# HSPS - AMBIGUOUS MEDIATORS OF IMMUNITY

EDITED BY: Stuart Keith Calderwood, Ayesha Murshid and Thiago J. Borges

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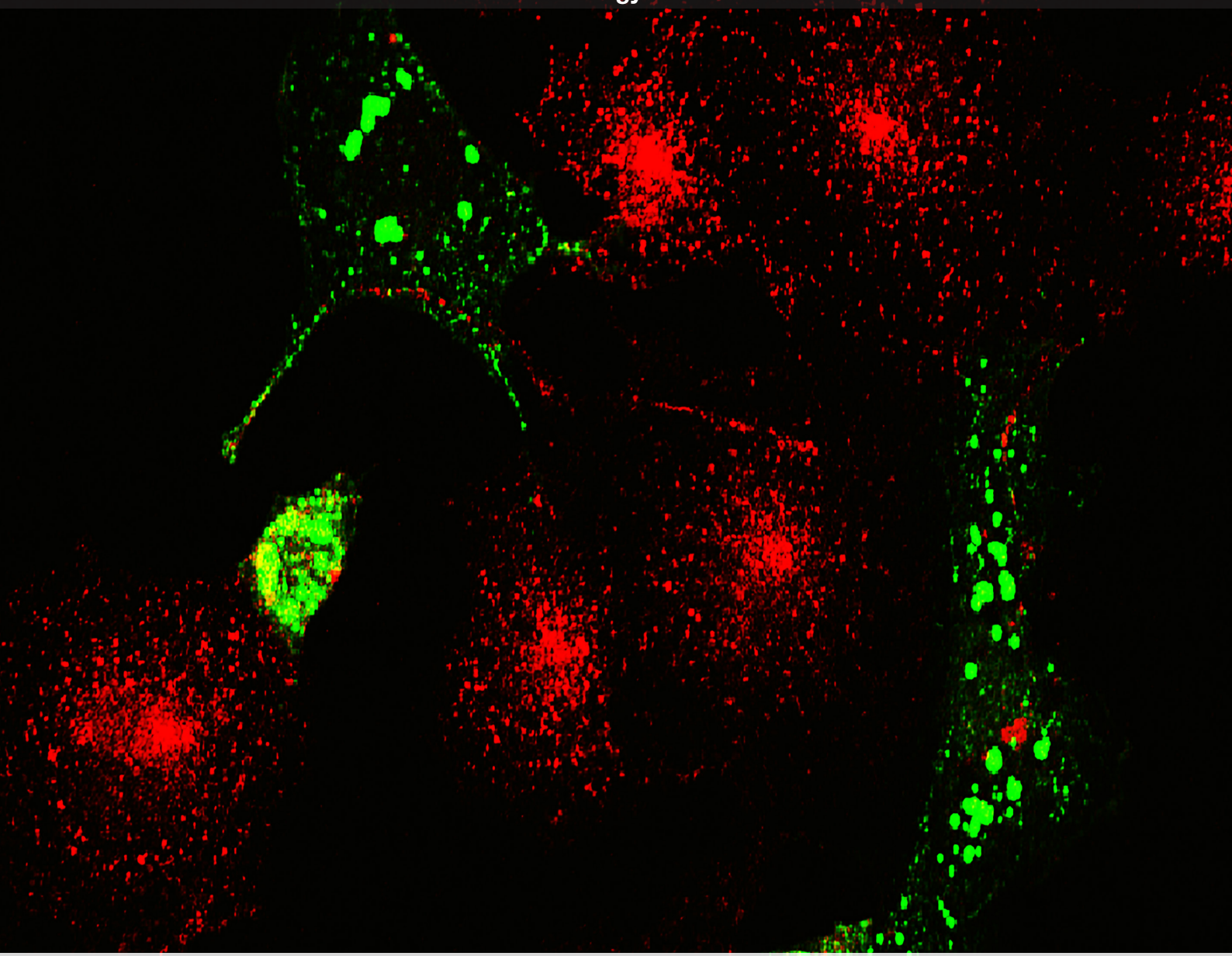

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# HSPS - AMBIGUOUS MEDIATORS OF IMMUNITY

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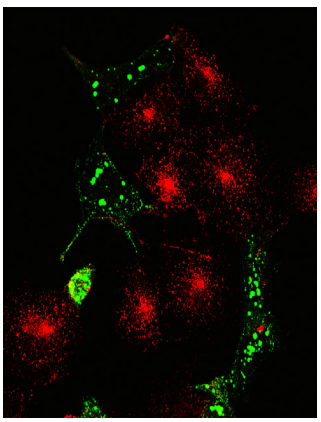

Dynamin dependent uptake of diI-oxLDL in LOX1 expressing CHO cells.

CHO stably expressing LOX1 cells were transfected with dynamin K44A-GFP for 18 hours. Cells were then incubated with 10ug/ml diI-oxLDL for 15 minutes. Cells were then fixed and mounted for Immunofluorescence study.

Figure by Ayesha Murshid

Heat shock proteins (HSPs) were discovered as polypeptides induced by stress that can be found in all kingdoms of cellular organisms. Their functions were, a first enigmatic and these proteins were thus classified by molecular weight, as in—Hsp27, Hsp70, Hsp90, Hsp110. More recently, each of these size-classified molecules has attributed a role in protein folding, and they thus came to be known, as a class, as molecular chaperones. However, the they possess properties beyond chaperoning. Indeed, their discovery in the extracellular spaces suggested roles in regulation of the immune responses.

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EDITORIAL
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# Editorial: HSPs—Ambiguous Mediators of Immunity

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**Keywords: heat shock protein, immunity, vaccine, mycobacterial Hsp65, transplant, arthritis**

**Editorial on the Research Topic**

**HSPs—Ambiguous Mediators of Immunity**

Heat shock proteins (HSPs) were discovered as polypeptides induced by stress that can be found in all kingdoms of cellular organisms. Their functions were, a first enigmatic and these proteins were thus classified by molecular weight, as in—Hsp27, Hsp70, Hsp90, Hsp110 (1). More recently, each of these size-classified molecules has attributed a role in protein folding, and they thus came to be known, as a class, as molecular chaperones—deterrents of unsuitable interactions between intracellular proteins (2). However, the HSPs possess properties beyond chaperoning. Indeed, their discovery in the extracellular spaces suggested roles in intercellular signaling and in the convoluted regulation of the immune responses.

A number of lines of investigation triggered interest in HSPs as mediators of immunity. Srivastava and others found that the molecular chaperone properties of HSPs could be harnessed in cancer vaccine design (3–5). They emphasized the role of HSPs in capturing tumor antigens and permitting their uptake and processing by antigen-presenting cells (APCs) prior to activation of cytotoxic lymphocytes. Others suggested that HSPs could behave like endogenous danger signals when flooding into the extracellular microenvironment after cell death (6). In another line of investigation, investigators studied the role of mycobacterial Hsp65 and Hsp70 in suppression of autoimmune diseases such as arthritis, diabetes, and prolongation of tissue grafts [Borges et al.; (7, 8)]. HSPs were thus implicated in contrasting and apparently opposed aspects of immunity.

The current volume contains articles dealing with these aspects of HSP biology. Four articles describe various roles of HSPs in tumor immunity and anticancer vaccine construction. In chapter 1, Zuo et al. describe tumor immunity strategies built around the "large HSPs"—Hsp110 and GRP170. These larger HSPs possess chaperoning power of remarkable strength leading to high avidity for antigens and effective vaccines. Shevtsov and Multhoff in chapter 4 review in detail Hsp70 and Hsp90 vaccines and their effectiveness in tumor therapy. The biological properties of Hsp90 are further discussed in chapter 5 by Tamura et al., emphasizing the role of this molecule in permitting antigens to cross plasma membranes, enter cells by endocytosis and cross the endosomal wall to the sites of antigen processing and acquisition by MHC Class I molecules. Most studies have indicated a role for surface receptors in mediating effects of extracellular HSPs. Murshid et al. in chapter 7 stress the role of scavenger receptors in the immune functions of such HSPs, concentrating on SRECI/*SCARF1* as an avid binder of most of the HSPs.

