove the misfolded mutant superoxide dismutase (mSOD1) [51] that is involved in the development of familial amyotrophic lateral sclerosis (ALS). ALS is a relatively common and fatal adult motor neuron (MN) disease with a prevalence of 2 per 100,000 individuals. mSOD1 misfolds within the MN endoplasmic reticulum (ER) causing protein aggregates that also contain the proapoptotic transcription factor C/EBP homologous protein (CHOP) and activated (phosphorylated) p38MAPK (pp38MAPK). These aggregates activate UPR, which disrupts the normal protein quality control function of the ER and plays a definitive role in disease initiation [52]. The mechanism whereby H11/HspB8 removes mSOD1 is still not fully understood and may involve interaction with the co-chaperone Bag-3, Hsc70, and CHIP (chaperone-associated ubiquitin ligase), thereby promoting autophagic removal by CHIP-mediated ubiquitylation [53]. However, its protective potential in mSOD1 transgenic animals is unclear. Interestingly, ICP10PK has strong protective activity in mSOD1 transgenic animals, as defined by significantly delayed disease onset and progression ($P < 0.001$ versus PBS), preserved neuromuscular junctions, and inhibition of MN degeneration (Laing et al., unpublished). Inhibition of mSOD1 aggregate formation (Figure 2(a)) is associated with protection, and studies of neuronally differentiated N2a cells that were stably transfected with the mSOD1 mutant G85R (N2aG85R) indicate that ICP10PK inhibits the formation of protein aggregates that contain mSOD1, pp38MAPK, and CHOP, apparently through its ability to inhibit p38MAPK phosphorylation (Figure 2(b)). The contribution of the α-crystallin domain, if any, is unclear, but H11/HspB8 and ICP10PK share the ability to inhibit UPR and regulate disease development.

7. H11/HspB8 Is a Signaling Protein with Multiple Functions

Theoretical predictions and results of circular dichroism spectroscopy studies suggest that H11/HspB8 is an intrinsically disordered protein (IDP) [4]. IDPs are functional proteins that do not fold into stable tertiary structures under physiological conditions and are characterized by conformational flexibility, which allows them to quickly fluctuate among various conformational substates. This property allows the IDPs to respond reliably and swiftly to environmental changes and cellular signals. Intrinsic disorder is very high in signaling and cancer-related proteins [54]. While it is still unclear whether ICP10PK is an IDP and the contribution of intrinsic disorder to the H11/HspB8 signaling function is still unknown, H11/HspB8 displays a dual and opposing role in cell survival that is cell-type specific. In human skin, it is expressed in keratinocytes with long-term growth potential and its inhibition (with antisense oligonucleotides) inhibited DNA synthesis and cell proliferation in cultured human keratinocytes [32]. H11/HspB8 has antiapoptotic activity in glioblastoma and breast cancer cells through cell-cycle regulation, potentially involving activation of the growth-associated transcription factor E2F and/or the cyclin-dependent kinase cdk4. In

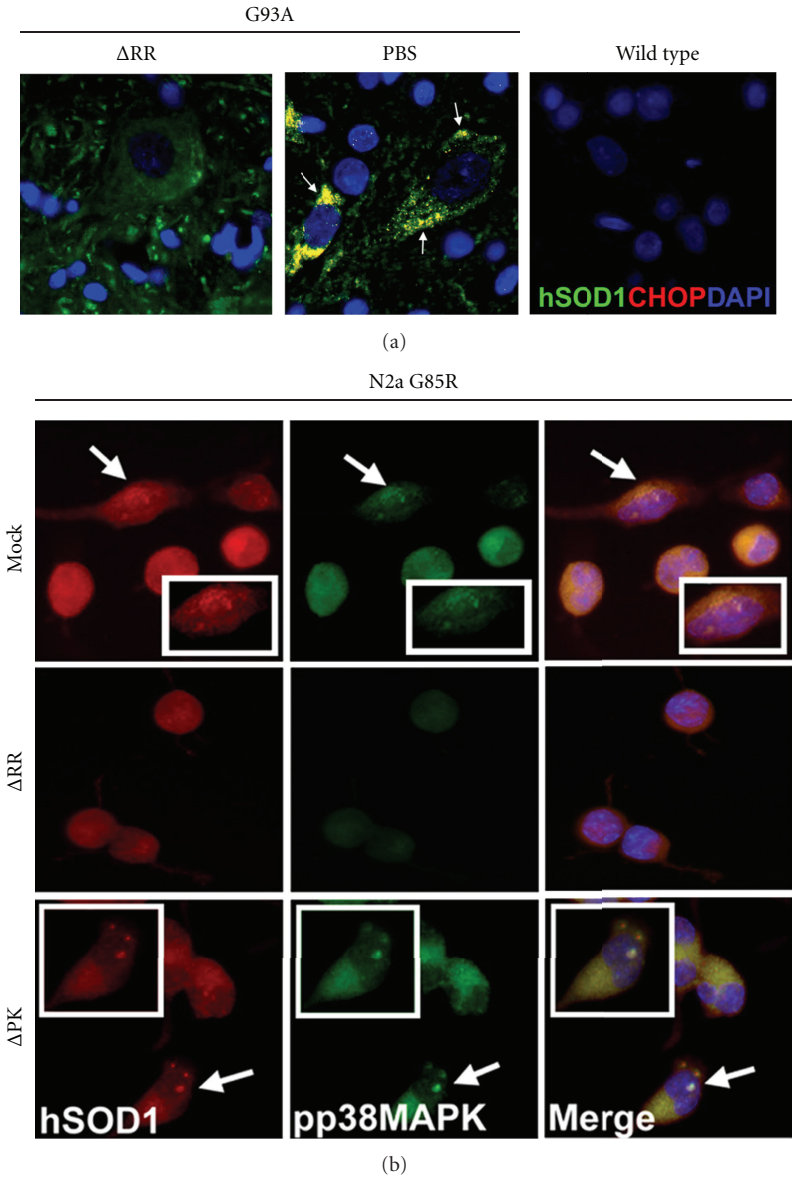

Figure 2: The ICP10PK vector ΔRR inhibits UPR. (a) mSOD1 (G93A) transgenic rats were treated with ΔRR (10^7 pfu; 100 μL) or an equal volume of PBS in both hind limbs by intramuscular injection beginning at age of 85 days. The ΔRR-treated G93A rats lived significantly longer than their PBS-treated counterparts with a median survival of 195 and 154 days, respectively ($P < 0.001$ by log-rank Mantel-Cox analysis). Serial sections from the L4-L5 region collected on day 153 were stained in double immunofluorescence with antibodies to human SOD1 (Alexa Fluor 488 conjugated secondary antibody; green) and CHOP (Alexa Fluor 594 conjugated secondary antibody; red). DAPI nuclear staining is blue. Only the PBS treated animals showed evidence of aggregates containing CHOP and mSOD1 co-localization. Scale bar = 10 μm. (b). Neuronally differentiated N2a cells that were stably transfected with another mSOD1 mutant (G85R) were mock infected with PBS or infected with ΔRR or a vector that lacks ICP10PK (ΔPK) and treated with H_2O_2 (100 μM; 2 hrs) to induce oxidative stress. N2a cells stably transfected with the wt SOD1 were studied in parallel and served as control. At 24 hrs after infection the cells were stained by double immunofluorescence with Alexa Fluor 594 conjugated SOD1 antibody (red) and Alexa Fluor 488 conjugated pp38MAPK antibody (green). DAPI nuclear stain is in blue. Scale bar = 10 μm. Aggregates are not seen in the ΔRR-treated cells, associated with the inhibition of p38MAPK phosphorylation (activation).

glioblastoma cells, H11/HspB8 expression is inversely correlated to Sam68, thereby increasing the S phase population and the expression of cell-cycle regulatory proteins such as cyclins E and A, ribonucleotide reductase, and proliferating cell nuclear antigen (PCNA), which are required for the transition from the G1 to the S phase of the cell-cycle [55]. In transgenic mice with increased cardiac expression, H11/HspB8 imparts myocardial hypertrophy accompanied by activation of Akt and p70S6 kinase [56, 57]. In these animals, H11/HspB8 directly interacts with bone morphogenetic protein (BMP) and activates both its "canonical" and "noncanonical" signaling pathways leading to regulation of

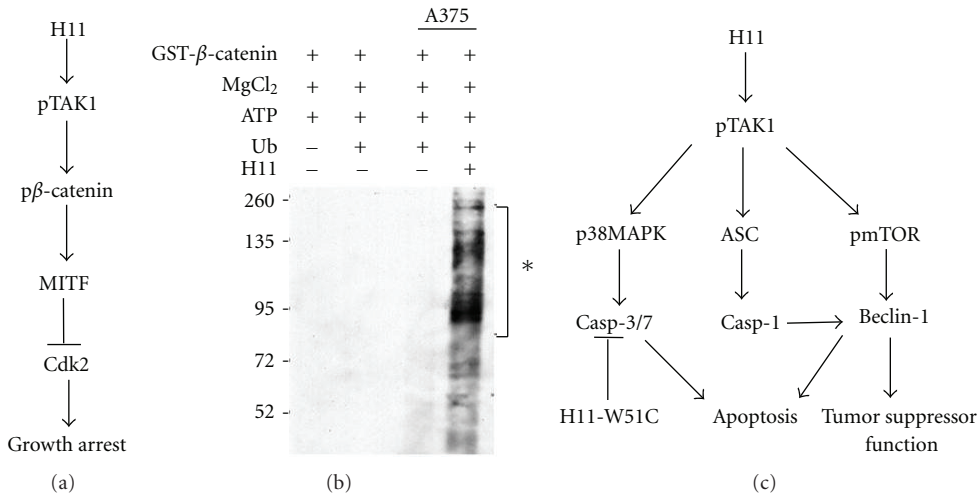

Figure 3: H11/HsPB8 causes growth arrest and melanoma cell death through distinct TAK1-regulated pathways. (a) Schematic representation of H11/HspB8-induced growth arrest involves β-catenin phosphorylation by the activated TAK1. (b) GST-β-catenin phosphorylated when mixed with cell extracts containing H11/HspB8-activated TAK1 is ubiquitylated causing its degradation ($*$). Ubiquitylation is not seen when GST-β-catenin is mixed with cell extracts that lack H11/HspB8 and therefore pTAK1. (c) H11/HspB8 induces different death pathways in the genetically diverse melanoma cells A375 and A2058 that initiate with TAK1 activation. In A375 cells, the TAK1/p38MAPK pathway activates caspase-3/7 to cause apoptosis. In A2058 cells, the TAK1/p38MAPK pathway activates caspase-3, but TAK1 also activates caspase-1 through ASC upregulation and upregulates Beclin-1 through mTOR phosphorylation at S2481 (pmTORS2481). Caspase-1 cleaves Beclin-1 to promote apoptosis, but Beclin-1 also contributes to cell death through still unknown tumor suppressor functions. The W51C mutant of H11/HspB8 has dominant-negative activity. It inhibits the death-inducing potential of the wild-type H11/HspB8 by inhibiting caspase activation through the activation of the B-Raf/MEK/ERK pathway [33].

activity and intracellular location of different PKs (including Akt, AMP-dependent protein kinase, and the ε-isoform of PKC) and inducible nitric oxide synthase [56–58]. However, its effect is dose dependent such that low doses (delivered with an adenovirus vector) increased the cell size, while high doses induced apoptosis of cardiac myocytes [59]. In these experiments, the hypertrophic effect was attributed to the activation of PI3-K and Akt and was independent of the H11/HspB8 kinase activity. By contrast, the proapoptotic effect was attributed to the inhibition of casein kinase 2 and it depended on the H11/HspB8 kinase activity [59]. The contribution of intrinsic disorder to these different functions is still unclear.

8. H11/HspB8 Is a Tumor Suppressor

In human melanocytes, H11/HspB8 causes growth arrest through β-catenin phosphorylation at the transcriptional activity site Ser^{552} and the resulting inhibition of the cell-cycle regulatory proteins cyclin E/Cdk2 [38]. Accordingly, H11/HspB8 expression is silenced through DNA methylation in a high proportion (77%) of melanoma tissues and cultures [38]. Significantly, H11/HspB8 is also silenced in a high proportion of atypical nevi, but not in most benign nevi, suggesting that its silencing contributes to tumor progression [60]. H11/HspB8 is also silenced by aberrant DNA methylation in prostate cancer and Ewing's sarcoma cells [36] and in hematologic malignancies [39] and its restored expression (e.g., by treatment with the demethylating agent Aza-C) induced cell death in all these cancers and inhibited tumor growth in xenograft models [36–39]. In melanoma, there is a good correlation between the levels of H11/HspB8 methylation, restored expression, and Aza-C-induced cell death, which is apparently related to the ability of high levels of restored H11/HspB8 to overcome the cell survival functions of the constitutively activated PI3-K/Akt pathway. Indeed, PI3-K inhibition increased Aza-C-induced cell death in the melanoma lines that had low/moderate levels of H11/HspB8 methylation and thereby restored expression [38]. While the effectiveness of Aza-C treatment in patients with metastatic melanoma is controversial, it is generally believed that future testing of Aza-C, in combination with other agents, is warranted [61]. However, the identification of molecular and prognostic markers remains a crucial aspect of patient selection for individual-targeted treatments. H11/HspB8 appears to be such a marker for Aza-C treatment of melanoma, with patients whose tumors have high levels of H11/HspB8 DNA methylation being seriously considered for demethylation therapy [38].

The cell-cycle regulatory potential of H11/HspB8 in normal cells, its dysfunctional state in cancer cells, and its ability to induce tumor cell death upon restored expression identify H11/HspB8 as a tumor suppressor. Also characteristic of tumor suppressors is the cell-type specificity of the H11/HspB8 effects and the finding that it can undergo single-site transforming mutation. Indeed, one of the H11/HspB8 missense mutants, W51C, causes anchorage-independent growth and it overrides the death-inducing capacity of the wild-type H11/HspB8 (Figure 3(c)) through activation of the B-Raf/MEK/ERK pathway [36], which is a notorious

contributor to melanoma development. The frequency of H1/HpB8 mutation is still unknown, but, given our finding that most studied melanoma cultures are sensitive to Aza-C-mediated cell killing, it is likely that such mutations are relatively rare.

9. Restored H11/HspB8 Expression Induces Tumor Cell Death through TAK1 Activation

Restored expression of H11/HspB8 causes growth arrest and inhibits tumor growth through the activation of programmed cell death pathways. It overrides the genetic diversity of melanoma tumors, a recognized obstacle to the development of effective therapeutic strategies, through the activation of distinct death pathways. However, all the inhibitory pathways initiate with the binding and direct phosphorylation (activation) of transforming growth factor β-activated kinase 1 (TAK1). TAK1 is not activated by the missense H11/HspB8 mutants P173H and W51C, which do not cause cell death. Growth arrest is through the ability of activated TAK1 (pTAK1) to bind and phosphorylate β-catenin (Figure 3(a)) promoting its ubiquitylation and proteosomal degradation (Figure 3(b)). The outcome is reduced nuclear accumulation and inhibition of transcriptional activity, as evidenced by decreased levels of the transcription factor MITF and its target CDK2 that functions in the G1/S and G2/M transitions (Figure 3(a)). This results in growth arrest in G1 and particularly G2 [37].