Four additional chapters concentrate on the immunoregulatory properties of HSPs, particularly emphasizing study of mycobacterial chaperones. In chapter 2, Manon et al. concentrate on mycobacterial Hsp70 and describe the generation of the first TCR transgenic mouse recognizing an anti-inflammatory Treg (regulatory T cell)-inducing Hsp70 peptides. The aim was to provide a model system for discovery of the mechanisms underlying generation of Hsp70-reactive $CD4^+CD25^+$ Treg and mediation of immunomodulation. In chapter 6, Moudgil et al. describe

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their interesting studies of the role of mycobacterial Hsp65 in controlling adjuvant arthritis in rodent models and potential development of novel treatments based on these findings. Along similar lines Borges et al. describe studies showing a potent role for mycobacterial HSPs in regulating alloimmunity and improving survival of tissue grafts. Finally, the O'Brien group (chapter 8) describes roles for the small HSP—Hsp27 in attenuating atherogenesis and other events in the extracellular spaces and in the circulation.

Finally, Calderwood et al. in chapter 3 attempt to synthesize some of theses apparently contrasting immunostimulatory and immunoregulatory effects of HSPs and develop an integrated understanding of potential *sequela* of HSPs encountering macrophages or dendritic cells.

Thus, from the analysis contained in this collection of articles, we appear to have come a long way in past 30 years in understanding "the other face of HSP biology"—the various families of HSPs escaping to the extracellular milieu and influencing immunity. However, much remains to be learned in terms of the recognition of HSPs by receptors on APC, in cell signaling and in understanding how these events are influenced by tissue context. In addition, the complex pathways undertaken by HSP-chaperoned peptides in the intracellular milieu and their influence on antigen presentation remain to be fully characterized. In terms of translation of HSP research to disease treatment, promising approaches to cancer immunotherapy, treatment of inflammatory disease such as arthritis and survival of tissue transplants appear to beckon. This area of HSP biology thus appears to have rich promise for the future.

## AUTHOR CONTRIBUTIONS

SC, AM, and TB each contributed equally to writing this editorial.

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frontiers in Immunology

REVIEW
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# Extracellular HSPs: The Complicated Roles of Extracellular HSPs in Immunity

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Extracellular heat-shock proteins (HSPs) interact with the immune system in a very complex manner. Many such HSPs exert powerful effects on the immune response, playing both stimulatory and regulatory roles. However, the influence of the HSPs on immunity appears to be positive or negative in nature – rarely neutral. Thus, the HSPs can act as dominant antigens and can comprise key components of antitumor vaccines. They can also function as powerful immunoregulatory agents and, as such, are employed to treat inflammatory diseases or to extend the lifespan of tissue transplants. Small modifications in the cellular milieu have been shown to flip the allegiances of HSPs from immunoregulatory agents toward a potent inflammatory alignment. These mutable properties of HSPs may be related to the ability of these proteins to interact with multiple receptors often with mutually confounding properties in immune cells. Therefore, understanding the complex immune properties of HSPs may help us to harness their potential in treatment of a range of conditions.

**Keywords: heat, shock, protein, immunity, immunosuppression, surface, receptors, scavenger**

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## INTRODUCTION

Molecular chaperones are proteins that permit the maturation and correct folding of most of the proteome (1, 2). As such, they are found in all cellular organisms and seem essential for cellular life. Protein folding seems to require chaperones from a number of different gene families that appear to function at various stages in a concerted folding cascade. These proteins belong to the small heat-shock protein (HSP) family including Hsp27 and the larger 70 kDa HSP family including Hsp70 as well as Hsp60, Hsp90, and Hsp110 families (3) (**Table 1**). We will discuss here, the mammalian immune responses to both prokaryotic (eubacterial) and eukaryotic HSPs under a range of contexts. The acronym HSP is derived from the early findings that some of these proteins are massively induced during proteotoxic stresses such as heat shock (4). Thus, the canonical functions of the HSP chaperones are in the folding of proteins during mRNA translation and in responding to protein unfolding crises in stressed cells (5).

However, HSPs also appear to possess functions outside the realm of protein folding, some of them acquired when they are released from cells to become extracellular HSPs (5, 6, 30, 31). HSPs have been observed in serum from human patients, pointing to their existence outside of cells, in living organisms (9). Among the first functions mooted for extracellular HSPs were in inflammation and immunity (23, 32). HSPs of each of the classes appeared to function in influencing the inflammatory

**TABLE 1 | Immune/inflammatory roles for extracellular chaperones.**

| Chaperone | Pro/anti-inflammatory | Adaptive immunity? | Reference |
|---|---|---|---|
| Hsp27 | Context | – | (5) |
| Hsp60 | Context | – | (1, 6–8) |
| Hsp70 | Context | + | (9–14) |
| Hsp90 | Context | ++ | (14–18) |
| Hsp110 | Pro | +++ | (19–22) |
| Grp94 | Pro | + | (23, 24) |
| Grp170 | Pro | +++ | (25–29) |
| Calreticulin | Pro | + | (23, 24) |

and immunological balance in tissues (**Table 1**). The hypothesis of a pro-immune function for extracellular HSPs was derived primarily from studies utilizing molecular chaperone vaccines in cancer treatment (10, 23, 33). It was shown that HSPs from a number of chaperone families could be extracted from cancer cells while they were associated with a range of tumor peptide antigens (11, 33, 34). These HSP–peptide complexes could then be injected into hosts as anticancer vaccines, delivering a range of tumor-derived antigens to the immune system and promoting antitumor immunity (19–22, 25–29). HSPs were, by the proponents of this approach, conventionally, viewed as playing a dominant role as promoters of immunity (32). In addition, a number of studies showed them to be pro-inflammatory mediators, and extravagant claims were made for molecular chaperones as activators of multiple facets of immunity. However, other investigators have demonstrated powerful anti-inflammatory roles for HSPs that we will discuss more fully, later in this manuscript (12, 35, 36). In addition, the properties of extracellular HSPs have now expanded to include powerful roles in processes outside the immune response. For instance, secreted Hsp90 has been shown to mediate wound healing and tumor metastasis (36, 37). Thus, extracellular HSPs appear to have come of age as major intercellular signaling molecules in biology and medicine.

Some of the issues discussed here, particularly the role of HSPs in antigen presentation, have been mentioned in a previous review (38). Here, however, we focus mainly on the potentially confound pro- and anti-inflammatory roles of HSPs and discuss how these properties can be manipulated toward clinically useful outcomes in both treatment of autoimmune conditions and in the deployment of chaperone anticancer vaccines.

## RELEASE OF HSPs INTO THE EXTRACELLULAR MICROENVIRONMENT

Structural considerations would tend to make one skeptical regarding the possibility of HSP secretion into the extracellular milieu. HSP family proteins lack an N-terminal hydrophobic signal sequence, characteristic of most secreted proteins, and thus, cannot be released from cells by the conventional secretion pathways. However, a number of non-canonical secretion pathways exist, many of which are employed by cytokines to gain access to the extracellular milieu. These eccentric mechanisms include release of the polypeptides *via* secretory lysosomes, a pathway utilized in the release of IL-1β from inflammatory cells (39). Hsp70 has been shown to be secreted from a number of cells in free form by a similar pathway, through a mechanism requiring the lysosomal pH gradient (31, 40). Indeed, Hsp70 is cosecreted from cells along with the lysosome resident protein LAMP1 (31). Hsp70 is also released from a range of other cells including tumor cells, reticulocytes, peripheral blood mononuclear cells, B cells, and dendritic cells in various types of lipid vesicles [reviewed by De Maio (41) and Vega et al. (42)]. These vesicles may include a variety of lipid-bounded structures, including ectosomes that are vesicles derived from the plasma membrane and that may contain cytosolic proteins as well as exosomes. Formation of exosomes is a complex process including the internalization of portions of the plasma membrane and subsequent release of exosomes containing a variety of previously intracellular proteins, including HSPs (43). The exosomal pathway is also utilized by some cells for IL-1β secretion (44). HSP-containing exosomes have a wide array of properties including both immunostimulatory and immunosuppressive functions, depending on the protein content of the exosome, cell of origin, and target cell (45–47).

Heat-shock proteins, therefore, can be secreted from a variety of cells in free form and in membrane-bounded particles. In addition, they can be released from cell undergoing necrotic death when membranes are disrupted, and the HSP can leak passively out of the cells (48). Hsp70 released in such a way has been shown to be strongly immunostimulatory.

## HSPs AS CARRIERS OF TUMOR ANTIGENS AND MEDIATORS OF IMMUNITY

### Adaptive Immunity

Molecular chaperones are unique immune modulators in that they can associate with a wide range of antigenic peptides and facilitate their delivery to antigen-presenting cells (APCs) (11, 13, 23, 33, 34). This property has proven to be desirable in the preparation of anticancer vaccines. Only a relatively small number of tumor antigens have been characterized, and we presume that this group represents a small minority of the real repertoire of unique cancer-derived antigens. Thus, chaperones, such as Hsp70, can be considered to "sample" the antigenic milieu of the malignant cell on encountering processed peptides *in vivo* and can be used to carry this sample into the APC during immunization (**Figure 1**). Such HSP-containing vaccines have proven to be highly effective in studies in experimental tumor systems in mice, in which they can lead to tumor regression associated with the generation of specific immunity (10, 13, 14, 20, 49–51). Issues in the preparation of the vaccines, which may influence the clinical effectiveness of vaccines, include the degree to which antigens can be retained by the chaperone and the affinity for the peptide during immunization and entry into APC (11, 14, 52).

Cross-presentation is a process by which extracellular antigens can gain access to the MHC class I pathway, a mechanism normally reserved for processing and presenting endogenous antigens (38). Efficient antigen cross-presentation is very important for vaccine effectiveness as MHC-I–peptide complexes permit recognition of cells bearing the complexes and killing by $CD8^+$ cytotoxic

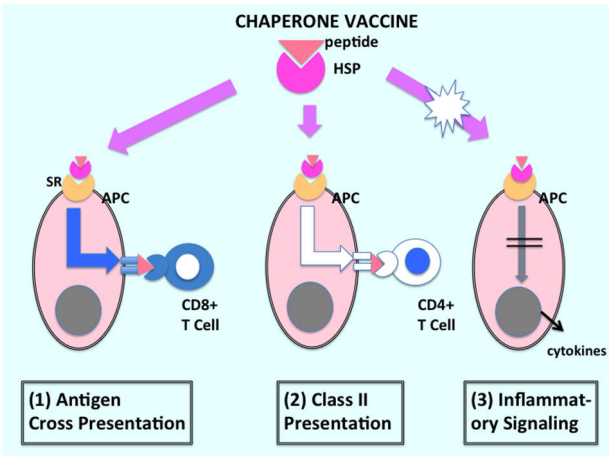


**FIGURE 1 | Immune activation by HSP-based anticancer vaccines.** The HSP–peptide complexes that comprise the anticancer vaccine are shown to interact with APC after vaccination of host. The vaccines can efficiently (1) cause cross-presentation of tumor antigen and, thus, prime $CD8^+$ T lymphocytes as well as activating $CD4^+$ T cells through the (2) class II pathway. However, many investigations suggest that HSPs may not have major effects on (3) inflammatory signaling and may require combination with agents with adjuvant activity or inflammatory cell killing. Gray spheres indicate nuclei.

T lymphocytes (53). Interestingly, Hsp90 appears to protect the integrity of internalized antigens associated with it, to trigger cross-presentation, and to carry antigens deep into the cell, penetrating the plasma membrane and endosomal membranes, and delivers chaperoned peptides to cytoplasmic proteasomes for processing (15, 16).

Although $CD8^+$ T cells can be triggered by DC to recognize antigens after cross-presentation, in the absence of further signals, such T cells are unable to kill their targets. Other inputs are required for full activation (54, 55). The principal pathway used by APC for sampling external antigens is the MHC class II pathway. MHC class II molecules are found only on the surfaces of immune cells. The class II pathway involves the uptake of antigens by receptors on DC, processing of such antigens in the lysosomal compartment, transport of vesicles containing antigen–MHC-II complexes to the cell surface, and presentation to $CD4^+$ T lymphocytes. Hsp90 is able to carry associated antigens into APCs and direct them into the class II pathway as well as facilitate cross-presentation. The choice of direction regarding entry of antigens into the class I/cross-presentation or class II pathways appears to usually depend upon the antigen-binding receptor that mediates triage between the two presentation systems (56, 57). However, Hsp90 appears neutral in this regard and increase penetration of associated peptides into either pathway (17, 18). One important activity governed by the MHC class II pathway is a process called dendritic cell licensing (54). In this mechanism, $CD4^+$ T cells that recognize the antigen on a particular DC produce a reaction in the APC that permits it to activate $CD8^+$ cells that interact with the same APC. Interaction of the T cell receptor on the $CD4^+$ T cell triggers the expression of CD40 ligand (CD40L) that can bind the CD40 counter receptor on the DC and induce expression of inflammatory cytokines, such as TNFα and IL-12 as well as stimulatory coreceptors, like CD80 and CD86 (58, 59). These coreceptors cooperate with MHC class I in fully activating the $CD8^+$ T cell through the T cell receptor. Thus, HSP–peptide complexes become internalized and trigger both the MHC class I and II pathways and may permit DC licensing to occur (17, 18). Our findings that HSPs can facilitate uptake of individual Ova antigens through the MHC-I and MHC-II pathways suggest the possibility of HSP–antigen complex could mediate DC licensing, although this has not yet been formally proven. Homing of $CD8^+$ T cells toward licensed DC may involve surface chemokine receptor CCR5, a process strongly stimulated by chemokines, CCL3 and CCL4 (54). It has been shown in Lewis lung carcinoma cells, *in vivo*, that antitumor immunity was activated along with release of chemokines CCL2, CCL5, and CCL10, by a mechanism dependent on Hsp70 and TLR4 (60).

## Inflammation and Innate Immunity

On exposure to prokaryotic cells or cell products, a separate branch of immunity known as innate immunity is stimulated. In this process, molecules characteristic of individual pathogens including contrasting types of viruses and bacteria, known as pathogen-associated molecular patterns (PAMPs) herald the infection and prime the immune response (61). Then, PAMPs interact with specific receptors on macrophages or DC, known as pattern recognition receptors (PRR), and trigger innate immunity. Best known among the PRR are the toll-like receptors (TLR) that can couple binding of individual PAMPs to intracellular signaling pathways and gene expression programs (62, 63). Most notable among the mechanisms triggered by PRR occupation are the NKK and MAP-kinase pathways that influence inflammatory transcription through activation of factors, such as NFκB and IRF3 (64). This process can lead to synthesis of costimulatory molecules, such as CD80, and activating cytokines such as TNFα and IL-12 that synergize with MHC class I signaling in generation of active and long lived CTL (54). It is not clear to what extent HSPs derived from prokaryotes might function as PAMPs, although their extreme conservation across all cellular species would seem to argue against this. HSPs derived from mycobacteria are, however, recognized by the mammalian immune response and invoke powerful immunity to the extent that they have been described as superantigens (65). The mechanisms by which prokaryotic Hsp60 activates immunity are not clear but could involve PRR, such as TLRs, or other mechanisms.

It has also been shown that some molecules released from damaged and dying cells, such as uric acid and high mobility group box 1 protein (HMGM1), may trigger a form of sterile innate immunity, and such molecules are referred to as damage-associated molecular patterns (DAMPs), in order to suggest a functional similar to PAMPs (66, 67). Thus, DAMPs are thought to trigger innate immunity by binding to PRR and triggering inflammatory signaling cascades. Hsp70 was widely reported to function as a DAMP and to trigger innate immunity through the TLR2 and TLR4 pathways (32). Although this field has run into some controversy, the majority of findings in studies carried out *in vivo* over the past 15 years suggested that Hsp70, through interaction with TLR4, could potentially act as

a DAMP (68). This area has been recently reviewed in depth (69). In addition to Hsp70, extracellular Hsp27 has recently been shown to cause both inflammatory and anti-inflammatory effects (5).