The death pathways initiated by pTAK1 include p38MAPK/caspases-3/7-dependent apoptosis, which is a major component of cell death in one of the studied melanoma cultures/xenografts (A375) and previously unrecognized death pathways in the other studied melanoma culture/xenografts (A2058) [37, 62]. In A2058 cells, the activated TAK1 caused upregulation of the inflammasome component apoptosis-associated speck-like protein containing a CARD (ASC) and it activated caspase-1 without producing IL-1β and IL-18, indicating that caspase-1 functions independent of the inflammasome-mediated death pathway (pyroptosis) [63]. Indeed, caspase-1 cleaved the haploinsufficient tumor suppressor Beclin-1 that was also upregulated by H11/HspB8 through a TAK1/mTORC1 pathway that involved mTOR phosphorylation at S2481, which is the site of intrinsic mTORC1-specific catalytic activity [64]. The cleaved Beclin-1 fragment translocated to the mitochondria and contributed to apoptosis, but the pronounced upregulation of Beclin-1 in A2058 xenografts, which have lower levels of H11/HspB8-induced apoptosis than their A375 counterparts, suggests that Beclin-1 also contributes to tumor growth inhibition through still unrecognized tumor suppressor functions (Figure 3(c)). These could include proliferative senescence, regulation of autophagic cell death networks, and cell-type-specific nonapoptotic cell death induced by deregulated Ras activation. Collectively the data identify TAK1 as a central player in the H11/HspB8-induced growth arrest/death of melanoma cells and underscore the therapeutic promise of H11/HspB8. It is still unclear whether the same pathways are used by the restored H11/HspB8 to cause cell death in prostate cancer, Ewing sarcoma, and/or hematologic malignancies.

10. H11/HspB8, Inflammation, and Autoimmune Disorders

H11/HspB8 was implicated in inflammatory processes based on its ability to activate dendritic cells through a Toll-like receptor-4- (TLR4-) dependent pathway [65]. Its restored expression in melanoma cells induces the proinflammatory cytokine TNF-α (Figure 4(a)), which likely contributes to tumor growth inhibition [66]. This pathway also initiates with H11/HspB8-mediated TAK1 activation. It includes pTAK1-mediated phosphorylation of receptor-interacting protein 2 kinase (RIP-2) (inhibited by the dominant-negative TAK1 vector K63W (Figure 4(c))) that activates NF-κB [67] causing its intranuclear localization (Figure 4(b)). NF-κB induces TNF-α by binding kB promoter elements [68] (Figure 4(d)). The data confirm that TAK1 activation is the pivotal factor in the ability of H11/HspB8 to regulate cell life/death decisions including inflammatory pathways other than pyroptosis (see also Section 8).

The ability of H11/HspB8 to induce cytokine production is consistent with findings for ICP10PK, except that the latter decreases the levels of TNF-α while increasing expression of the anti-inflammatory cytokine IL-10, at least in microglia [27]. Notwithstanding their apparently different inflammation modulatory functions and the contribution of the cell type, the similarity between ICP10PK and H11/HspB8 suggests that they may contribute to the onset of autoimmunity through "molecular mimicry." Indeed, linear peptide epitopes, processed from viral proteins, mimic normal host self-proteins, thus leading to an immune cross-reaction between the virus and host cell antigens, with inflammation as a typical sign of autoimmune diseases [69, 70]. In addition, because chaperones can broadly associate with other proteins including autoantigens and recruit antigen presentation pathways, they can work as endogenous adjuvants in immune responses, for example, those previously induced by viral infection. However, additional studies are needed in order to better understand the possible interaction between H11/HspB8 and ICP10PK in inflammation and autoimmunity.

11. H11/HspB8 and the Origin of ICP10PK

H11/HspB8 and ICP10PK are atypical PKs that lack some of the catalytic motifs of the eukaryotic PKs [9, 10] and share 32% identity and 59% homology [14, 31]. They share multifunctional activity that encompasses signaling, UPR, and the regulation of life-cycle potential. However, their exact relationship remains unclear. The level of sequence homology between H11/HspB8 and ICP10PK is similar to that seen for viral Bcl-2 homologues and their cellular counterparts, supporting the interpretation that the two proteins are members of the same protein family. However, if we accept the premise that the presence of an

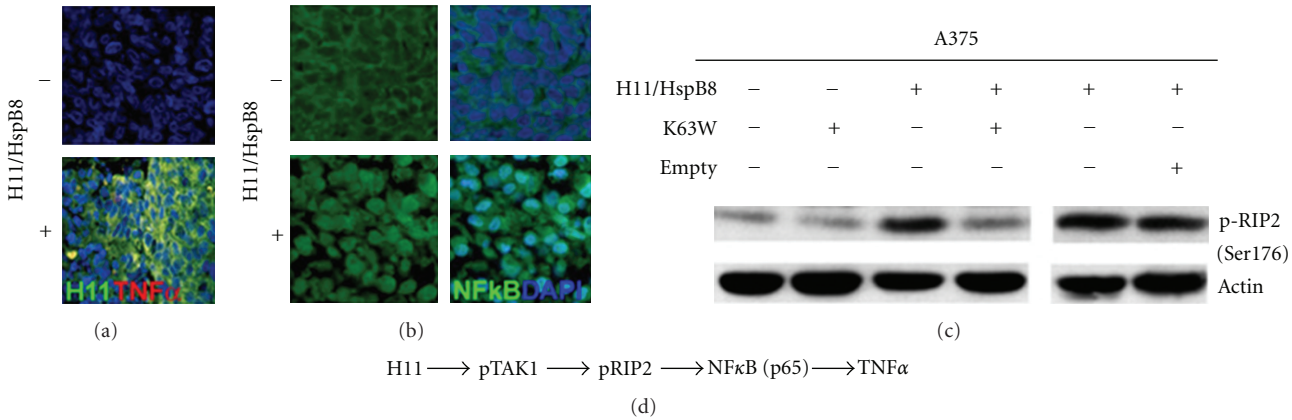

Figure 4: H11/HspB8 induces TAK1-dependent TNF-α expression. (a) Serial sections from A2058 and A375 xenografts collected at the end of the follow-up period from mice subjected or not to restored H11/HspB8 expression were stained with antibodies to H11/HspB8 (Alexa Fluor 488 conjugated secondary antibody; green) and TNF-α (Alexa Fluor 594 conjugated secondary antibody; red). (b). Serial sections from the A2058 and A375 xenografts as in (a) were stained with Alexa Fluor 488 conjugated NF-κB p65 antibody. Restored H1/HspB8 activates NF-κB (intranuclear localization). (c) A375 cells stably transfected with tet-inducible H11/HspB8 were given Dox to restore H11/HspB8 expression alone or with the TAK1 dominant-negative mutant K63W or empty vector. Extracts collected 3 days later were immunoblotted with antibody to pRIP2 (Ser176). They were stripped and reprobed with antibody to actin (loading control). (d) Schematic representation of the pathways involved in H11/HspB8-induced TNF-α induction.

α-crystallin motif, even if degenerate, is a *sine qua non* criterion for evolutionary-based inclusion into the sHsp family [1], we must infer that ICP10PK is evolutionarily related to H11/HspB8. According to this interpretation, ICP10PK is likely to have evolved from H11/HspB8, which was originally captured by HSV-2 in order to provide functional advantages favorable to virus survival, such as the inhibition of neuronal apoptosis. The finding that ICP10PK is required for HSV-2 growth and latency reactivation [16–19] supports this interpretation. Presumably, once it was captured, H11/HspB8 fell under the control of the viral R1 promoter, losing the regulatory constraints that define its cell-type-specific death-inducing potential while retaining kinase and ATPase-independent chaperone activity and the ability to inhibit UPR. This interpretation is supported by the finding that a missense mutation (namely, W51C) can convert H11/HspB8 from a proapoptotic to a dominant antiapoptotic protein that induces cell proliferation through the B-Raf pathway independent of the cell type. It is consistent with current understanding of virus evolution, which recognizes viruses as "gene robbers" that have evolved after cellular species [71]. However, the presence of a TM domain and its contribution to the ICP10PK kinase activity are distinct from the processes used by H11/HspB8, and the possibility cannot be excluded that H11/HspB8 evolved from ICP10PK captured by the cell from HSV-2 for a specific function. Indeed, recent studies have shown that cells contain genes of viral origin such as the syncitins, which are of retroviral origin and function in placentation. In simians, *syncytin-1* and *-2* entered the primate genome 25 and >40 Mya, respectively, and retained their coding capacity in all of the subsequent lineages. They display placenta-specific expression, are fusogenic, and may have immunosuppressive activity. A pair of env genes from endogenous retroviruses that have a completely distinct origin are also essential for placentation in the mouse, suggesting that, on several occasions in the course of mammalian evolution, retroviral infections have resulted in the independent capture of genes that have been positively selected for a convergent physiological role [72]. However, HSV DNA does not integrate, presumably reducing the likelihood of gene capture. Notwithstanding, the finding that HSV-2 is the only virus that contains Hsp homologues suggests that it has likely evolved differently than its closely related homologue, HSV-1.

12. Conclusion and Perspectives

The involvement of H11/HspB8 in the regulation of multiple cellular processes is unquestionable, but, despite the finding that it inhibits UPR and stimulates signaling, the molecular mechanism(s) of its multivariate activities remain largely unknown. Its similarity to ICP10PK, which is expressed by a common viral pathogen, underscores the evolutionary importance of these functions, particularly as it pertains to human disorders and therapeutic strategies. Future investigation should include the potential interaction of H11/HspB8 with ATP-dependent chaperones and their co-chaperones, as well as different components of proteasomal and autophagosomal systems. Further analysis of the signaling pathways modulated by H11/HspB8 should emphasize the contribution of the cell type, the criteria that define new methylation patterns in some tumors, and the genetic diversity of distinct tumor lines. Better understanding of the ability of H11/HspB8 to modulate regulatory protein families (namely Bcl-2 and IAP), its mutation frequency, and its contribution to disease should also be emphasized. The mechanism of the H11/HspB8-induced death of tumor cells other than melanoma (namely prostate) and the role

of TAK1 need further investigation, as does the contribution of H11/HspB8 to oncolytic virotherapy. Because H11/HspB8 is involved in different cellular processes, it appears to be an attractive therapeutic target. The use of H11/HspB8 as a marker for the selection of patients to be included in Aza-C treatment protocols should be further explored, particularly within the context of panels of hypermethylated genes and combinatorial therapy that includes PI3-K inhibition. The identification of hypermethylated genes that may contribute to H11/HspB8-induced cell death is particularly important. Finally, better understanding of the inflammatory potential of H11/HspB8 and the contribution that molecular mimicry with ICP10PK may have towards the development of autoimmune disorders should also be given further attention. Such understanding will provide important new tools to advance the development of much needed therapeutic strategies.

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Research Article

HSP70 and HSP90 Differentially Regulate Translocation of Extracellular Antigen to the Cytosol for Cross-Presentation

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Antigens (Ag) from cancer or virus-infected cells must be internalized by dendritic cells (DCs) to be presented to CD8$^+$ T cells, which eventually differentiate into Ag-specific cytotoxic T lymphocytes (CTLs) that destroy cancer cells and infected cells. This pathway is termed cross-presentation and is also implicated as an essential step in triggering autoimmune diseases such as Type I diabetes. Internalized Ag locates within endosomes, followed by translocation through a putative pore structure spanning endosomal membranes into the cytosol, where it is degraded by the proteasome to generate antigen peptides. During translocation, Ag is believed to be unfolded since the pore size is too narrow to accept native Ag structure. Here, we show that paraformaldehyde-fixed, structurally inflexible Ag is less efficient in cross-presentation because of diminished translocation into the cytosol, supporting the "unfolded Ag" theory. We also show that HSP70 inhibitors block both endogenous and cross-presentation. ImageStream analysis revealed that the inhibition in cross-presentation is not due to blocking of Ag translocation because a HSP70 inhibitor rather facilitates the translocation, which is in marked contrast to the effect of an HSP90 inhibitor that blocks Ag translocation. Our results indicate that Ag translocation to the cytosol in cross-presentation is differentially regulated by HSP70 and HSP90.

1. Introduction

The majority of extracellular Ag internalized by DC is processed and presented by MHCII molecules to activate CD4$^+$ T cells, but some fraction is integrated into the conventional MHCI antigen-processing pathway to prime CD8$^+$ T cells [1, 2]. The latter pathway is termed cross-presentation [3] and is indispensable for elimination of transformed and/or virus-infected cells [4, 5]. In addition to its role in host defense, cross-presentation certainly operates in autoimmunity. Thus, DCs are believed to uptake even self-antigens from cells and organs including mesenchymal tissues, process them, and present antigenic peptides in the context of MHCI molecules to prime self-Ag-specific CD8$^+$ T cells. Under certain conditions this can lead to a pathological attack on self-tissues. Several CD8$^+$-T-cell-mediated autoimmune diseases have been identified, such as Type I diabetes [6–9], multiple sclerosis [10, 11], nephritis [12, 13], and psoriasis vulgaris [14]. To prevent the development of such diseases, secondary lymphoid organs generally serve as the site for elimination of CD8$^+$ T cells recognizing self-Ag with high affinity, a process that has been referred to as cross-tolerance [15–17]. Both cross-priming and cross-tolerance are based on the same cross-presentation mechanism, but the outcome is dictated by many factors in the microenvironment. The details concerning circumstances that maintain or break cross-tolerance were comprehensively discussed recently in an excellent review [18].

In cross-presentation, the mechanism by which exogenous Ag translocates from the endosome/phagosome into the cytosol has long been a mystery. However, we recently have shown that cytosolic HSP90 participates in the translocation by pulling Ag out into the cytosol [19, 20]. Available HSP90 inhibitors initially led us to investigate its role in cross-presentation. Importantly, the results elucidated from pharmacological inhibition paralleled those obtained from experiments using mice and cells in which HSP90α was genetically ablated [20]. Thus, the use of chemical inhibitors to molecular chaperones such as HSP90 is a reasonable approach to dissect antigen-processing mechanisms. Based on this experience, we tested the effect of HSP70 inhibitors on antigen presentation when they recently became commercially available. We surprisingly found that the HSP70 inhibitor, VER, completely blocked both endogenous and cross-presentation in a dose-dependent manner. Our results presented here together with our previous studies lead to a model in which HSP70 downregulates and HSP90 upregulates Ag translocation during cross-presentation.

2. Materials and Methods

2.1. Mice. C57BL/6 (B6) mice were purchased from Clea. OT-I (H-2K^b restricted, anti-OVA TCR transgenic) mice [21] were kindly provided by Dr. Heath (The Walter and Eliza Hall Institute, Melbourne, VIC, Australia). All mice were maintained under specific pathogen-free conditions in the RIKEN RCAI animal facility according to institutional guidelines.

2.2. Cells. OT-I CD8$^+$ CD11b$^-$ T cells were purified from splenocytes by depletion of CD11b$^+$ cells followed by positive selection of CD8$^+$ cells by magnetic separation with the IMag system (BD Biosciences, Franklin Lakes, NJ, USA). The DC2.4 DC cell line was kindly provided by Dr. K. L. Rock (University of Massachusetts Medical School, Worcester, MA, USA).

2.3. Antibodies and Reagents. Mouse anti-OVA serum was generated in our laboratory. OVA (Sigma) was used as a model antigen. The proteasome inhibitor MG115 was from Biomol. The HSP70 inhibitors, VER ((5′-O-[(4-cyanophenyl)methyl]-8-[[(3,4-dichlorophenyl)methyl]amino]-adenosine)) and PIF (2-phenylethynesulfonamide), were purchased from TOCRIS bioscience and StressMarq Biosciences Inc., respectively. Endo-Porter was purchased from Gene Tools, LLC. All other reagents including radicicol were purchased from Sigma.