## SOME ANTI-INFLAMMATORY AND IMMUNOREGULATORY EFFECTS OF HSPs

Although prokaryotic HSPs can trigger a powerful immunodominant response in animals, most reports indicate that their effects are generally not pro-inflammatory, and the antibodies and T cells activated in the response had anti-inflammatory properties (69–71). Curiously, the epitopes that T cells respond to in mycobacterial Hsp60 were conserved with mammalian HSPs, and such cells recognized and responded to epitopes in the mammalian proteins. Prokaryotic Hsp60, therefore, did not seem to act as a PAMP (7). In addition, although HSPs were shown to interact with TLRs, such PRR often provoked anti-inflammatory signaling (8). For instance, Hsp60-derived peptides interacted with TLR2 on regulatory T cells (Tregs) leading to an immunosuppressive response. In addition, purified mycobacterial Hsp70 inhibited the maturation of DC (72, 73). Intracellular HSP levels were shown to increase in inflamed tissues and HSP-derived peptides expressed on the cell surface and appeared to activate Treg responses, thus mediating immunoregulatory functions (74). No studies, to date, have shown direct interactions between HSPs and TLRs, and in fact, attempts to show such binding have been negative (73, 75). The extracellular influences on TLR activity that have been reported may, therefore, be indirect and likely dependent on primary interactions of the HSP with other receptors on immune cells, such as the scavenger receptors (SR), followed by recruitment of TLR (64). The powerful immune effects of non-mammalian Hsp60 may also involve mechanisms independent of TLRs, and it has been suggested that the immune response may be genetically programmed to respond to such chaperones (76).

Interestingly, some of the studies applying HSP vaccines to cancer therapy indicated that, although there was significant activation of antitumor CTL by these agents, these were followed by a delayed Treg response. These findings suggest contrasting effects of the vehicle (HSP) and cargo (antigenic peptide) components of chaperone vaccines on immunity. These data might be interpreted as, suggesting that, while tumor antigens chaperoned by the HSPs trigger antitumor immunity, processed peptides from the HSP component of the vaccine led to a suppression of immunity. Using the chaperone vaccines at lower doses appeared to favor induction of CTL over the immunoregulatory response, perhaps by reducing the levels of HSP-derived peptides below a threshold (77, 78). It would seem that most chaperone vaccines, although efficiently triggering external tumor antigen presentation, do not deliver the inflammatory signal required to overcome antigenic tolerance (**Figure 1**). Such vaccines might be improved by use of adjuvants or pro-inflammatory forms of therapy, as discussed below.

## WHEN HSPs BECOME PRO-INFLAMMATORY FACTORS

One notable finding observed in multiple investigations of HSPs was that, even in studies where an inflammatory response to HSPs was not detected, the chaperones could strongly amplify responses to PAMPs, such as LPS (79). Mycobacterial Hsp65, a protein discussed in the last section as provoking generally immunomodulatory responses, when covalently fused to antigenic polypeptides produced a potent vaccine that generated effective CTL even in the absence of adjuvant (80). In addition, the combination of Hsp70 elevation in target tissues with therapies leading to necrotic cell killing led to a profound stimulation of inflammation and CTL killing that could lead to tumor rejection (81). This approach involved, after elevation of tissue Hsp70, targeting the normal tissues of origin with treatments that led to inflammatory modes of cell killing. This combined treatment resulted in the regression of distant, transplanted tumors (81). The findings observed in these studies were that, for instance, in prostatic tissue, cell killing by fusogenic viruses in the presence of elevated Hsp70 led to induction of the cytokines IL-6 and TGF-β, resulting in generation of highly inflammatory IL-17 and tumor rejection by antigen-specific CTL (82). This effect seemed to depend on generation of IL-6 by the combination of high tissue levels of Hsp70 and inflammatory death. However, in similar studies on pancreatic tissues, combination of Hsp70 and lytic virus failed to generate IL-6 and led to generation of a Treg response and continued growth of pancreatic carcinoma (82). Thus, the balance between immunoregulatory and immunogenic responses of Hsp70 appears to be poised on a knife-edge, influenced by the tissue type and mode of cell killing.

It is well known that the mode of cell death has a powerful influence on inflammation and immunity in interacting APC (83). For instance, when cells die by an apoptotic mechanism, their intracellular contents remain enveloped by an external membrane and, thus, are not released into the environment to trigger inflammation (**Figure 2**). In addition to this, many apoptotic cells expose "eat me" signals, such as the phospholipid phosphatidylserine on the surface, triggering engulfment by macrophages and leading to immunosuppression (84, 85). Additional anti-inflammatory signals emanating from apoptotic cells may include the release of AMP from the apoptotic cell (86). In necrotic cell death, cell membranes become permeabilized, the intracellular milieu becomes externalized, and DAMPs, such as HMGB1, urate, and nucleic acids, released in this way become accessible to detection by neighboring macrophages or DC (85). It should be noted that the response of phagocytic cells to apoptotic bodies is complex and depends on the nature of the dead or dying cells and the surface densities of "eat me" or "don't eat me" signaling molecules that are cell specific. In addition, in late apoptotic cells that have failed to be phagocytosed at an early stage, membranes become permeabilized. Therefore, such late apoptotic cells acquire some of the properties of necrotic cells, permitting release of DAMPs and switching the effects of the cell corpses on engulfing phagocytes toward a more immunogenic influence (85, 87).

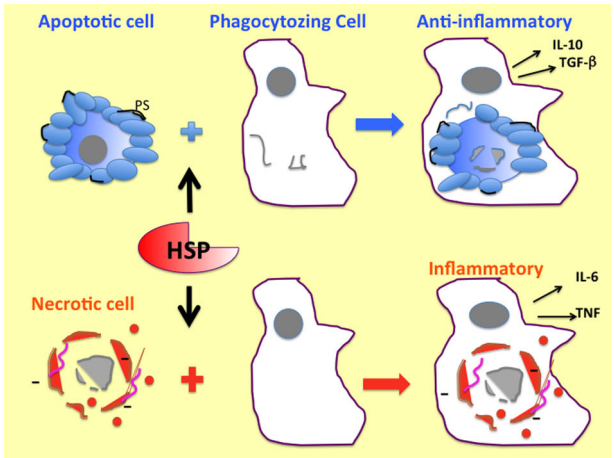


**FIGURE 2 | Contrasting effects of cell corpses resulting from apoptotic and necrotic cell killing on immunity**. Apoptotic cells, depicted here with surface blebs, externalize the phospholipid phosphatidylserine to the outer leaflet of the plasma membrane leading to anti-inflammatory signaling and suppression of immunity. Necrotic cells depicted here with compromised membranes release DAMPS, such as HMGB1 (small orange spheres), and acquire net negative charge. Nucleic acids (pink lines) become located to the cell surface. Necrotic cells often trigger release of inflammatory cytokines, such as IL-6 and TNFα. HSPs, such as Hsp70, trigger the phagocytosis of dead mammalian cells as shown here, although the mechanisms involved in such stimulation are currently not clear. Gray spheres indicate nuclei.

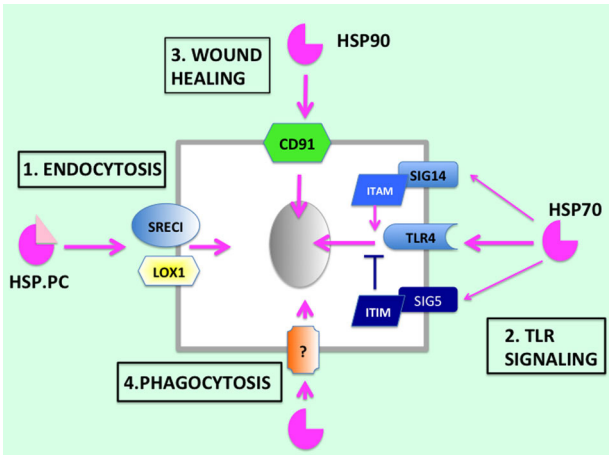


**FIGURE 3 | HSP receptors**. HSP receptors include SREC-I and LOX-1 that mediate (1) endocytosis and, thus, presentation of antigens to APC. Hsp70 can also (2) trigger signaling through the TLR4 pathway in a range of cells. (It is not clear whether HSPs can interact directly with TLRs or whether the primary interactions are through other receptors.) Effects of Hsp70 on TLR4 signaling can be modulated by binding to either Siglec-14 that activates TLR4 signaling or other Siglec family members, such as Siglec-5, that activate the pathway. Hsp90 can also (3) activate wound healing responses, which may play key roles in inflammation, through binding to CD91. Hsp70 triggers (4) phagocytosis, a property that may be important in its immune functions, through currently unknown mechanisms. Gray ovals indicate nuclei.

It is not clear in which way extracellular HSPs might synergize with the extruded contents of necrotic cells in activating immunity, although increased capacity for engulfment by immune cells which is triggered by Hsp70 might amplify inflammatory functions (88–90). The earlier studies of Todryk et al., in fact, indicated that extracellular Hsp70 decrease DC maturation and increase capacity for engulfment of antigenic materials by the immature APC (91). Phagocytosis is a complex process and involves the formation of a broad synapse type structure between the engulfing cell and the mammalian cell body of prokaryotic cells. Multiple receptors appear to make contact between the cells. Interestingly, in *C. elegans* this process has been shown to require the surface receptor CED-1 (92). Both, mammalian scavenger receptor associated with endothelial cells (SREC-I) and cluster of differentiation 91 (CD91) possess sequence similarities to this protein, suggesting some involvement in phagocytosis and a potential mechanism for HSPs in this process (93). In addition, necrotic cells can supply signals to augment the immune effects of HSPs through release of HMGB1, a ligand for TLR4, and externalized nucleic acids that might interact with TLR3 or TLR9 and trigger inflammatory signaling (85) (**Figures 2** and **3**).

## POTENTIAL MECHANISMS – CELL SURFACE RECEPTORS AND CELL SIGNALING PATHWAYS

Most evidence suggests that the biological effects of extracellular HSPs are mediated through cell surface receptors (24, 94) (**Figure 3**). Thus, some of the clues to the properties of extracellular HSPs may lie within the interactions of these chaperones with these receptors and the unleashing of their embedded signaling cascades. The first surface receptor reported to bind to HSPs was CD91 also known as Low density lipoprotein receptor-related protein 1 (LRP1), the alpha-2-macroglobulin receptor (A2MR), and apolipoprotein E receptor (APOER) (24). This high molecular weight receptor was shown originally to bind to LDL and, as its possession of multiple names suggests, a wide range of (over 30) other extracellular structures (94). CD91 is expressed most abundantly in vascular smooth muscle cells and in hepatocytes. There has been some skepticism regarding its expression in APC, such as DC, and, therefore, its potential role in immune responses involving the HSPs (95). However, there seems little doubt that CD91/LRP1 is a *bona fide* HSP receptor, and this protein appears to play key roles in, for instance, responses to Hsp90α in the wound healing response and in tumor metastasis (36, 37).

It was next shown that Hsp70 could associate with lectin-type oxidized LDL receptor (LOX-1), a receptor found on the surfaces of human DC and that such binding could mediate cross-presentation of peptide cargo associated with this HSP, leading to $CD8^+$ lymphocyte-mediated immunity (75). LOX-1 is the product of the oxidized low-density lipoprotein receptor 1 (*ORL1*) gene expressed most abundantly in vascular endothelial cells, macrophages, and DC (96). Although LOX-1 belongs to the C-type lectin receptor family, it also clusters with the SR, a group of proteins that have, in common, the ability to bind to covalently modified LDL molecules, such as oxidized LDL and

acetylated LDL (97). Furthermore, exploration of the SR family indicated significant interaction of Hsp70 with at least two other members, including SREC-I, encoded by the *SCARF1* gene and Stabilin-1/FEEL-1 (encoded by the *STAB1* gene) (73, 98). SREC-I/*SCARF1* was able to bind to Hsp70 and Hsp90 in DC and mediated cross-presentation of associated tumor antigens, leading to activated CTL (13, 17). Murine bone marrow derived DC appeared to utilize both LOX-1 and SREC-I/*SCARF1* in interacting with Hsp90–peptide complexes (17, 75). In addition, SREC-I was also shown to mediate the uptake of antigens chaperoned by Hsp90 into the Class II pathway and stimulate activation of $CD4^+$ T lymphocytes (18). The SR are regarded as receptors that respond to cellular debris including cell bodies, the remains of endogenous proteins spilled into the extracellular milieu, and the residue from invading pathogens. As such, the SR might be regarded as good choices for receptors with which APC might sample the extracellular environment (93). Stabilin-1 appears to be only sparsely expressed on the surface of DC and appears to function mostly in intracellular trafficking (99). Thus, there appeared little doubt that that the SR could bind to HSP-PC and mediate presentation of peptide cargo (17, 18). However, the nature of the receptors that might respond to HSPs and modify the inflammatory response still required investigation.

Many reports suggested that TLRs, particularly TLR2 and TLR4, might mediate inflammatory responses to HSPs in an up or down manner (32, 76). However, as mentioned above, attempts to demonstrate direct binding of HSPs to TLRs have not been generally successful (73, 75). Thus, HSP interaction with TLRs is likely to be an indirect one. It was shown, recently, that SREC-I/*SCARF1* could interact with TLR4 on the surface of mouse macrophages (64). Interestingly, both LPS and Hsp90 could mediate this complex interaction and lead to the sequestration of TLR4 in lipid raft domains (64). In the case of LPS, encasement of SREC-I–TLR4 complexes within lipid rafts was required for activation of signaling through the NFκB and MAP-kinase cascades, resulting in inflammatory cytokine secretion. Significantly, exposure to Hsp90, although leading to SREC-I–TLR4 colocalization, failed to trigger the inflammatory cascade through this mechanism. Alternative pathways of inflammatory suppression by Hsp90 may, thus, be involved (64).

Recent studies have also suggested that another receptor family, sialic acid-binding immunoglobulin-like lectins (Siglecs) may participate in inflammatory responses resulting from binding to HSPs (100). These receptors have been shown to bind to conjugated sugar residues in the cell coats of adjacent cells and generally suppress inflammation. Suppression of inflammation by these receptors involves immunoreceptor tyrosine-based inhibitory motif (ITIM) sequences in the intracellular domain that recruit anti-inflammatory proteins after ligand-triggered tyrosine phosphorylation (100). Upon activation, Siglec receptors have been shown to directly associate with TLRs and inhibit TLR-mediated activation of inflammatory signaling cascades, such as the NFκB pathway (101). Interestingly, recently, it was shown that two human Siglecs, Siglec-5 and Siglec-14 each, bind to Hsp70 (102). What was most intriguing about these interactions was that, while Siglec-5 contained the expected ITIM domain and repressed anti-inflammatory signaling through quenching NFκB, Siglec-14 appeared to have acquired a domain that binds proteins, such as DAP12, containing an intracellular immunoreceptor tyrosine-based activating motif (ITAM) that stimulated release of inflammatory cytokines (102, 103). Differential expression of members of the Siglec family might, thus, either amplify or suppress the HSP regulated activities of cell surface TLR and inflammatory signaling through mediation of the NFκB pathway. A further property of extracellular HSPs mentioned above, that may play a key role in their immune functions, is their ability to trigger phagocytosis. However, the mechanisms and receptors involved in this process have not been well characterized and further experiments will be required to understand the mechanisms involved (**Figure 3**).

Thus, responses to extracellular HSPs appear to involve the cooperative or confounding outputs of a range of cell surface receptors that together may determine their influence on immune reactions within tissues. To date, however, we have not been able to identify a dedicated high-affinity HSP receptor. Dedicated receptors, such as the insulin receptor, are often able to pick up tiny concentrations of ligands, often with affinities in the range of $10^{-9}$–$10^{-10}$M, with exquisite selectivity. For HSPs, we have identified, so far, only "hand me down" receptors that also bind a large range of other ligands with moderate affinity. It remains to be determined if dedicated HSP receptors will be found in immune cells of mammalian species.