2.4. Paraformaldehyde Fixation of OVA. OVA were treated with 2% paraformaldehyde for 24 hours and dialyzed against PBS extensively. The proteins were applied to endotoxin removal PD10 column and used for AF-647 labeling or cross-presentation assay. All procedures were performed in a 4°C cold room.

2.5. Antigen Presentation Assay. The direct and cross-presentation assay for OVA is described in our previous reports [19, 20]. To analyze endogenous antigen presentation, plasmid encoding OVA protein (pcDNA3-myc-TEV-flag-OVA) was transfected into DC2.4 cells using a Nucleofector device (Amaxa Biosystems). Transfected cells were incubated with or without chemical inhibitors for 3 hr and then fixed with 0.5% paraformaldehyde (PFA) and 10^4 cells/well were cultured with 10^5 cells/well OT-I CD8$^+$ T cells. Culture supernatants were collected at the indicated hours of culture and the amount of IFNγ released was measured by ELISA. In the cross-presentation assay, DC2.4 cells were pretreated with chemical inhibitors for 15 minutes at 37°C and then pulsed with soluble OVA protein (0.5 mg/mL, unless otherwise indicated) in the presence of inhibitors for 60 min at 37°C. After washing out the free OVA, cells were cultured for another 2 hr in medium for antigen processing and then fixed with 0.5% PFA and quenched with 0.1 M Glycine. Fixed cells were cultured with purified OT-I CD8$^+$ T cells in 96-well U-bottom plates (DC 10^4: T cell 10^5 cells/well) for the indicated hours. The amount of IFNγ released into the culture medium by CD8$^+$ T cells was measured by ELISA.

2.6. Membrane Labeling and Uptake of Alexa Flow (AF) 647-OVA for ImageStream. To visualize and quantify the localization of pulsed OVA, the ImageStream system was used in this study following the protocol supplied by the manufacturer. The membranes of DC2.4 were labeled with PKH67 and the cells were then incubated with AF647-OVA (0.5 mg/mL) for 5 min followed by washing three times. The cells were then subjected to ImageStream analysis.

2.7. ImageStream Data Acquisition and Analysis. Details can be found in our previous report [20]. PKH67-labeled DCs (2×10^6 cells) were incubated with AF647-OVA (0.5 mg/mL) for 5 min and washed 3 times. The intact, single cells were analyzed by ImageStream. Double-positive cells, consisting of PKH67-labeled DCs and AF647-OVA (R2), were gated in the plots of PKH67 versus AF647 by comparison with the single color plots for each fluorophore (Figures 4(a) and 4(b), left panel). The double-positive cells included two populations: the DCs that had internalized AF647-OVA (R5 and R6) and those to which AF647-OVA was attached on the cell surface and not internalized (R4), as shown in Figures 4(a) and 4(b), right panel. The internalization score is adjusted such that a ratio value of 0.5 (half of the OVA is inside the cell) has a score of zero, so that a ratio value less than 0.5 (more than half of the OVA is outside the cell) has a negative score (R4) and a value greater than 0.5 (more than half is inside) has a positive score (R5 and R6). The phagocytic ratio was calculated by the counts of cells with internalized OVA (R5 + R6) divided by the count of total PKH67-positive DCs (R2 + R3). Gating for colocalized events was based on visual inspection of histogram bins. Representative images of R5 and R6 are shown in Figure 6. R5 was identified as the DC population with a significant proportion of internalized OVA dislocated

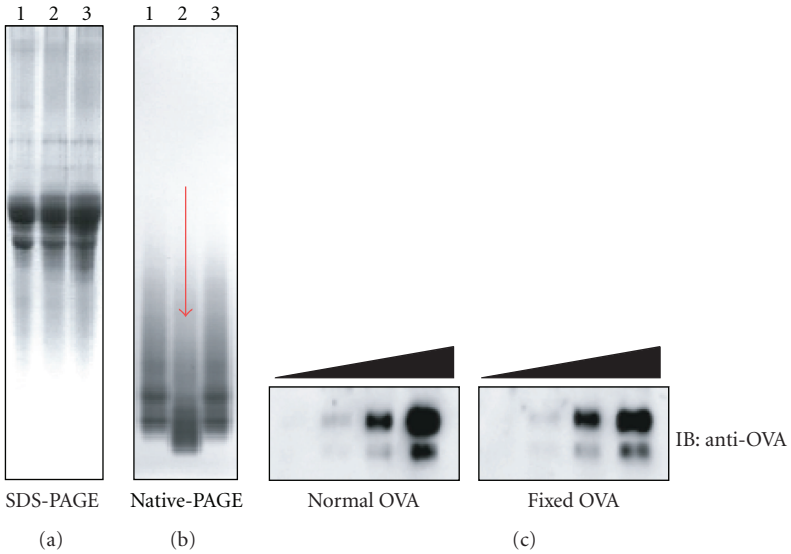

Figure 1: Paraformaldehyde-fixed OVA migrates faster than native OVA in native-PAGE but retains its immunoreactivity. Native OVA (lane 1), paraformaldehyde-fixed OVA dialyzed against PBS (lane 2), and native OVA dialyzed against PBS (lane 3) were analyzed by SDS-PAGE (a) and native-PAGE (b). (c) Native OVA and fixed OVA (1, 0.2, 0.04 μg/lane each) were run on SDS-PAGE, and western blot was performed using anti-OVA polyclonal antibodies.

from the endosomes into the cytosol. R6 was identified as the population in which most of the internalized OVA was still located within the endosomes.

2.8. Fluorescence Microscopy for Visualization of Vesicles. DC 2.4 cells, grown for approximately 16 hr on a glass bottom dish, were treated with MG115, with/without 10 μM inhibitors for 15 min. The cell membranes were stained with PKH67 on ice according to the protocol provided by the manufacturer, then pulsed with 0.5 mg/mL AF647-labeled OVA for 5 min at 37°C and fixed. The fluorescent images of the PKH67-labeled DC (green) and AF647-OVA (red) were observed with a KEYENCE BZ-9000 fluorescence microscope using GFP and Cy5 filter sets, respectively. The individual images were overlaid on image-processing software BZ-II analyzer. AF647-OVA, PKH67-DC, and their merged vesicles were defined as red, green, and yellow, respectively, by the software.

3. Results

3.1. Less Efficient in Cross-Presentation of Fixed Ovalbumin (OVA). In order to examine nature of Ag required for efficient cross-presentation, we treated OVA with 2% paraformaldehyde for 24 hours and then extensively dialyzed it against PBS. The treated OVA is hereafter referred to as fixed OVA. The fixed or native OVA were indistinguishable by conventional SDS-PAGE analysis (Figure 1(a)). However, fixed OVA were distinguishable by native-PAGE analysis as it migrated significantly faster than native OVA (Figure 1(b), lane 2). Both proteins were detected equally well with antibodies specific to OVA in western blot analysis (Figure 1(c)) and could be labeled to a comparable level with AF647 (Figure 2(a)). To test internalization efficiency, cells of the DC-like cell line DC2.4 were pulsed with AF647-labeled OVA for 5 to 30 minutes. After washing, the cells were analyzed by flow cytometry. As shown in Figure 2(b), internalization efficiency of the two proteins was nearly equal. Based on these results, we proceeded to carry out Ag cross-presentation assays using the fixed and native OVA. Graded doses of both proteins were pulsed onto DC2.4 cells for three hours and unbound OVA was washed off. The DC2.4 cells were then fixed to halt Ag processing, quenched, and incubated with OTI-CD8$^+$ T cells (specific to OVA$_{257-264}$-MHCI K^b complex) for 48 hours. Ag presentation efficiency was evaluated by quantification of IFNγ produced by OTI-CD8$^+$ T cells following recognition of MHCI K^b-OVA$_{257-264}$ complex on cell surface of DC2.4 cells. Notably, the amount of IFNγ produced by OTI-CD8$^+$ T cells cultured with fixed-OVA pulsed DC2.4 cells was significantly lower than that with native OVA (Figure 3). Thus, Ag presentation efficiency was apparently lower in DC2.4 cells pulsed with fixed OVA than with native OVA.

3.2. Less Efficient Translocation of Fixed OVA from Endosome to Cytosol. Since uptake efficiency by DC2.4 cells was equal between native and fixed OVA, we examined translocation of the two proteins following internalization by using the ImageStream system, which can dissect subtle differences in fluorescence distribution within cells and provide a statistical view by analyzing 10,000 cells, as previously we reported [20]. AF647-labeled native or fixed OVA (red fluorescence) was pulsed onto DC2.4 cells whose cell membranes were labeled with PKH67 (green fluorescence). 5 minutes later, the cells were washed and subjected to ImageStream. Intact and single cells were first selected, and then cells double

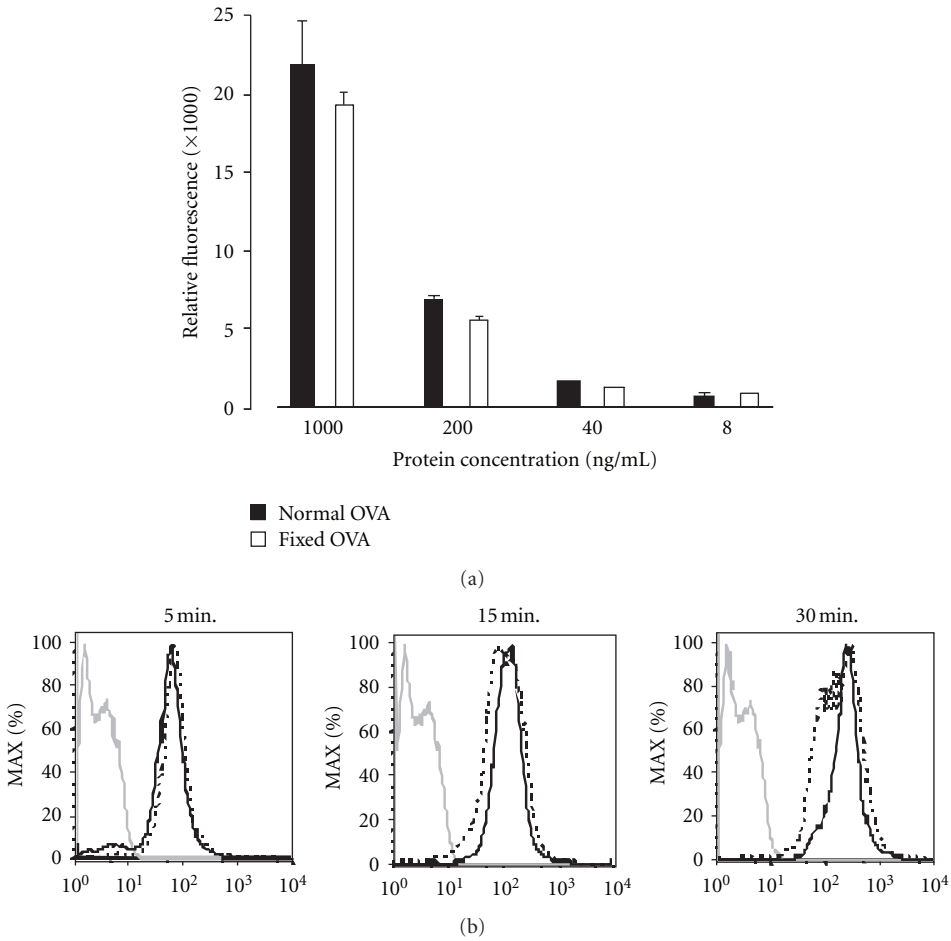

Figure 2: Internalization efficiency by DC2.4 cells is comparable between native and fixed OVA. (a) Labeling-efficiency with AF647 was equal between native and fixed OVA. Native and fixed-OVA were labeled with AF647 and graded doses were analyzed for their relative fluorescence incorporation. (b) DC2.4 cells were incubated with 1 mg/mL AF647-labeled native OVA or fixed OVA for the indicated times and internalization was analyzed by flow cytometer. (- - - -): native OVA, (—): fixed OVA. Gray line: unpulsed DC2.4 cells. Note: MFI = 114 (5 min), 159 (10 min), and 306 (15 min) with normal OVA and 77.3 (5 min), 160 (10 min), and 310 (15 min) with fixed OVA. The results were confirmed in two independent experiments.

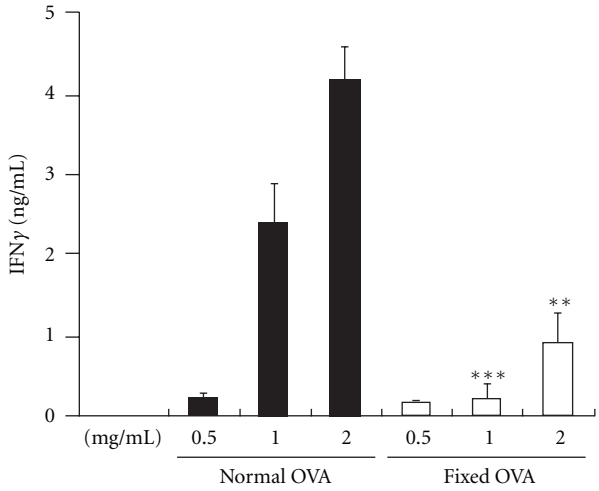

Figure 3: Cross-presentation of fixed OVA is less efficient than native OVA. Graded doses of native and fixed-OVA as indicated were pulsed onto DC2.4 cells for three hours. The cells were then fixed and incubated with OTI-CD8$^+$ T cells for 48 hours. The production of secreted IFNγ by the T cells was evaluated by ELISA. $^{**}P < 0.01$; $^{***}P < 0.001$. The results were confirmed in at least two independent experiments.

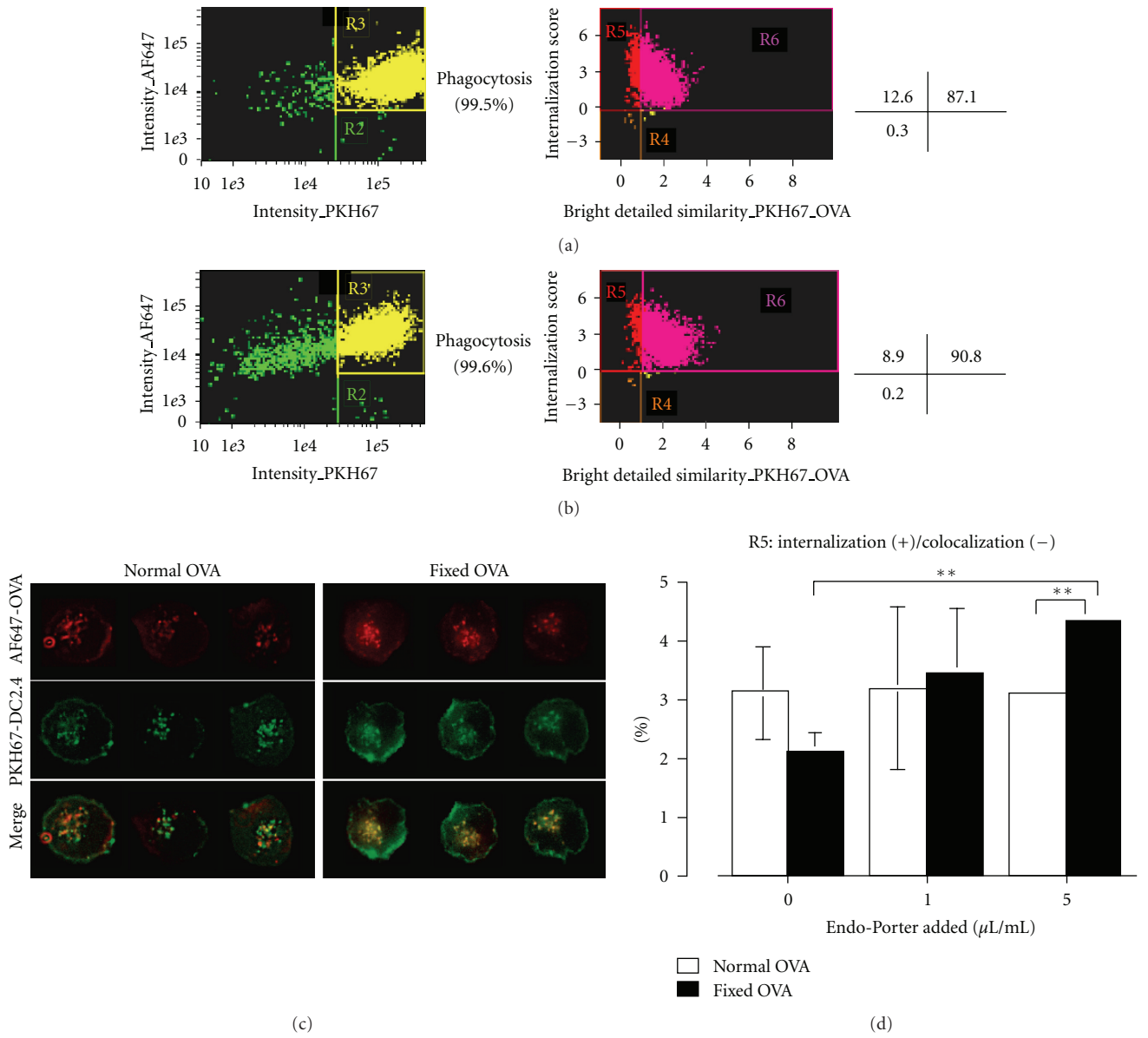

Figure 4: Translocation of fixed-OVA to the cytosol is less efficient than native OVA. 0.5 mg/mL AF647-labeled native OVA (a) and fixed OVA (b) were pulsed onto PKH67-labeled DC2.4 cells for 5 minutes, washed, and subjected to ImageStream analysis. The relative frequency of cells in R5 of native and fixed OVA, thus, internalization (+)/colocalization (−), was 12.6% and 8.9%, respectively. (c) AF647-labeled native or fixed-OVA was pulsed onto PKH67-labeled DC2.4 cells for 5 minutes, washed and subjected to fluorescence microscopy analysis. (d) The same experiments were done as in (a), except that DC2.4 cells were treated with or without Endo-Porter as indicated. The percentage of cells in R5 of native and fixed-OVA were plotted as a bar graph. Note: DC2.4 cells used in ((a)–(c)) were pretreated with 10 μM MG115 15 minutes prior to and during incubation with OVA in order to prevent proteasome activity. Data are mean ± SD of two separate experiments. $^{**}P < 0.01$.

positive for PKH67 and AF647 (identified as cells internalizing OVA, R3 in Figures 4(a) and 4(b), left panels) were further dissected according to bright detailed similarity (BDS) and internalization score. (The detailed principle is described in Section 2.) Populations in R5 and R6 were identified as cells where internalized OVA and membranes were not colocalized and colocalized, respectively. Thus, R5 identified cells where internalized OVA was translocated into the cytosol while R6 identifies cells in which internalized OVA is still retained within the endosome/phagosome. The representative fluorescence images were similar to those in Figure 6(a) (data not shown). Phagocytosis efficiency was comparable, thus, 99.5% and 99.6% with native or fixed OVA, respectively (Figures 4(a) and 4(b)). However, translocation efficiency was lower with fixed OVA (8.9%) compared to native OVA (12.6%), as seen in Figures 4(a) and 4(b), right panel. To further confirm this observation, we used fluorescence microscopy with a focal depth of

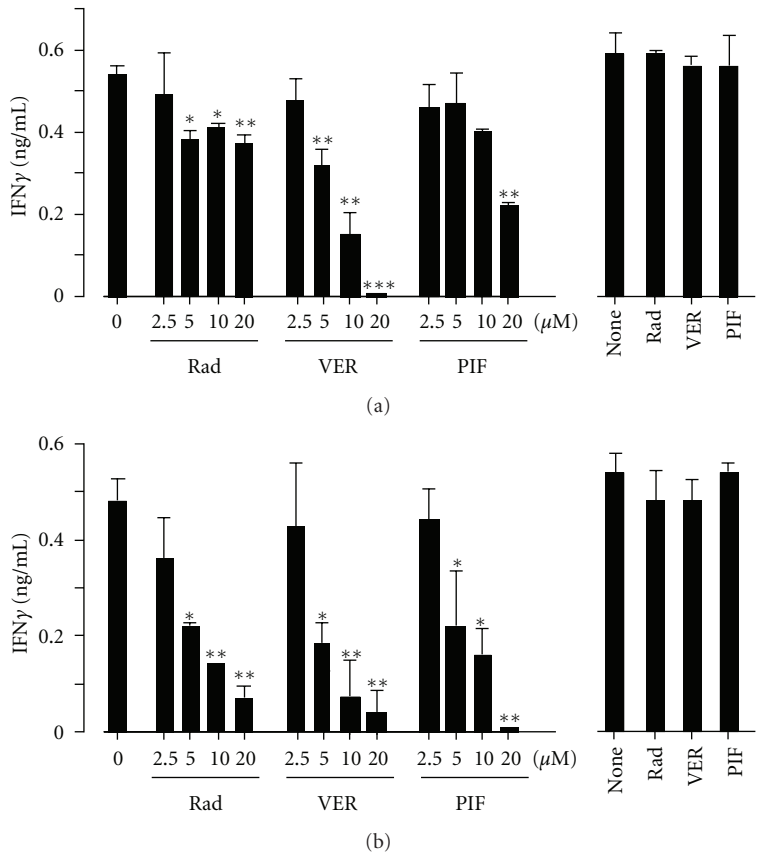

Figure 5: HSP70 inhibitor blocks both endogenous- and cross-presentation. (a) DC2.4 cells were transfected with a plasmid containing cDNA encoding OVA by electroporation and immediately incubated with the indicated inhibitors. Three hours later, the cells were fixed and incubated with OTI-CD8$^+$ T cells for 24 hours (left panels). Also, DC2.4 cells transfected with an empty plasmid were pulsed with a suboptimal dose of OVA$_{(257-264)}$ peptide (10^{-9} M) for three hours in the presence of inhibitors (20 μM each), fixed and were used as positive controls in the same assay (right panel). (b) DC2.4 cells were incubated with 0.5 mg/mL OVA in the presence of the indicated inhibitors for three hours and fixed. The cells were washed and incubated with OTI-CD8$^+$ T cells for 24 hours (left panel). Also, DC2.4 cells were pulsed with 0.5 mg/mL OVA in the presence of the indicated inhibitors (20 μM each) for three hours, fixed and were used as positive controls in the same assay (right panel). The production of secreted IFNγ by T cells in the supernatants was evaluated by ELISA.$^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. The results were confirmed in at least two independent experiments.

0.3∼0.4 μm compared to 4 μm in ImageStream. Red fluorescence, representing translocated OVA, was observed much more frequently in cells pulsed with native OVA compared with cells pulsed with fixed OVA (Figure 4(c)). These results indicate that fixed OVA is less efficient in translocation from endosome to the cytosol after internalization.

The imaging analysis suggests that structural flexibility of OVA is important in translocation; thus, mainly unfolded OVA undergoes translocation. We wondered if a chemical reagent Endo-Porter, which is believed to make transient artificial pores within endosomal membranes so that internalized molecules can be translocated into the cytosol, could affect translocation efficiency following internalization. We treated DC2.4 cells with Endo-Porter during endocytosis and then applied ImageStream analysis. Interestingly, we found that translocation efficiency of fixed OVA was increased by Endo-Porter in dose-dependent manner, whereas that of native OVA was unaffected (Figure 4(d)).

3.3. Differential Inhibition of Endogenous and Cross-Presentation by HSP70 and HSP90 Inhibitors. We previously reported that HSP90 inhibitors completely block cross-presentation but only partially block endogenous presentation and that Ag translocation into the cytosol depends on cytosolic HSP90 [19, 20]. With respect to the cytosolic molecular chaperones, HSC/HSP70 is an abundant heat shock protein, along with HSP90. Since HSC/HSP70 inhibitors became available recently, we investigated their effects on both endogenous and cross-presentation. Among HSP70 inhibitors, VER binds to the ATP binding site of HSC/HSP70 to block its chaperone activity [22–24], while PIF interacts with inducible HSP70 and disrupts its association with cochaperones and substrate proteins [25]. In addition to these two inhibitors, radicicol, an inhibitor to HSP90 was used as a control because we previously used it to dissect the role of HSP90 in cross-presentation [19, 20].

Interestingly, we found that VER completely blocked both endogenous presentation and cross-presentation in

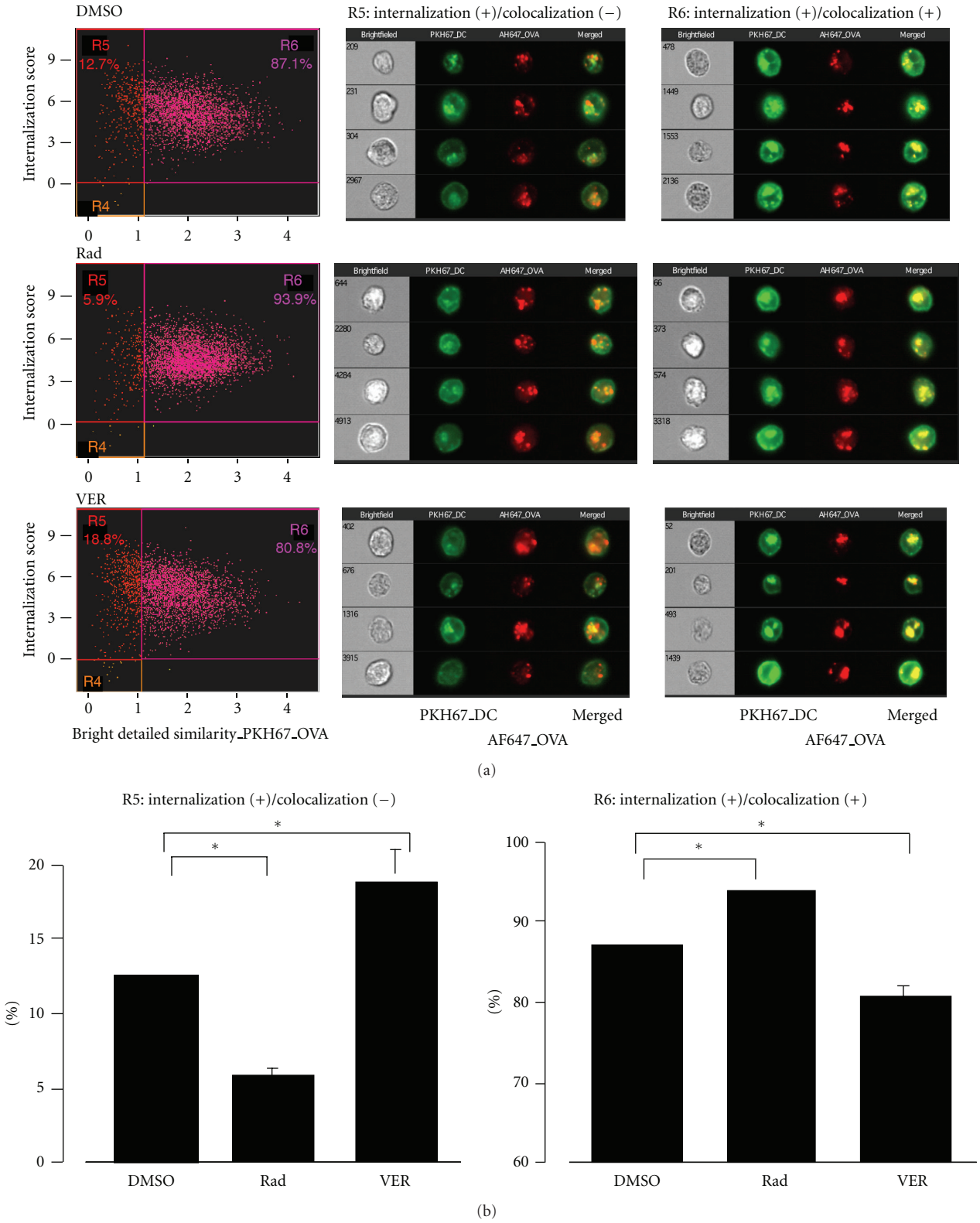

Figure 6: HSP70 inhibitor does not block translocation of OVA to the cytosol following internalization. (a) 0.5 mg/mL AF647-labeled OVA was pulsed onto PKH67-labeled DC2.4 cells for 5 minutes, washed and subjected to ImageStream analysis. DC2.4 cells were treated with the indicated inhibitors (10 μM) 15 minutes prior to and during the five-minute incubation with OVA. The cells were washed and subjected to ImageStream analysis. (b) Data in (a) were plotted as bar graph. Percentage of DC2.4 cells treated with the indicated inhibitors in R5 and R6 are shown. Data are mean ± SD of two separate experiments. $^{*}P < 0.05$.