## CONCLUSION

The intracellular roles of HSPs in protein folding have been conserved since the dawn of cellular life (3). However, the HSPs also appear to have acquired key roles in the immune systems of animals early during evolution, and these roles are preserved in modern animal species (104). Such HSPs can capture intracellular antigens and present them to APC, mediating the cross-priming of recipient cells. In addition, HSPs, when processed and presented on the APC cell surface, can activate Treg cells and inhibit immunity and inflammation. Their effects on the immune system are, thus, bivalent. In the presence of PAMPs or tissues undergoing necrosis, Hsp70, in particular, becomes a strong inflammatory agent. The precise nature of the responses elicited by extracellular HSPs may, therefore, depend upon the particular tissue milieu within which they are released and the identities of the receptors on the surfaces of immune cells that encounter them.

## OUTSTANDING QUESTIONS AND POTENTIAL ADVANCES

(1) Many of the immune-active HSPs are members of different gene families, with only a modicum of structural similarity (**Table 1**). However, each chaperone appears to be recognized by similar families of receptors, including SR and the LDL receptors, despite their sequence dissimilarity (94). Understanding the structural basis for extracellular HSP

recognition by these and potentially new classes of receptors would be an advance in determining the roles of the chaperones in immunity and predicting novel recognition structures. However, this has been an open question for a number of years now and research is ongoing.

(2) It is becoming apparent that members of individual HSP gene families have distinct properties. For instance, only Hsp90α is secreted from cells and the other major isoform, Hsp90β, is mostly retained within the cell (36). It will be enlightening to learn whether such specificity in extracellular function holds for other HSP families.

(3) We have, in recent years, acquired some understanding of how these "leaderless" proteins gain access to the extracellular microenvironment in intact cells (see Release of HSPs into the Extracellular Microenvironment), although it will be invaluable to gain a more concerted understanding of mechanisms of HSP secretion.

(4) We will continue the search for HSP receptors in immune cells, their expression patterns and their connection to inflammatory and anti-inflammatory responses to both endogenous HSPs and prokaryotic paralogs (94). It is likely that a major key to understanding how chaperones trigger immune responses and the direction of such responses depends on understanding the combinatorial effects of the multiple receptor families.

(5) It seems clear that many HSPs, although capable of efficient transport of antigenic peptides within chaperone vaccines, do not on their own deliver a second signal for APC maturation, thus reducing their effectiveness as stand-alone agents. In order to break immune tolerance to tumor antigens, chaperone vaccines might be best deployed in cancer therapy in combination with PAMPs, such as double stranded RNA or unmethylated CpG motifs or checkpoint inhibitors such as anti-CTLA4 (105, 106). Alternatively, vaccines could be used with agents, such as ionizing radiation, that can cause necrotic killing and subsequent inflammatory effects in tumors (including release of intracellular DAMPs) thus leading to immune rejection of the cancers (10).

## AUTHOR CONTRIBUTIONS

SC, JG, and AM contributed in writing the text of the review.

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# Heat Shock Protein–Peptide and HSP-Based Immunotherapies for the Treatment of Cancer

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Intracellular residing heat shock proteins (HSPs) with a molecular weight of approximately 70 and 90 kDa function as molecular chaperones that assist folding/unfolding and transport of proteins across membranes and prevent protein aggregation after environmental stress. In contrast to normal cells, tumor cells have higher cytosolic heat shock protein 70 and Hsp90 levels, which contribute to tumor cell propagation, metastasis, and protection against apoptosis. In addition to their intracellular chaperoning functions, extracellular localized and membrane-bound HSPs have been found to play key roles in eliciting antitumor immune responses by acting as carriers for tumor-derived immunogenic peptides, as adjuvants for antigen presentation, or as targets for the innate immune system. The interaction of HSP–peptide complexes or peptide-free HSPs with receptors on antigen-presenting cells promotes the maturation of dendritic cells, results in an upregulation of major histocompatibility complex class I and class II molecules, induces secretion of pro- and anti-inflammatory cytokines, chemokines, and immune modulatory nitric oxides, and thus integrates adaptive and innate immune phenomena. Herein, we aim to recapitulate the history and current status of HSP-based immunotherapies and vaccination strategies in the treatment of cancer.

**Keywords: HSP70 heat shock proteins, HSP90 heat shock proteins, cancer vaccine, innate immunity, adaptive immunity**

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## BACKGROUND

Heat shock proteins (HSPs) with the molecular weights of approximately 70 and 90 kDa have the capacity to stimulate antitumor immune responses either as carriers for antigenic peptides, which can be cross-presented by major histocompatibility complex (MHC) class I molecules, or as natural immunogens (1–3). Depending on the availability of ATP and ADP, members of the heat shock protein 70 (HSP70) family have the capacity to release and bind tumor-specific antigens, respectively. Following cross-presentation on MHC class I antigens, a CD8+ cytotoxic T cell response is initiated. Preclinical models revealed that vaccination with HSP–peptide complexes purified from tumor, but not normal cells, are able to mediate specific and protective immunity against autologous tumors. In recent years, a large number of receptors, including the alpha-2 macroglobulin receptor CD91 (4), lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) (5, 6), scavenger receptor expressed by endothelial cells-1 (SREC-1) (7), toll-like receptors-2/4 (TLRs-2/4) (8–10), their cofactors CD14 (11, 12), fasciclin EGF-like, laminin-type EGF-like and link domain-containing scavenger receptor-1 (FEEL-1), common lymphatic endothelial and vascular endothelial receptor-1 (Clever-1), stabilin-1

(Stab-1), and CD40 (13), have been found to be involved in the uptake of HSPs and HSP-chaperoned exogenous peptides into antigen-presenting cells (APCs).

Despite their high degree of homology certain HSP sequences are not conserved and thus act as immunogenes, which can be recognized as foreign by the host's innate and adaptive immune system, especially when they are presented in a tissue/tumor-specific manner (14). In combination with pro-inflammatory cytokines, including interleukin 2 (IL-2), IL-12, or IL-15, the major stress-inducible Hsp70 (HSPA1A) or non-conserved sequences derived thereof has been found to activate the cytolytic, proliferative, and migratory capacity of natural killer (NK) cells (15). This activation was accompanied by an upregulated expression density of activatory C-type lectin receptors CD94/NKG2C and NKG2D on NK cells (16). Furthermore, an intra-tumoral infusion of free, recombinant Hsp70 has been shown to increase the infiltration of NK cells and CD8+ cytotoxic T cells into tumors and the secretion of interferon gamma (IFN-γ) (17, 18).

## HSP–PEPTIDE COMPLEXES AS A VACCINE STRATEGY

In 1986, the group of Old et al. firstly described a glycoprotein with a molecular weight of 96 kDa (gp96), which was found to act as a tumor rejection antigen. Gp96 was isolated from mouse fibrosarcomas that were chemically induced by the carcinogen methylcholanthrene A (1). Li and Srivastava (19) characterized gp96 as an ER-residing member of the HSP90 family, which contains an ATPase activity. In addition to gp96, members of the HSP70 family that also possess an ATPase domain appeared to be equally immunogenic for the adaptive immune system such as gp96 when tumor-specific antigens were bound to them (20, 21). Since ATP sepharose columns are widely used for the purification of HSP–peptide complexes from tumor cells, there is a risk that the immunogenic peptides are dissolved from the HSP–peptide complexes during purification due to their ATPase activity (22). Therefore, a novel purification method, which was based on ADP-affinity chromatography, has been established for the isolation of intact HSP–peptide complexes (23).

Immunogenic peptides chaperoned by gp96 and HSP70s were not only found to elicit specific immune responses against cancer (24, 25) but also against infectious diseases (26, 27), indicating the broad applicability of HSP-based vaccines. It was also found that following oxidative stress the immunogenicity of HSP-based vaccines was found to be increased (28). This finding might be explained, on the one hand, by the fact that the amount and the repertoire of immunogenic peptides might differ in stressed and non-stressed cells. On the other hand, it is possible that stress-inducible members of HSP families might be better qualified for chaperoning immunogenic peptides than their constitutively expressed correlates. Since HSP-chaperoned peptides only mediate protective immunity against autologous, but not allogeneic tumors (24), and HSP–peptide complexes eluted from healthy tissues were found to be inefficient in stimulating T cell-mediated immunity and was assumed that HSP-chaperoned peptides are tumor cell type specific.