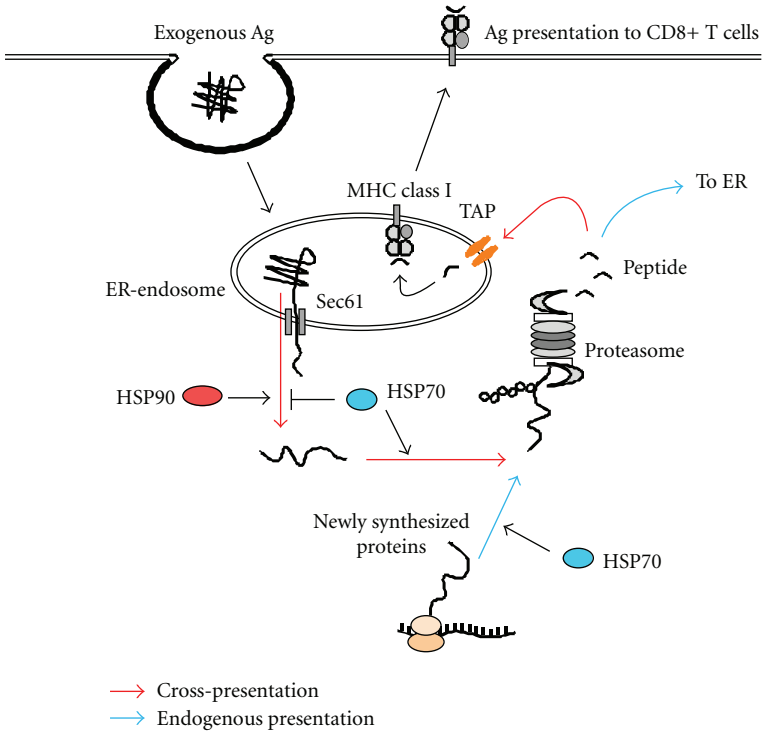

Figure 7: Model illustrating how HSP70 and HSP90 regulate cross-presentation and endogenous Ag presentation. In cross-presentation, internalized exogenous Ag is unfolded within the endosome and translocated through a putative translocon Sec61 complex into the cytosol. The translocation is facilitated by HSP90 but limited by HSP70. The translocated proteins are forwarded to the proteasome for degradation in HSP70-dependent manner. Ag-derived peptides generated by the proteasome enter the same endosome from which it dislocates to the cytosol through TAP molecules and associate with MHCI molecules. TAP molecules and MHCI molecules within the endosome are probably recruited from the endoplasmic reticulum (ER) in a Sec22b-dependent manner. In endogenous Ag presentation, a portion of newly synthesized, unfolded proteins are transported to the proteasome for their degradation by HSP70. Generated peptides enter the ER to associate with MHCI molecules.

dose-dependent manner (Figures 5(a) and 5(b), left columns). PIF also blocked cross-presentation completely and showed partial but significant blocking of endogenous presentation (Figures 5(a) and 5(b), left columns). As expected based on our previous results [19, 20], radicicol completely blocked cross-presentation, but there was only partial or marginal inhibition of endogenous presentation (Figures 5(a) and 5(b), left columns). The three inhibitors did not block presentation by DC2.4 cells pulsed with suboptimal dose (10^{-9} M) of $OVA_{257-264}$ peptides (Figures 5(a) and 5(b), right panels).

3.4. Differential Effect of HSP90 and HSP70 Inhibitors on Translocation of OVA from Endosome to Cytosol. The mechanism by which VER blocks cross-presentation was investigated in the context of OVA translocation following internalization by ImageStream. In the solvent (DMSO) control experiment, 12.7% cells were identified as internalization (+)/colocalization (−), thus, translocation (+) (Figure 6(a), upper, left panel, R5). Radicicol, as reported previously [20], downregulated translocation (5.9%) (Figure 6(a), middle, left panel). By contrast, VER did not inhibit translocation but surprisingly increased it (18.8%) (Figure 6(a), bottom, left panel). Fluorescence images of cells treated with each inhibitor (R5 and R6) are shown in Figure 6(a), middle and right panels. Bar graphs summarizing the results of Figure 6(a) are shown in Figure 6(b). These results indicate that an HSP70 inhibitor, VER, rather facilitates or at minimum does not inhibit Ag translocation, which is in marked contrast to the effects of radicicol.

4. Discussion

The present study focused on two important issues—the structural characteristics of Ag required for efficient cross-presentation by DC and the involvement of HSP70 in cross-presentation. These two issues may be seemingly distinct; however, they share a common thread—antigen translocation from endosome to the cytosol.

We showed that paraformaldehyde-fixed OVA was significantly less efficient in translocation from endosome to cytosol following internalization, thus, in cross-presentation, compared to native OVA. As internalization efficiency was nearly comparable between two forms of the protein, the decreased ability of fixed OVA is, at least in part, likely caused by its inflexible structure, which makes it difficult to translocate through a putative translocon in the endosomal membrane. In other words, the pore structure

of the translocon is too narrow for native proteins to pass through while keeping their original 3D structure, as previously suggested [1]. Artificial creation of pores in the endosomal membranes with Endo-Porter allowed fixed OVA to translocate to the cytosol, whereas normal OVA translocation was unaffected (Figure 4(d)), suggesting that the translocation mechanism of fixed OVA may be different from that of native OVA. It is likely that translocation of fixed OVA depends simply on the pore size of the translocon. Diffusion to the cytosol in an Ag dose-dependent manner might be a main mechanism operating in this case. By contrast, unfolding of an internalized Ag occurs within the endosome in the case of native OVA, which might provide an opportunity for HSP90 or other molecules to capture and pull the molecule out to the cytosol through the narrow window of the translocon. This concept should be further examined in future's experiments.

We showed that an HSP70 inhibitor, VER, completely blocked cross-presentation as well as endogenous Ag presentation (Figure 5). By contrast, the HSP90 inhibitor, radicicol, blocked cross-presentation completely but showed only partial inhibition of endogenous Ag presentation (Figure 5, [19, 20]). These results suggest that the molecular mechanism inhibited by VER is distinct from that inhibited by radicicol. Radicicol mainly blocks translocation of Ag from endosome to cytosol [20], whereas VER might block a mechanism common to both cross-presentation and endogenous Ag presentation. The common mechanism might be the transport of unfolded Ag to the proteasome. Thus, unfolded Ag emerging across the endosomal membrane or newly synthesized proteins on the ribosome that have become unfolded due to various reasons are captured by HSP70 and forwarded to the proteasome for degradation [26]. Indeed, HSP70 was shown previously associated with Bag1- and HSP70-bound proteins to be degraded are transported to the proteasome [27]. A postproteasomal event might also be a target of VER, since a similar HSP70 inhibitor deoxyspergualin (DSG) blocked MHCI antigen presentation due to dissociation of peptides chaperoned by HSP70 [28].

In contrast to radicicol, VER facilitates translocation of AF647-labeled OVA to the cytosol, indicating that HSP70 has a suppressive effect on translocation of exogenous Ag to the cytosol. HSP70-dependent, constitutive suppression of Ag translocation would therefore result in downregulation of cross-presentation. This scenario may seem contraindicated in terms of effective host defense. However, blocking of Ag translocation into the cytosol results in Ag retention in endosome, which in turn stimulates fusion with lysosomes, eventually followed by enhanced MHCII antigen presentation. In this context, we recently observed that HSP90α-deficient mice showed enhanced T-cell-dependent antibody production [29], whereas T-cell-independent antibody production was unchanged [29]. We believe that this phenotype is caused by a spontaneous increase in the knockout mice of MHCII presentation because compared to DC of normal mice, splenic DC of HSP90α-deficient mice showed increased ability to present Ag to CD4$^+$ T cells after pulsing with graded doses of OVA [29]. A model depicting these pathways is shown in Figure 7.

In conclusion, extracellular Ag to be translocated from endosome to the cytosol needs some structural flexibility, and translocation of Ag to the cytosol is regulated by two distinct cytosolic molecular chaperones, HSP70 and HSP90. The dichotomy of Ag presentation (MHCI versus MHCII presentation of exogenous Ag) regulated by HSP70 and HSP90 might be associated with cross-priming and cross-tolerance, which would give rise to differential outcomes in the onset of autoimmune disease as well as in host defense against cancer and infectious disease.

Authors' Contribution

Y. Kato and C. Kajiwara contributed equally to this work.

Acknowledgments

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Research Article

The Role of *M. leprae* Hsp65 Protein and Peptides in the Pathogenesis of Uveitis

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Experimental autoimmune uveitis (EAU) is a well established model for immune-mediated organ-specific disease. Our group has recently shown that the *M. leprae* Hsp65 aggravated the uveitis in mice; in the present study, we evaluated the action of *M. leprae* K^{409}A mutant protein and the synthetic peptides Leader pep and K^{409}A pep (covering amino acids residues 352–371 of WT and K^{409}A proteins of *M. leprae* Hsp65, resp.) on the pathogenesis of EAU. Mice received the 161–180 IRBP peptide and *B. pertussis* toxin followed by the intraperitoneal inoculation of K^{409}A protein or the Leader pep or K^{409}A pep. The Leader pep aggravated the disease, but mice receiving the K^{409}A pep did not develop the disease and presented an increase in IL-10 levels by spleen cells and a decrease in the percentage of CD4+ IFN-γ+ T cells. Moreover, animals receiving the Leader pep presented the highest scores of the disease associated with increase percentage of CD4+ IFN-γ+ T cells. These results would contribute to understanding of the pathogenesis of EAU and support the concept that immune responses to Hsp are of potential importance in exacerbating, perpetuating, or even controlling organ-restricted autoimmune diseases, and it is discussed the irreversibility of autoimmune syndromes.

1. Introduction

Experimental autoimmune uveitis (EAU) is an organ-specific, T-cell-mediated disease that targets the posterior pole of the eye [1] and is characterized by granuloma formation in the neural retina, destruction of photoreceptor cells, and blindness [1, 2].

The Hsp60 family comprises molecules that are immunodominant in several infectious processes [3]. The *Mycobacterium leprae* Hsp65 is part of this family-denominated chaperonins and one of the major immuno-reactive proteins in mycobacteria [3]. It is noteworthy that this protein shares approximately 50% amino acid identity with its mammalian homologue, suggesting their participation in the autoimmune diseases by molecular mimicry mechanism between self and exogenous Hsp molecules [4, 5]. The role of the Hsp65 has been intensively investigated in the pathogenesis of arthritis and diabetes [6, 7], murine systemic lupus erythematosus [8], ocular manifestations of Behcet's disease, and acute anterior uveitis [9, 10]. High anti-Hsp65 antibodies titers were correlated to the retinopathy in type I diabetes patients [11] and with the development and progression of atherosclerosis lesions [12, 13].

Moreover, in animals with acute EAE, lesions were accentuated by increased expression of Hsp60, primarily by infiltrating cells. In chronic EAE, Hsp60 was found predominantly on CNS components, particularly oligodendrocytes

and astrocytes, showing the participation of Hsp in CNS [14]. In addition, the 180–188 mycobacterial Hsp65 epitope, which is cross-reactive with a self-antigen in joint cartilage, is able to initiate and induce resistance to subsequent attempts to produce the adjuvant arthritis [6, 15].

Our group previously exposed that the passive administration of wild-type *M. leprae* Hsp65 interfered with endogenous equilibrium by enhancing the entropy of the immunobiological system, as expressed by the early death of the (NZB×NZW)F_1 experimental lupus mice [8] and aggravation of the ocular disease in experimental autoimmune Uveitis [16]. Following these studies, the biological effects and the primary sequence of *M. leprae* Hsp65 Leader pep and K^{409}A pep synthetic peptides, which cover residues 352–371, were shown [17].

In this context, in the present study, we evaluated the effects of the K^{409}A mutant Hsp65 protein and the Leader pep and K^{409}A pep peptides administration in the pathogenesis of EAU. We showed that K^{409}A and Leader pep were able to aggravate the disease. On the other hand, mice injected with K^{409}A pep did not develop the disease when compared to controls, K^{409}A, and Leader pep.

2. Methods

2.1. Animals. Six- to eight-week-old B10.RIII mice were obtained from the animal facilities at the University of São Paulo, Brazil. All animals were housed under specific pathogen-free conditions and handled under ethical conditions. The Animal Care Committee of the Institute of Biomedical Sciences at the University of São Paulo approved all the procedures used in this study.

2.2. Expression of the Recombinant K^{409}A Mutant M. leprae Hsp65 in Escherichia coli and Purification. Expression and purification of the recombinant K^{409}A mutant *M. leprae* Hsp65 was done as described in [8].

2.3. Peptide Synthesis. Leader pep (ENSDSDYDREKLQERLAKLA) of *M. leprae* Hsp65 and K^{409}A pep (ENSDSDYDREALQERLAKLA) of the mutated form K^{409}A, both covering residues 352–371, were synthesized using the Fmoc (N-(9-fluorenyl)methoxycarbonyl) procedure [18] in a Shimadzu PSSM8 peptide synthesizer (Shimadzu, Tokyo, Japan). The Fmoc-amino acids were purchased from Novabiochem (Nottingham, UK). The synthetic peptides were purified by preparative reversed-phase chromatography (reversed-phase HPLC), and the purity and identity of the peptides were confirmed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry on Ettan MALDI-TOF/Pro instrument (Amersham Biosciences, Buckinghamshire, UK) and by analytical reversed-phase high profile liquid chromatography (HPLC) (Shimadzu Inc., Tokyo, Japan).

2.4. Induction of EAU and In Vivo Treatment with Hsp65 Molecules. Mice [n = 4–6/group] were immunized subcutaneously (s.c.) at the base of the tail with 40 μg of interphotoreceptor retinoid binding (IRBP) 161–180 peptide emulsified in 0.2 mL of complete Freund's adjuvant (CFA) (v/v). At the same time, mice were injected intraperitoneally (i.p.) with 0.5 μg of *Bordetella pertussis* toxin (PTX) in 0.1 mL as an additional adjuvant and followed by single-dose inoculation of 2.5 μg of K^{409}A, Leader pep, or K^{409}A pep i.p.

2.5. Histopathology EAU. Eyes were collected and prepared for histopathological evaluation at the end of each experiment (day 21 after immunization). The eyes were immersed for 1 h in phosphate-buffered glutaraldehyde 4%, transferred into phosphate-buffered formaldehyde 10% for 24 h, and replaced with ethanol 70% until processing. Fixed and dehydrated tissue was embedded in paraffin wax, and 4–6 μm sections were cut through the papillary-optic nerve plane. Sections were stained by hematoxylin and eosin. Presence or absence of disease was evaluated in a double-blinded fashion by examining six sections cut at different levels for each eye. Severity of EAU was scored on a scale of 0 (no disease) to 4 (maximum disease) in half-point increments, according to a semiquantitative system described previously [1], according to lesion type, size, and number. In brief, the minimal criterion to score an eye as positive by histopathology was inflammatory cell infiltration of the ciliary body, choroids, or retina (EAU grade 0.5). Progressively higher grades were assigned for the presence of discrete lesions in the tissue such as vasculitis, granuloma formation, retinal folding and/or detachment, and photoreceptor damage [1].

2.6. Determination of Cytokine Production. Spleen cells harvested 21 days after immunization were cultured in 24-well plates (10^6 cells/well) and stimulated with 30 μg/mL IRBP. Supernatants were collected for cytokine analysis after 72 h and stored at $-80°$C until assayed. The level of IL-10 was assessed by ELISA using kit from BD/ Pharmingen (La Jolla, CA), and the level of IL-17 was assessed by ELISA using kit from eBioscience (San Diego, CA). All kits were used according to the manufacturer's instructions.

2.7. Intracellular Cytokine Staining and FACS Analysis. For detection of intracellular expression of IFN-γ, cells from draining lymph nodes and spleens collected at day 21 after immunization were labeled with anti-CD4 monoclonal antibody, fixed and permeabilized, stained intracellularly, and analyzed by flow cytometry evaluating the CD4 and IFN-γ expression. Cells were left unstimulated or stimulated overnight with IRBP (30 μg/mL) in the presence of either Golgiplug or Golgistop at the recommended concentrations (BD Pharmingen). All samples were acquired on a FACSCalibur (BD Biosciences), and data were analyzed with FlowJo software (TreeStar).

2.8. Statistical Analysis. Data are expressed as mean ± SD. Statistical analyses were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California, USA). Parametric Student's t-test was employed, and P values <0.05 were considered significant.