Furthermore, an efficient rejection of tumors in preclinical models requires the presence of CD8+ T cells in the priming phase and that of CD4+ helper, CD8+ cytotoxic T cells, and M1 macrophages in the effector phase (21). Exogenous antigens, which are typically presented by MHC class II antigens, can be channeled by HSPs into the endogenous pathway and thus can be presented on MHC class I molecules (29). This HSP-mediated switch of peptides from the endogenous MHC class II to the MHC class I pathway is also termed as antigen cross-presentation (29–31).

For a while, the mechanism how exogenous HSP–peptide immune complexes are taken up by APCs remained elusive because HSP-specific receptors had not been identified and characterized. The group of Binder et al. classified the interaction of HSP–peptide complexes with APCs as specific and saturable. These attributes are typical for a receptor–ligand interaction (32). The same group was among the first who identified CD91 as a receptor for immunogenic peptides complexed with HSP90 and HSP70 families and for calreticulin (33). CD91, which is also termed low-density lipoprotein-related protein, was initially described as a receptor for alpha-2 macroglobulin (4). Until today, a large variety of different receptors, such as LOX-1 (6), SREC-1, FEEL-1, Clever-1, Stab-1 (5, 7, 34, 35), TLRs-2/4, and their cofactor CD14 (11, 12, 36) and CD40 (13), have been shown to be involved in the uptake and signaling of HSP70 and HSP90 complexes with APCs (37).

It is important to note that the capacity of HSPs or HSP–peptide complexes to elicit antitumor-specific immunity is highly dependent on the dose. Although low doses of HSP–peptide complexes have been found to be efficient in the stimulation of antitumor immune responses, a 5- to 10-fold higher dose than the optimal stimulatory dose turned out to be ineffective or even immunosuppressive (38). High doses of gp96–peptide complexes were found to induce immune tolerance and thus were applied to treat autoimmune diabetes and encephalomyelitis in preclinical models (39, 40). The mechanisms, which are involved in the induction of tolerance by HSP70s, have been found to be associated with TLR2 and TLR4. The TLR2/MyD88 signaling pathway, which is induced after binding of exosomal Hsp70 to TLRs, has been found to mediate protection of the myocardium against ischemic reperfusion injury (10), and the TLR4/ERK1,2/p38/MAPK pathway has been found to initiate pStat3-mediated immunosuppressive activity in myeloid-derived suppressor cells (9).

Based on the knowledge on the molecular characteristics and functions of HSPs and HSP–peptide-based vaccines, the stimulation of antitumor immune responses initiated clinical applications (41). Between 2000 and 2014, gp96 and HSP70–peptide-based vaccines derived from autologous tumor lysates were clinically applied in phase I to phase III clinical trials in different tumor entities including late stage melanoma (42) either alone or in combination with GM-CSF and IFN-γ (43–48), metastatic colon carcinoma (49), renal cell carcinoma (50), gastric carcinoma (51, 52), pancreatic carcinoma (53), chronic myeloid leukemia (54), and glioblastoma (55) (**Table 1**). The outcome of these trials showed the induction of immunological responses in a large number of patients treated with HSP–peptide complexes;

**TABLE 1 | Phase I–III clinical trials using HSP-based vaccines.**

| HSP vaccine | Tumor entity | Study | Reference |
|---|---|---|---|
| Gp96 | Late stage melanoma | Pilot | (42) |
| Gp96 + sPD-1 | Malignant melanoma | Phase I–III | (46–48) |
| Gp96 + GM-CSF | Malignant melanoma | Phase I–II | (44) |
| Gp96 + GM-CSF + IFN | Malignant melanoma | Phase I–II | (45) |
| Hsp70 | Malignant melanoma | Phase I | (44) |
| Gp96 | Metastatic colon carcinoma | Phase I | (49) |
| Gp96 | Gastric carcinoma | Phase I | (51, 52) |
| Gp96 | Pancreatic carcinoma | Phase I | (53) |
| Gp96 | Hodgkin lymphoma | Phase I | (54) |
| Hsp70 | Chronic lymphatic leukemia | Phase I | (84) |
| Hsp70 | Advanced solid tumors | Pilot | (76) |
| Gp96 | Glioblastoma | Phase I–II | (55) |
| Hsp70 | Glioblastoma | Phase I | (76) |
| Hsp70-activated NK cells | Colon carcinoma, NSCLC | Phase I–II | (72, 73) |
| Hsp70 | HIV | Phase I | (26) |
| Hsp70 mRNA | HCC | Phase I | (85) |

however, clinical responses (CRs) were observed only in certain patient subgroups.

## HSP70 IN THE STIMULATION OF INNATE IMMUNITY

Heat shock protein 70 has been found to be overexpressed in tumor cells. Hsp70 is presented on the cell membrane of a large variety of solid tumors, including lung, colorectal, breast, squamous cell carcinomas of the head and neck, prostate and pancreatic carcinomas, glioblastomas, sarcomas, and hematological malignancies, but not on corresponding normal tissues (56, 57). A membrane Hsp70+ phenotype has been determined either directly on single cell suspensions of freshly isolated tumor biopsies by cell surface iodination/biotinylation (58, 59) and flow cytometry using cmHsp70.1 monoclonal antibody (60) or indirectly in the serum of patients using a novel lipHsp70 ELISA (61). In contrast to commercially available ELISA systems, the lipHsp70 ELISA specifically detects free and lipid-bound, exosomal Hsp70 which is actively released by viable tumor cells. Therefore, it is assumed that the quantification of exosomal Hsp70 in the serum serves as a measure for viable tumor mass in a patient and thus might provide a diagnostic/prognostic biomarker in the future (62). A membrane Hsp70+ tumor phenotype has been found to be associated with highly aggressive tumors, causing invasion and metastases and resistance to cell death (57, 63, 64). However, NK cells, but not T cells, were found to kill membrane Hsp70+ tumor cells after preactivation with naturally occurring Hsp70 or an Hsp70–peptide (TKD) derived thereof in combination with low dose IL-2 (TKD/IL-2) (65). Since the induction of the cytolytic activity of NK cells with Hsp70–peptide is dose dependent and saturable, it was assumed that the interaction of NK cells with the peptide might also be receptor mediated. By antibody and protein/peptide blocking assays, the C-type lectin receptor CD94 was identified as a potential receptor that mediates the interaction of NK cells with Hsp70–peptide. CD94 forms a heterodimer either with the coreceptor NKG2A or NKG2C and thus can act as an inhibitory or activation receptor complex (66–69). Following incubation of NK cells with Hsp70 protein or Hsp70–peptide + IL-2, the density of CD94 was found to be upregulated concomitant with an increased cytolytic and migratory activity against membrane Hsp70+ tumor cells (70). In addition, also other activatory NK cell receptors, such as NKG2D, and natural cytotoxicity receptors (NCRs), but not inhibitory killer-cell immunoglobulin-like receptors (KIRs), were found to be upregulated on NK cells upon stimulation with Hsp70–peptide + IL-2.

A summary of major activities of Hsp70 in inducing adaptive and innate antitumor immune responses is illustrated in **Figure 1**. On the one hand, Hsp70 either alone or in combination with immunogenic peptides is able to induce the maturation of dendritic cells (DCs), activate the cytolytic, proliferative, and migratory capacity of NK cells, stimulate the antigen-dependent T cell activation and IFN-γ secretion, induce the release of pro- and anti-inflammatory cytokines, on the other hand, membrane-bound Hsp70 acts as a tumor-specific antigen, which is recognized by preactivated NK cells. As a carrier for HSP-chaperoned tumor-specific antigens members of the HSP70 and HSP90 family have been found to support antigen uptake, processing, and presentation on MHC class I to CD8+ cytotoxic T lymphocytes and on MHC class II molecules to CD4+ helper T cells.