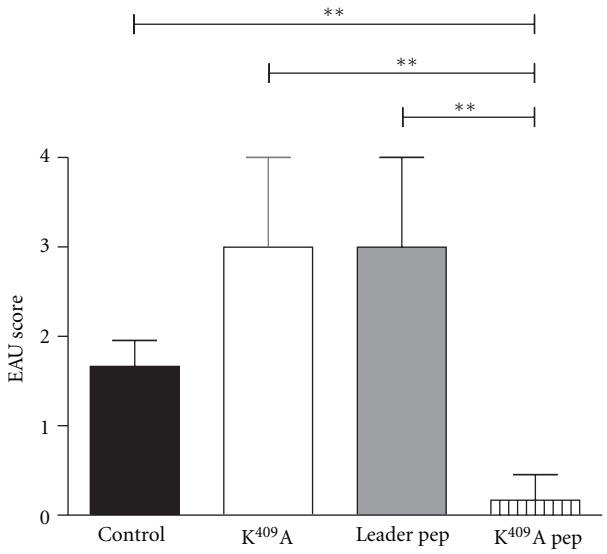

Figure 1: Administration of $K^{409}A$ protein and Leader pep increased EAU scores, and injection with $K^{409}A$ pep decreased EAU scores. B10.RIII mice were immunized with 40 μg 161–180 IRBP peptide on day 0 and followed by a single injection with 2.5 μg of $K^{409}A$, Leader pep, or $K^{409}A$ pep (i.p.). Eyes were collected for histopathology 21 days after immunization. EAU scores were assigned by histopathologic examination of the eyes on a scale from 0 to 4 according to the extent of inflammation and tissue damage. $^{**}P < 0.01$: control *versus* $K^{409}A$ pep; $K^{409}A$ *versus* $K^{409}A$ pep; Leader pep *versus* $K^{409}A$ pep. Disease incidence: control group: 75% of animals presented grade 1.5; $K^{409}A$: 75% of animals expressed grade 3; Leader pep: 75% of animals presented grade 3; $K^{409}A$ pep: 75% of animals expressed no disease.

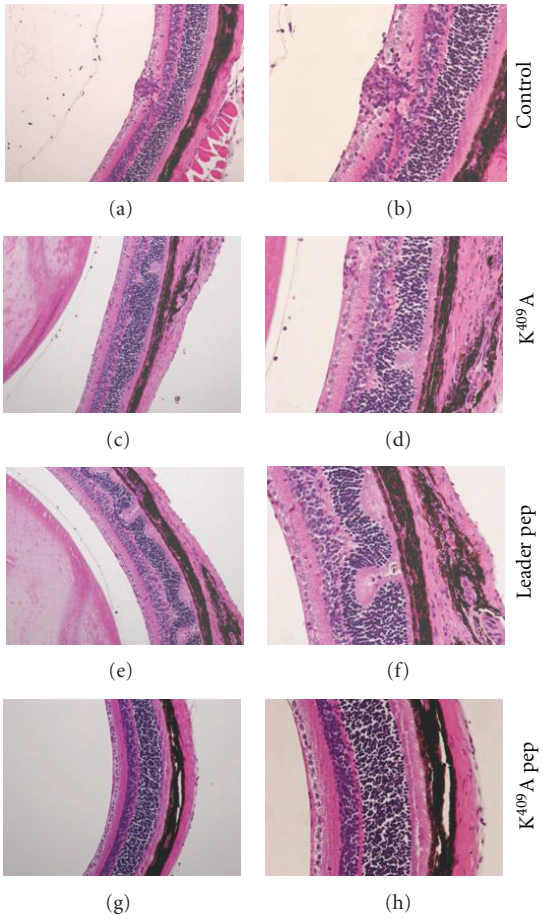

Figure 2: Histopathologic features representative of the EAU scores. Mice control shows ocular lesions characterized by cells infiltrating the vitreous and vasculitis (a) and (b); in mice injected with $K^{409}A$, it is possible to observe inflammatory cells in the vitreous and retinal folds (c) and (d); mice injected with Leader pep show cells in the vitreous and retinal folds (e) and (f); mice injected with $K^{409}A$ pep exhibit a normal retinal architecture corresponding to nonimmunized naïve mice (g) and (h). Representative photographs (hematoxylin-eosin staining). Original magnifications: 100× (left); 400× (right).

3. Results

3.1. $K^{409}A$, Leader Pep, and $K^{409}A$ Pep in the Disease Development in Mice. The animals were immunized and injected with $K^{409}A$, Leader pep, or $K^{409}A$ pep. We observed that $K^{409}A$ and Leader pep aggravated the development of the disease when compared with $K^{409}A$ pep and control group (Figure 1). On the other hand, animals injected with $K^{409}A$ pep did not develop the disease (Figure 1). The histopathologic examination shows vasculitis and presence of inflammatory cells in the vitreous in mice with EAU on day 21 (Figures 2(a) and 2(b), controls). In animals injected with $K^{409}A$ (Figures 2(c) and 2(d)) and Leader pep (Figures 2(e) and 2(f)), it is possible to observe the presence of inflammatory cells in the vitreous and retinal folds. On the other hand, in animals injected with $K^{409}A$ pep we can observe a normal organization of the retina (Figures 2(g) and 2(h)).

3.2. Decrease of T CD4+ IFN-γ+ Cell Population in Lymph Nodes and Spleens of Mice Injected with $K^{409}A$ Pep. It is known that autoreactive Th1 cells mediate EAU, and thus its induction correlates with the production of IFN-γ by T cells. In this work, we observe a significant increase in the frequency of CD4+IFN-γ+ T cells in lymph nodes (Figure 3(a)) of animals injected with Leader pep when compared to those of $K^{409}A$, $K^{409}A$ pep, and control groups. Also, there is a decrease of CD4+IFN-γ+ T cells in lymph nodes and spleen of the $K^{409}A$ pep mice group compared to the other experimental groups (Figures 3(a) and 3(b)). These results corroborate with our score results where we showed that mice injected with $K^{409}A$ pep presented lower scores of the disease when compared to mice injected with $K^{409}A$, Leader pep, and controls.

3.3. Analysis of IL-10 and IL-17 Levels in Spleen. The immunosuppressive cytokine IL-10 regulates EAU susceptibility and may be a factor in genetic resistance to EAU [19]. In this work, we showed an increase in IL-10 levels by mice injected with $K^{409}A$ pep when compared to other groups. This result corroborates the EAU score presented by $K^{409}A$ pep mice, in which the animals did not develop the disease

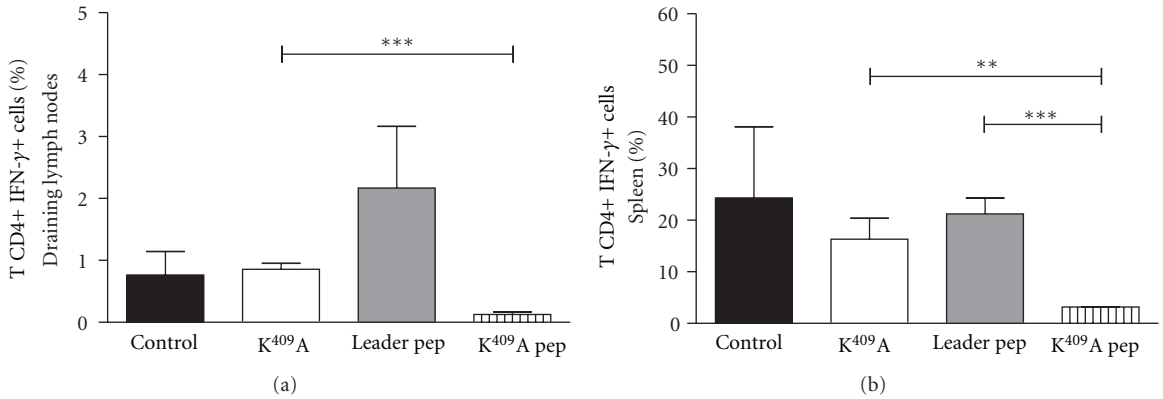

Figure 3: Expansion of CD4+IFN-γ+ T cells in lymph nodes (a) and spleen (b) after K^{409}A, Leader pep, and K^{409}A pep inoculation. Cells from draining lymph nodes and spleen collected at day 21 after immunization were labeled with anti-CD4 monoclonal antibody, fixed and permeabilized, stained intracellularly, and analyzed by flow cytometry evaluating the CD4, IFN- expression. Results are expressed in mean and SD. $^{***}P < 0.001$: K^{409}A *versus* K^{409}A pep (A); $^{**}P < 0.01$: K^{409}A *versus* K^{409}A pep (B); $^{***}P < 0.001$: Leader pep *versus* K^{409}A pep (B).

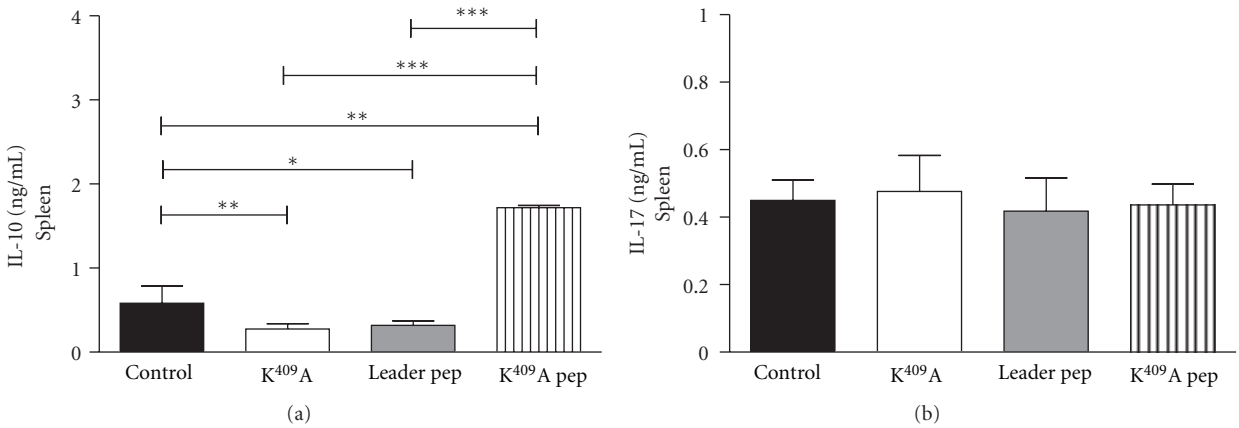

Figure 4: Analyses of IL-10 and IL-17 levels. Spleen cells from K^{409}A, Leader pep, and K^{409}A pep inoculated, or control mice were harvested at day 21 and stimulated *in vitro* with 30 mg/mL IRBP. After 72 hours, IL-10 (a) and IL-17 (b) levels were determined by ELISA. Results are expressed as mean ± SD. $^{**}P < 0.01$: control *versus* K^{409}A (A); $^{*}P < 0.05$: control *versus* Leader pep (A); $^{**}P < 0.01$: control *versus* K^{409}A pep (A); $^{***}P < 0.001$: K^{409}A *versus* K^{409}A pep (A); $^{***}P < 0.001$: Leader pep *versus* K^{409}A pep (A).

(Figure 4(a)). In regard of IL-17 production, it was observed that spleen cells of animals injected with K^{409}A, Leader pep, and K^{409}A pep produced the same amount of cytokines as the control animals (Figure 4(b)).

4. Discussion

Recently, we showed that EAU scores are increased in IRBP-immunized animals and inoculated with recombinant wild-type *M. leprae* Hsp65. Interestingly, this was associated with a higher expansion of CD4+IFN-γ+ (Th1) and CD4+IL-17+ T cells (Th17), and with higher levels of IFN-γ [16]. Moreover, Marengo and collaborators still demonstrated that WT Hsp65 inoculation accelerates death in mice which spontaneously develop the Systemic Lupus Erythematosus (SLE) [8].

In the present work, we showed higher EAU scores in animals injected with K^{409}A and Leader pep. However, animals injected with K^{409}A pep did not develop the disease. Histopathological analysis showed that animals injected with K^{409}A pep did not show lesions of retina when compared to controls, otherwise K^{409}A and Leader pep presented retinal folds, vasculitis, and inflammatory infiltrating cells in the vitreous.

To evaluate the effect of K^{409}A, Leader pep, and K^{409}A pep in the periphery, it was evaluated the CD4+ T cells producing IFN-γ in spleen and lymph nodes of mice induced to EAU. In this model, the increase in CD4+ IFN-γ + T-cell population in lymph nodes and spleens was associated with the increased scores observed in the K^{409}A, Leader pep, and control groups. Friedland and collaborators demonstrated that the secretion of proinflammatory cytokines from monocytes activated by mycobacterial 65 kDa Hsp may be important in the host immune response and in the development of antigen-specific T-cell-mediated immunity [20].

Th17 cells were recently described as crucial for the development of EAU [21–25]; however, it was not observed differences in IL-17 levels by splenocytes at day 21 after-immunization between the experimental groups. Thus, we

may assume that, at this phase, the Th17 cells are in the eye. On the other hand, different from that we observed in the present work, it was previously shown an expansion of $CD4^{+}IL\text{-}17^{+}$ T cells in mice injected with rHsp65 [16].

It is known that the immunosuppressive cytokine IL-10 regulates EAU susceptibility and may be a factor in genetic resistance to EAU [19]; the results showed that spleen cells from animals injected with $K^{409}A$ pep produced higher levels of IL-10. The increase of IL-10 levels can be associated with the absence of the disease, as IL-10 is a potent suppressive cytokine. Moreover, as we did not evaluate Tregs, it is also possible that higher levels of IL-10 observed in Leader pep mice group may be derived from this population such as Tr1 cells or IL-10 secreting $Foxp3^{+}$ cells.

In 2006, our group showed that rGal-1 administration was able to decrease the disease in mice with EAU, and this fact was associated with increased levels of IL-10 and decreased levels of IFN-γ [26]. A study with experimental arthritis showed that antibodies against the Hsp molecule suppress inflammation by inhibiting the proinflammatory effect of the Hsp on the innate immune system. The increase in IL-10 secretion in the inflammatory site can skew the local cytokine profile from an inflammatory to an anti-inflammatory response and thus explain the mechanism of protection against inflammation by these antibodies [27].

The opposite effects of the $K^{409}A$ protein and their correspondent peptide which covers the 352–371 residues, the $K^{409}A$ pep, in EAU score were unexpected. In $(NZB\times NZW)F_1$ mice, which present the $H\text{-}2^{d/z}$ alleles, the inoculation of the Leader pep and $K^{409}A$ pep shows resembled effects to their respectively proteins in the survival time, showing a amplified effect [17]. Here, in the EAU susceptible B10.RIII mice, the $H\text{-}2^{r}$ allele can explain at least in part their divergent effect. We can speculate that an important participation of the genetic background and immunobiological factors occurs. EAU is a organ-specific disease mediated by T-cells commitment, and SLE is a systemic disease characterized mainly by humoral self-response. Furthermore, differences at the H-2 loci in B10.RIII and $(NZB\times NZW)F_1$ mice suggest different or selective antigen-binding core and presentation to the immune system. Also, we can consider that the short length of the $K^{409}A$ pep facilitates its antigen processing of these cells, being more effective than the $K^{409}A$ protein. Some theoretical experiments based on molecules interaction by structural modeling studies can clarify the MHC molecules peptide binding to better comprehend the *in vivo* findings observed to the mutant forms.

Figure 5 is based on the linear equation $y = a + \blacktriangle_i$: this theoretical model presupposes that in anautoimmune process, it must be considered the entropy production [▲] during the disease progression where the slope a is the intensity and degree of the process aggravation; ▲, although variable to each individual, is a constancy for everyone, increasing at all illness episode [$_i$]. It was formulated the principle that *the immunological history of an individual is unique and irreversible*, being cumulative in an autoimmune process, staying progressively far from the physiological equilibrium [8]; thus, as for other autoimmunities syndromes, the uveitis

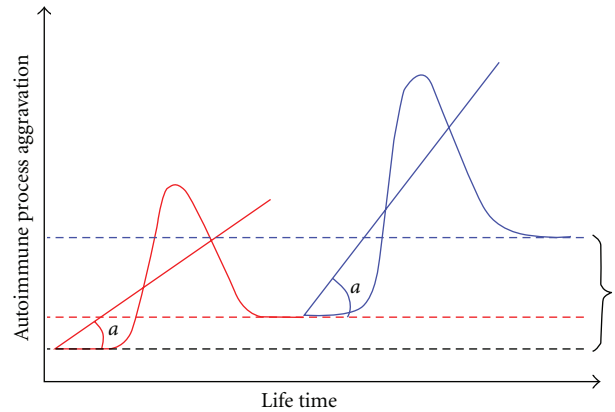

Figure 5: Autoimmune disease aggravation: Y: the individual state of an irreversible immune responsiveness; a: disease degree; $\blacktriangle_i$: entropy production, where i represents the number of autoimmune episodes being a time-dependent discrete variable.

is not reversible in the sense that the original immune state before the beginning of the disease cannot be reacquired. The general physiological conditions could be disrupted by similar homologous proteins, such as the *M. leprae* Hsp that, along the life time, proceeds performing a pathological role. At each new episode, the entropy production is higher, and the disease progression could only be controlled or delayed.

In conclusion, $K^{409}A$ and Leader pep were able to aggravate EAU development when administrated at the same time with the immunization. Moreover, mice injected with Leader pep presented high scores of the disease associated with increase percentage of CD4+IFN-γ+ T cells. However, $K^{409}A$ pep was able to inhibit the development of EAU, and this fact can be associated with an increase observed in IL-10 levels by spleen cells and with a decrease in the percentage of CD4+IFN-γ+ T cells. The results of this study would contribute significantly to understanding of the role of the Hsp molecules in the pathogenesis of ocular autoimmune diseases. They also support the concept that immune responses to Hsp have potential importance in exacerbating and perpetuating organ-restricted autoimmune diseases.

Conflict of Interests

All the authors declare that they have no conflict of interests.

Authors' Contribution

A. G. Commodaro, E. B. Marengo, O. A. Sant'Anna and L. V. Rizzo contributed equally to this work.

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Clinical Study

Improved Metabolic Control in Diabetes, HSP60, and Proinflammatory Mediators

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The diabetes-atherosclerosis relationship remains to be fully defined. Repeated prolonged hyperglycemia, increased ROS production and endothelial dysfunction are important factors. One theory is that increased blood levels of heat shock protein (HSP)60 are proinflammatory, through activation of innate immunity, and contribute to the progression of vascular disease. It was hypothesized that improvement of diabetes control in patients presenting with metabolic syndrome would lower HSP60, and anti-HSP60 antibody levels and decrease inflammatory markers. Paired sera of 17 Italian patients, before and after intensive treatment, were assayed for cytokines, HSP60 and anti-HSP60 antibodies. As expected, intensive treatment was associated with a decrease in HgbA1C ($P < 0.001$) and BMI ($P < 0.001$). After treatment, there was a significant decrease in IL-6 ($P < 0.05$). HSP60 levels were before treatment $-6.9 + 1.9$, after treatment $-7.1 + 2.0$ ng/mL (P = ns). Overall HSP60 concentrations were lower than published reports. Anti-HSP60 antibody titers were high and did not decrease with treatment. In conclusion, improvement of diabetic control did not alter HSP60 concentrations or antiHSP60 antibody titers, but led to a reduction of IL-6 levels.

1. Introduction

Atherosclerosis is more widespread and severe in diabetics [1]. It is characterized by a greater inflammatory involvement, exposing patients to a high risk of cardiovascular (CV) events [2, 3]. Myocardial infarction is 2 to 4 times more frequent, and increased cardiovascular risk remains even after controlling for other concomitant factors like hypertension and dyslipidemia. Diabetics' cardiovascular mortality exceeds 70%. Epidemiological studies demonstrated that even a small reduction of glycosylated hemoglobin corresponds to a reduction of CV risk, but tight control has been associated with no reduction in CV mortality [4–6].

Despite a great deal of research, the cellular and molecular mechanisms underlying the glucose-atherosclerosis relationship are not fully understood. There are multiple potential pathways to endothelial injury and the vascular complications of diabetes, including chronic inflammation, increased oxidative stress, and activation of the immune response—both innate and adaptive [3, 7, 8]. Once the initial dysfunction in the endothelium occurs, chronic inflammation and an immune response contribute to the progression of vascular disease. Autoimmunity is one mechanism of vascular injury in diabetes. A key-identified antigen is heat shock protein (HSP)60, a protein that has been found on the surface of stressed endothelial cells [9, 10]. Anti-HSP60 antibodies have been found in the serum of many individuals, and they are thought to increase in a number of disease states. HSP60 is also an important endogenous inflammatory mediator. Toll-like receptors (TLR), part of the innate immune response, are present on the endothelial cell membranes and recognize HSP60 present in the circulation. The binding of HSP60 to endothelial cell TLRs will result in the activation of NFκB, leading to increased expression of inflammatory genes and the release of pro-inflammatory cytokines, including TNFα and IL-6.

We hypothesized that in diabetes there would be a decrease in serum HSP60, HSP60 antibodies and in inflammatory cytokines with improved glycemic control. We investigated the effect of good metabolic control on serum HSP60,

HSP60 antibodies, and inflammatory cytokines in type-2 diabetic patients to evaluate the influence of hyperglycemia on autoimmune and inflammatory indicators.

2. Methods

2.1. Patient Data. Diabetic patients from one of our clinics (CB) were enrolled in a study to determine the effect of glycemic control on inflammatory endpoints and the release of HSP60 into the serum. Paired sera were collected from 17 diabetic patients (10 women and 7 men, mean age 62.3 ± 2.1 years), before and after having intensive treatment for glycemic control. Local committee approval was obtained and patients gave informed consent. Subject characteristics are shown in Table 1. Samples were stored at −80°C until use.

HOMA (homeostasis model assessment, an index of insulin-resistance) was calculated based on the following formula: (blood glucose [mmol/L] × insulin level [μU/mL])/22.5.

Glycosylated hemoglobin(HgbA1C) was measured by the hospital clinical laboratory. The routine assay involves an automated analytical system based on a cation-exchange HPLC method. The procedure is the reference DCCT method.

2.2. Serum HSP60. A commercial ELISA (Assay Designs) was used to measure serum HSP60 levels. Samples were diluted 1 : 20 before analysis based on pilot studies.

2.3. Serum Anti-HSP60. Slot-Blots—a standard method in immunology, were used to assess anti-HSP60 antibody titers. This method is distinct from the slot blot method, which is a rectangular version of dot blots. We used an apparatus manufactured by BioRad, which provides 20 vertical slots, designed for testing small serum samples for antibodies. The apparatus is the size of a small SDS PAGE gel. We ran a set of molecular weight markers and a gel-wide band of recombinant human HSP60 (StressGen) on gel, and then transferred to nitrocellulose. The membrane was stained with Ponceau Red (Sigma) to verify the presence of the single *HSP60 protein band across the width of the membrane. A copy of the membrane was made to allow comparison of the final film with the distribution of protein on the membrane*. The prestained molecular weight markers (New England Biolabs) also provided another reference point. We then placed the membrane in the slot-blotting apparatus, and added diluted serum samples based on the range seen in pilot studies. The slot blot is incubated in the apparatus on a rocker panel so that the sample goes back and forth in its long, vertical slot the height of the blot. After incubation, the sample is aspirated out, and the slots washed several times. Then the blot is removed from the apparatus and incubated with anti-human IgG-peroxidase as with a standard western. The end result after development is tiny bands which one can test for aligning with the blot wide band of HSP60. It is not uncommon to see non specific bands at other sizes, which would be read as a positive in a 96 well plate.

This device has 20 vertical slots that permit loading of 20 different serum samples to develop the membrane. Samples were diluted 1 : 100 and 1 : 250 in PBS (phosphate-buffered saline) based on pilot studies. Following incubation for 1 hour on an orbit shaker, serum was aspirated out of the device, the slots washed with TBST, and the membrane removed. The membrane was incubated with anti-human IgG-HRP (Amersham) at 1 : 1000. The blot was then developed with ECL (Pierce) as previously described [11]. The 20 blot lanes were then each interpreted as positive or negative based on the presence or absence of a band matching the location of the recombinant human HSP60 on the membrane. In contrast to dot blotting for antibody titers, this approach confirms the *specificity* of a positive result.

2.4. Cytokine Assay. An inflammatory human cytokine cytometric bead array (BD Biosciences) was used to measure IL-1β, IL-6, IL-8, IL-10, IL-12 and TNFα following the directions of the manufacturer.

2.5. Statistics. Data is reported as mean values ± the standard error of the mean (SEM). A $P < 0.05$ was considered to be significant. Paired data from before treatment to optimized treatment was compared using a paired T test or Wilcoxon Signed Rank test, where indicated. Multi-variate analysis was performed by Pearson Correlation (SigmaStat). A $P < 0.05$ was considered to be significant.

3. Results

3.1. Baseline Diabetes Indices. HgbA1C demonstrated a significant drop with optimized treatment, as expected ($P < 0.001$, Figure 1(a)). Significant reductions of BMI ($P < 0.001$, Figure 1(b)), HOMA ($P < 0.001$) (Figure 1(c)), and waist circumference ($P < 0.001$, Figure 1(d)) occurred, demonstrating significantly improved control of diabetes.

3.2. HSP60. HSP60 was present in the serum of both before and after treatment patients (Figure 1(e)). Absolute HSP60 levels before treatment were −6.9 ± 1.9, after −7.1 ± 2.0 ng/mL (P = ns). Three patients had no HSP60 in their sera either before or after treatment. There was no correlation between diabetes duration and HSP60 levels, nor was there a correlation with the medication(s) used for diabetes treatment.

3.3. Anti-HSP60 Antibodies. Anti-HSP60 antibodies were detected by slot blotting. The graph summarizes the results of 1 : 100 and 1 : 250 dilutions of plasma (Figure 2(a)). With a 1 : 100 dilution, anti-HSP60 antibodies were present in 76.5% before, and 88.2% after (P = ns). This decreased to 58.8 and 58.8% at 1 : 250. A representative slot blot is shown in Figure 2(b).

3.4. Cytokine Levels. As shown in Figure 3, IL-6, IL-8, IL-10, IL-12 and TNFα were detected in the serum both before and after intensive treatment. IL-1β was undetectable. Only IL-6 showed a significant reduction with treatment ($P < 0.05$).

Table 1: Characteristics of patients enrolled in the study.

Patient	Age	Sex	BMI		HgbA1C (n.v. 6,6)		HOMA		Waist circum (cm)		Diabetes treatment: drugs (+diet and physical activity)	Lenght of treatment (years/months)	Comorbidties
			Before	After	Before	After	Before	After	Before	After			
1	51	M	35	34	8.5	7.2	2.9	3.6	136	112	Gliclazid	0/3	HTN, DL
2	66	M	30	30	15.6	6.0	6.0	6.0	104	100	Repaglinid	0/6	HTN, DL
3	52	F	31	31	7.9	6.3	7.3	6.9	98	89	Metformin	0/6	HTN, DL
4	67	F	36	35	7.3	6.7	5.0	5.2	113	105	Metformin	1/2	HTN, DL, CVA
5	64	M	28	24	7.3	5.6	1.3	0.9	104	93	Metformin	0/6	HTN, DL
6	65	F	27.5	25.5	9.5	6.7	5.1	1.9	113	104	Metformin	1/0	HTN, DL
7	67	M	31	27	7.9	5.8	4.8	2.3	107	96	Metformin	0/5	HTN, DL
8	60	M	31	30	9.4	7.2	5.6	3.9	103	96	Metformin	0/2	HTN, DL
9	74	F	37	33	7.7	6.6	3.2	1.8	124	107	Metformin + Repaglinid	1/2	HTN, DL, Afib
10	67	F	38	34	8.4	6.4	8.7	2.5	136	128	Metformin + Glimepirid	0/6	HTN, DL
11	50	M	29	27	12.0	6.9	3.8	1.6	96	106	Metformin + Glimepirid	0/6	HTN, DL
12	71	F	40	38	8.4	6.5	5.6	3.9	133	127	Metformin	0/8	HTN, DL
13	71	F	43	39	8.2	6.7	5.7	2.6	109	109	Metformin	0/5	HTN, DL
14	73	F	32	29	8	6.9	4.5	3.8	102	102	Metformin	0/8	HTN, DL
15	59	F	41	40	9.1	6.7	10.9	4.0	121	118	Metformin	1/5	HTN, DL
16	55	F	41	39	8.4	6.9	6.0	2.3	123	102	Metformin	0/2	HTN, DL
17	47	M	31	29	8.8	6.6	9.5	3.5	104	96	Metformin	1/9	HTN, DL

Pat: patient; n.v: normal value; circum: circumference; treatment: Treatment; comorbidities: cardiovascular comorbidities; HTN: hypertension; DL: dyslipidemia; CVA: cerebral vascular accident; Afib: atrial fibrillation.

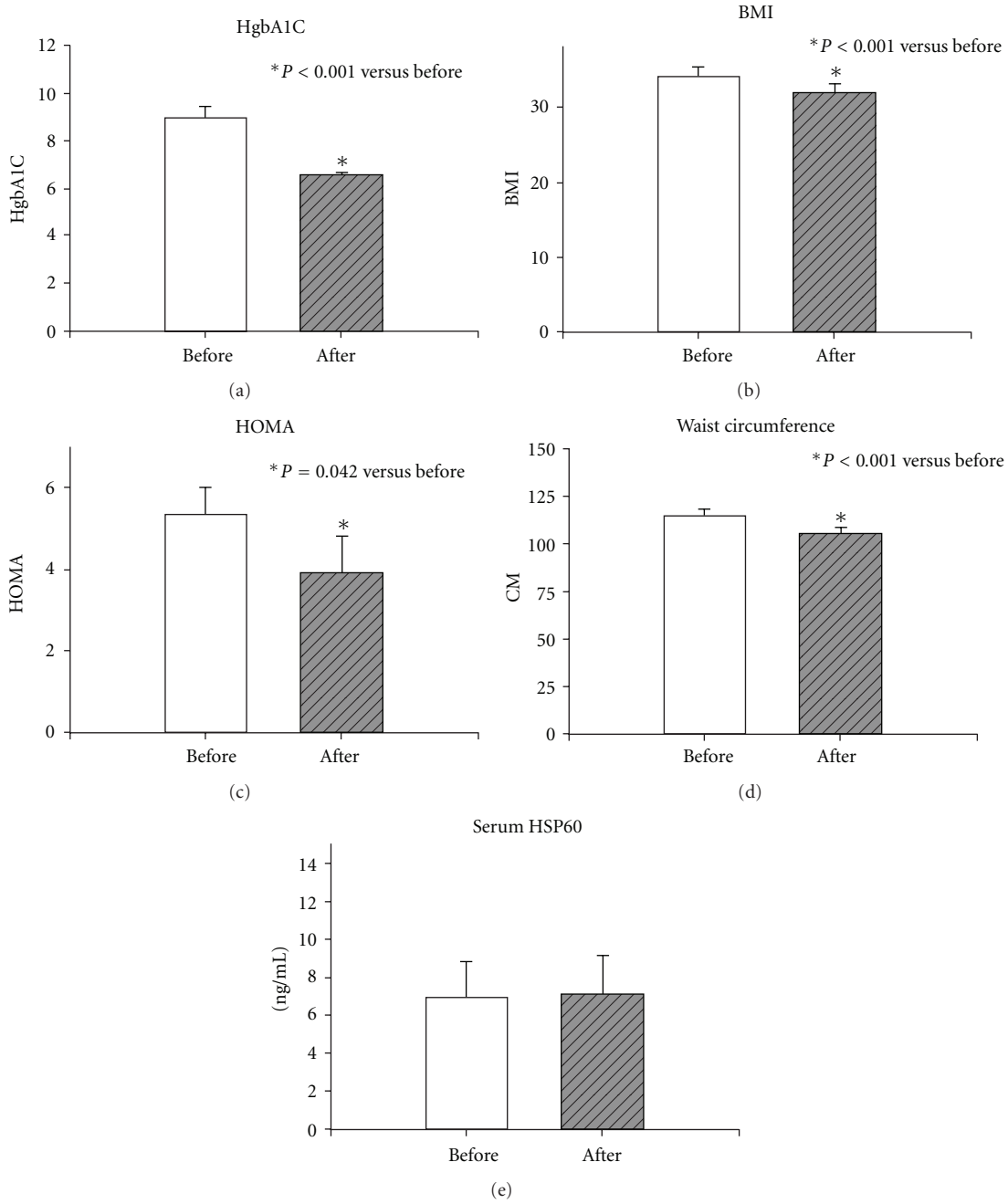

Figure 1: (a) HgbA1C before and after intensive treatment. (b) BMI before and after intensive treatment. (c) HOMA before and after intensive treatment. (d) Waist circumference before and after intensive treatment. (e) Serum HSP60 levels by ELISA before and after intensive treatment. $^*P < 0.001$ except c, where $P < 0.05$.

Overall, IL-8 levels dropped, but there was considerable variation in levels and therefore the change did not reach significance.

3.5. Correlation Analysis. A Pearson product moment correlation analysis was performed to identify correlation amongst the clinical findings and inflammatory markers analyzed. As shown in Table 2, before treatment, there was a negative correlation between HSP60 and IL-12. There were borderline correlations between HSP60 and IL-10 and IL-6. As would be expected, there was a positive correlation between BMI and waist circumference. After intensive treatment, again a correlation between BMI and waist circumference was found, and in addition a negative correlation between HOMA and IL-10 was also found. TNFα levels showed a correlation with IL-12 levels ($P = 0.023$). There was a borderline correlation of HSP60 and TNFα levels, but this did not reach significance ($P = 0.055$). In the present study, leptin and adiponectin were not tested because, unlike cytokines, they are not considered markers of inflammation and are predominantly of adipocytic origin.

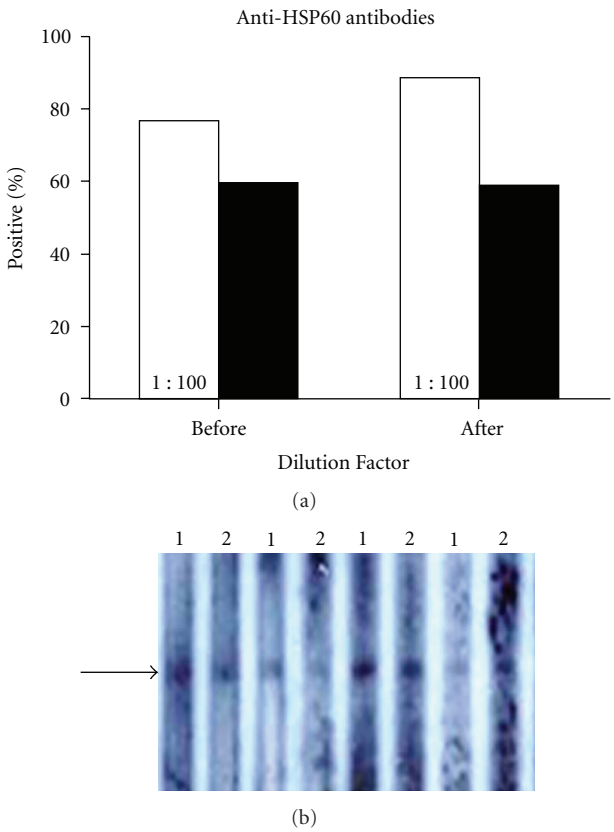

Figure 2: (a) Graph summarizes anti-HSP60 antibody titers before and after intensive treatment. Antibody levels were tested at two different dilutions: 1 : 100 (white bars) and 1 : 250 (black bars). (b) Representative slot blot. Arrow marks location of HSP60 on membrane. Lanes were read as positive or negative at a given dilution based on the presence of a band at the level of the arrow, which marks the location of the recombinant human HSP60 on the membrane. 1—1 : 100; 2—1 : 250.

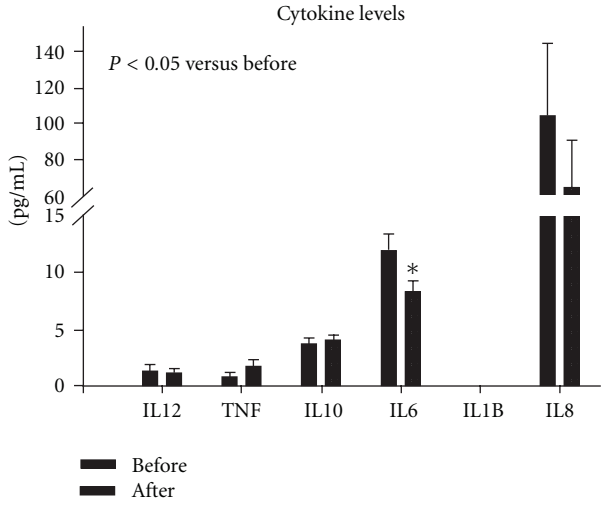

Figure 3: Multiple inflammatory cytokines were measured by flow cytometry using a cytometric bead array. Graph summarizes results. Black bar is before intensive treatment and grey bar after intensive treatment. No IL-1β was detected in any samples. $^*P < 0.05$ versus before treatment.

4. Discussion

The underlying mechanisms for the far greater severity of vascular disease in diabetics remain to be fully defined [3, 8]. Endothelial dysfunction, characterized by increased adhesion molecules, increased proinflammatory markers, increased pro-thrombotic factors, increased ROS and loss of normal regulation of vascular tone, occurs in diabetes, likely a result of the convergence of multiple proinflammatory mechanisms [2]. Endothelial dysfunction is accompanied by the development of an autoimmune reaction that appears to play an important role in the inflammatory evolution of atherosclerosis [9, 12]. There are a number of molecules, which are thought to contribute to this process, including oxidized(ox) LDL, advanced glycation end-products, and HSP 60, with oxLDL and HSP60 acting as autoantigens [9]. The oxLDL likely are a result of ROS action on LDL, which have penetrated the subendothelial space. Activation of the immune system through chronic infection or exposure of intracellular proteins, such as HSP60, which are then recognized as danger associated molecular patterns (DAMPs), can lead to inflammation and cell apoptosis and contribute to the development and progression of vascular disease [3, 13]. HSP60 is primarily a mitochondrial protein, where it is critical for proper folding of key metabolic proteins, but HSP60 is also found in the cytosol, where in the heart it has an antiapoptotic role [14]. Diabetes has been reported to be associated with very high levels of HSP60 in blood [15]. HSP60 associates with the cell membrane under stress conditions, and in heart failure localized to the surface of cardiac myocytes, which correlated with myocyte apoptosis [10, 16]. HSP60 has been identified as a potential ligand for TLR4. We have recently shown that TNFα and HSP60 each drives the expression of the other, which could explain the correlation between HSP60 and TNFα levels [17]. HSP60 has been shown to activate endothelium, smooth muscle cells, and macrophages [18]. TLR4 activation and expression increase in diabetes in response to hyperglycemia, and this was not prevented by insulin treatment [19, 20]. TLR4 mediates vascular inflammation and insulin resistance in diet induced obesity. We have previously reported that extracellular HSP60 causes cell apoptosis via TLR4 activation and production of TNFα [21]. Chlamydial and other bacterial HSP60 (HSP65), which have high homology to human HSP60, have also been implicated in vascular disease, but because these infectious agents are so commonplace, it has been difficult to irrefutably prove [18, 22, 23]. Cellular (T cells) and humoral (anti-HSP60 antibodies) immune responses potentially also play a fundamental role in triggering the inflammatory process that fuels atherosclerosis [13]. Thus, serum HSP60, which is usually an intracellular protein, can contribute to the inflammatory state seen in diabetes through multiple mechanisms.

Clinically, elevated HSP60 plasma levels have been found to correlate with increased carotid stiffness in middle-aged individuals [33]. Most significantly, antibodies to HSP60, either mammalian or bacterial (HSP65) were found to correspond to increased intima-media thickness (IMT) in young males (17-18 years.) [34]. These studies support

Table 2: Significant Pearson correlations for clinical factors and inflammatory markers change with treatment.

Before intensive treatment				
HSP60	IL-10	IL-12	TNFα	BMI
−IL-12 0.040	−HSP60 0.051	−HSP60 0.040	+ IL-12 0.014	+ Waist 0.005
−IL-10 0.051	+IL-12 0.030	+IL-10 0.030		
+IL-6 0.055		+TNFα 0.014		
After Intensive treatment				
TNFα	HOMA	BMI		
+HSP60 0.055	−IL-10 0.009	+Waist 0.010		
+IL-12 0.023				

+ is positive correlation, − negative correlation. Number on right is *P* value.

Table 3: Summary of serum cytokine values reported from recent studies.

Reference	IL-1β pg/mL	IL-6 pg/mL	IL-8 pg/mL	IL-10 pg/mL	IL-12 pg/mL	TNFα pg/mL	Diabetes duration (Years)	Nationality
Mol et al. 1997 [24]	0.816					1.19	10 ± 7*	Dutch
Adachi et al. 2004 [25]						2.4	Unknown	Japanese
Doganay et al. 2002 [26]	<0.05	<0.05	8.3			6.6	6.0 ± 0.6	Turkish
Lee et al. 2008 [27]	0.94	1.47	5.11	0.77		3.95	9.0 ± 5.4	Korean
Mishra et al. 2011[1] [28]		6.12			118	38.6	newly diagnosed	Indian
Mishra et al. 2011[2] [28]		9.55			147	50.8	newly diagnosed	Indian
McGee et al. 2011 [29]		7.03				16.9	1 yr. Minimum	British
Park et al. 2011 [30]		1.78					5.1	Korean
Hu et al. 2009[3] [31]		21.8				0.065	Unknown	Chinese
Hu et al. 2009[4] [31]		38.5				0.111	Unknown	Chinese
Ozturk et al. 2009 [32]		4.01	104.8	8.13			9.84 ± 7.13	Turkish
Current Study	0	12.05	104.6	3.78	1.41	0.83	6.0 ± 5.3	Italian

Values ± SEM except where indicated. Mishra et al. and Hu et al. both divided their diabetic populations into 2 groups: one by CRP and the other by presence or absence of evidence of atherosclerosis. Last row is baseline values for current study. *Standard deviation; [1]CRP< 3, [2]CRP > 3; [3]no evidence of atherosclerosis; [4]evidence of atherosclerosis (increased carotid IMT).

a role for HSP60 and anti-HSP60 antibodies in atherosclerotic disease in the nondiabetic. Correlation may be more difficult to identify in older individuals as a multitude of factors, which increase with age, and contribute to the progression of established atherosclerotic disease.

4.1. HSP60 Antibodies. A very high titer of HSP60 antibodies was present in our patients, and it did not change with optimization of metabolic control. A number of studies have implicated HSP60 antibodies in endothelial apoptosis and dysfunction [35, 36]. A recent study demonstrated using a randomized double-blind, placebo-controlled cross-over design that simvastatin could lower anti-HSP60 antibody titers [37]. Only 15% of these patients were diabetic, and the anti-inflammatory properties of simvastatin may have been a critical attribute for reduction in anti-HSP60 antibodies [38]. High titers of anti-HSP60 antibody were still present after months of treatment, and these antibodies can interact with HSP60 on the surface of endothelial cells, leading to monocyte recruitment and further inflammation.

4.2. Cytokines. Six different cytokines were measured, and of these only IL-6 was significantly reduced after intensive treatment. This could be secondary to the effect of reduced glucose levels on the inflammatory state of endothelium. However, IL-6 also reflects the low-grade chronic inflammatory state that characterizes diabetes per se, the increased level of insulin resistance, and the increase in visceral adipose

tissue, all of them present in the metabolic syndrome [39]. The observed decrease in IL-6 could be due to the reduction of blood glucose as well as of BMI, waist circumference and insulin resistance reached by our patients. This study is one of the few that show this effect in diabetic patients in a context of metabolic syndrome. IL-8 levels were increased, but the decrease in IL-8 with treatment did not reach significance. Other cytokines including TNFα, IL-10, and IL-12 did not change with diabetes control. Growing evidence supports a cause-effect relationship between systemic inflammation related to diabetes or obesity, and endothelial inflammation. This is important, as systemic inflammation may make plaques unstable and rupture prone [1–3]. Therefore, any reduction of the systemic inflammation could have a positive effect on atherosclerosis-related disease.

Table 3 compares serum cytokine levels measured at baseline in the current study with recent studies in the literature. These studies covered different populations from different countries. All patients were type-2 diabetics, but the duration of diabetes varied from newly diagnosed to 10 years. Little to no IL-1β was detected in any of the studies [24, 26, 27]. IL-6 levels ranged from undetectable in one study to over 38 pg/mL [26–32]. The IL-6 levels for the patients in the current study were in the middle of this range. IL-8 levels in the literature were split with two groups finding levels less than 10 pg/mL, and two other studies, including the current one, finding levels of just over 100 pg/mL [26, 27, 32]. Similarly, IL-10, which was only measured in three studies, was very low in one, 3.78 (current study) and 8.13 pg/mL in the other studies [27, 32]. IL-12 levels were only measured by Mishra et al. with these investigators finding 100 times the levels found in the patients in the current study [28]. TNFα levels also had a wide range, but most of the studies, including the current one, detected levels between 0.065 to 6.6 pg/mL, while the remaining 3 had average serum values as high as 50.8 pg/mL [24–29, 31]. Thus, blood cytokine levels vary widely in diabetic populations.

5. Conclusions

Improved diabetes control was not associated with a decrease in either HSP60 or anti-HSP60, even though IL-6 decreased, suggesting less inflammation. Although diabetes control was significantly improved, as evidenced by a decrease in HOMA, this does not mean that hyperglycemia, which can cause endothelial dysfunction, did not occur intermittently, and that other factors driving inflammation in diabetics were eliminated. Diabetes is a chronic inflammatory disease, and even with good control of blood sugar, there is increased vascular disease. HSP60 and anti-HSP60 antibodies are not reduced by months of improved glucose control and may contribute to the increased incidence of vascular disease in diabetic patients with good glucose control. Finally, IL-6, a metabolic syndrome and atherosclerosis-related inflammatory marker could be of value in clinical practice as an indicator (together with HgbA1c) of the efficacy of the treatment as well as of the efficacy of preventive action on atherosclerosis.

Conflict of Interests

The authors disclaim any conflict of interest.

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