The mechanism how Hsp70 preactivated NK cells lyse membrane Hsp70+ tumor cells could be identified as granzyme B-mediated apoptosis. The cell death-inducing serine protease granzyme B has been found to directly interact with membrane Hsp70 on tumor cells, as determined by different methods including matrix-laser desorption ionization time to flight mass peptide finger printing (MALDI-TOF), Western blot, and flow cytometry (71). NK cells that have been prestimulated with Hsp70–peptide + IL-2 showed a significantly upregulated production of granzyme B, whereas the intracellular levels of perforin were found to be upregulated only moderately (16, 70). Since tumor cells that lack an Hsp70 membrane expression are not lysed by granzyme B, as demonstrated in isogenic tumor cell systems that differ in their membrane Hsp70 expression levels, it was concluded that Hsp70–peptide + IL-2 preactivated NK cells predominantly kill their target cells *via* granzyme B-mediated apoptosis (71).

Safety and tolerability of *ex vivo* TKD/IL-2 stimulated, autologous NK cells have been demonstrated in patients with metastasized colorectal and NSCLC in a Phase I clinical trial (72). Based on promising clinical results of this Phase I trial, a Phase II randomized clinical study was initiated in 2015 (73). The primary objective of this multicenter proof-of-concept trial is to examine whether an adjuvant treatment of NSCLC patients after platinum-based radiochemotherapy (RCT) with Hsp70–peptide TKD + IL-2-activated, autologous NK cells is clinically effective. Only membrane Hsp70+ tumor patients will be recruited into the trial since membrane Hsp70 was identified as the tumor-specific target for Hsp70–peptide + IL-2 preactivated NK cells. The primary endpoint of this study is the progression-free survival that will be compared between patients who received RCT or RCT+ an NK cell-based immunotherapy. As secondary endpoints overall survival, toxicity,

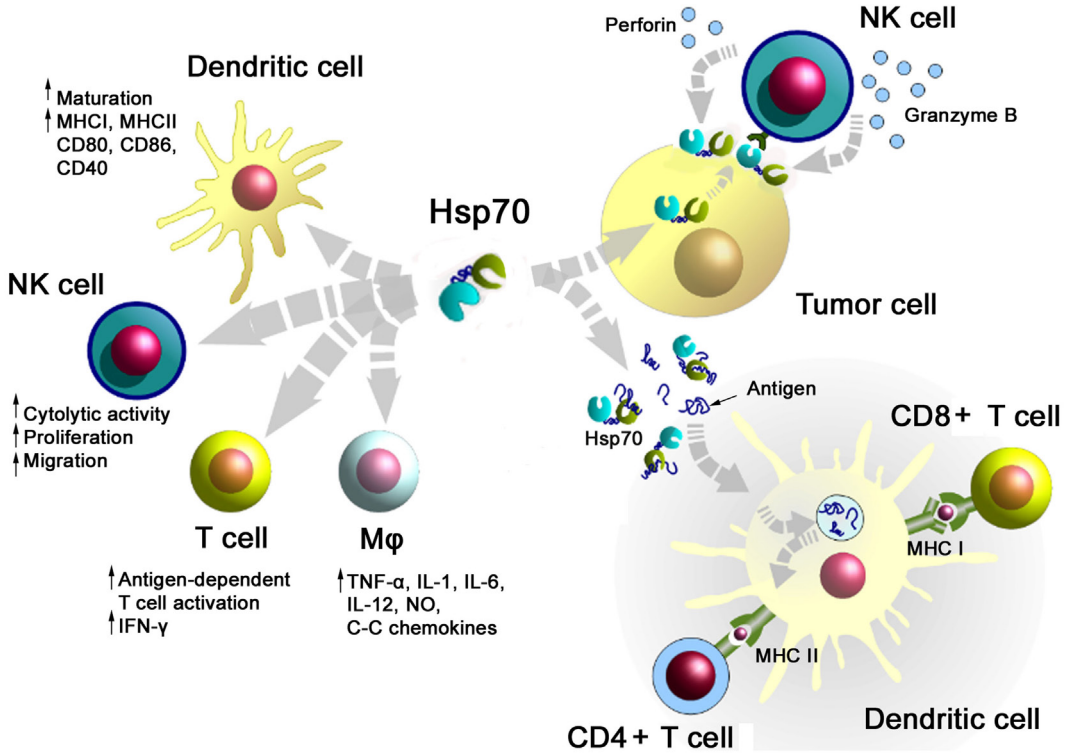


**FIGURE 1 | Major immune modulatory functions of heat shock protein 70 (Hsp70) either alone bound to exosomes or in combination with tumor-derived peptides**. Abbreviations: IFN-γ, interferon gamma; IL, interleukin; Mφ, macrophages; MHC, major histocompatibility complex; NK cells, natural killer cell; NO, nitric oxide.

quality-of-life, and biological responses will be determined in both study groups.

## HSPs AS ADJUVANTS FOR THE STIMULATION OF ANTITUMOR IMMUNE RESPONSES

Heat shock proteins, especially the major stress-inducible Hsp70, can provide cytokine function, which initiate both, innate and adaptive immunity (74–77). In parallel, these HSPs can act as classical chaperones that facilitate uptake, processing, and presentation of tumor antigens into APCs. Moreover, exogenously delivered, purified Hsp70 was shown to sensitize cancer cells to lymphocyte-mediated cytotoxicity due to triggering the translocation of its intracellular analog to the tumor cell surface and due to an increased release of Hsp70 into the extracellular milieu (18). For these reasons, the aforementioned immunomodulatory activities of Hsp70 have been widely exploited for therapeutic approaches in recent years either as single treatment or in combination with other treatment modalities to generate an effective antitumor immunity. The intra-tumoral injection of Hsp70 protein or an upregulation of Hsp70 within the tumor by an *hsp70.1* gene transfer was shown to have a significant therapeutic potential in preclinical studies (18, 78–81). Thus, prolonged intra-tumoral delivery of exogenous Hsp70 in a rat glioblastoma model caused a significant inhibition of tumor progression, which as accompanied by an increased cytotoxic activity of NK cells and CD8+ T lymphocytes (82). A comparable therapeutic efficacy was previously reported by Rafiee et al. (79) who showed a complete tumor eradication following transfection of the *hsp70.1* gene sequence into mouse tumor cells. The systemic antitumor immune response was found to be mediated by CD4+ and CD8+ T cells (79). Presumably, a combination of Hsp70-based therapies with other immunological approaches, such as immune- and T cell check-point inhibitors, might further increase the therapeutic efficacy. In another approach, the intra-tumoral injection of Hsp70 was combined with mild local hyperthermia and magnetite cationic liposomes (MCLs). This strategy demonstrated great potential in the treatment of mouse melanoma (81). With regard to these results, our group coupled Hsp70 to nanocarriers such as superparamagnetic iron oxide nanoparticles (SPIONs) (83). Hsp70-SPIONs were shown to effectively deliver immunogenic peptides from tumor lysates to DCs and thus stimulated a tumor-specific, CD8+ cytotoxic T cell response in experimental glioma models (83). Up-to-date several clinical trials clearly demonstrate that the application of Hsp70 either as a single treatment regimen or in combination with other therapies is feasible and can stimulate antitumor immunity in patients (18, 84, 85). Clinical efficacy could be demonstrated in patients with brain tumors who received surgery and intra-tumoral delivery of recombinant

Hsp70. Out of 12 patients with late stage brain tumors, one patient showed a complete CR and another patient showed a partial response (PR) (18). The CRs were accompanied by an enhanced $Th_1$-cell-mediated immune response and a reduction of immunosuppressive Treg cells. In the Phase I clinical trial reported by Maeda et al., DCs transfected with Hsp70 mRNA (HSP70-DCs) were applied in 12 patients with non-resectable or recurrent HCV-related hepatocellular carcinoma (HCC) (85). The authors demonstrated that 7 out of 12 patients had either a CR or stable disease (SD), suggesting the efficacy of the proposed therapy. In another study, an intra-tumoral vaccination with recombinant oncolytic type-2 adenovirus that overexpresses Hsp70 was found to inhibit primary and metastatic tumors *via* an enhanced oncolytic activity and Hsp70-mediated immune responses (84). Presumably, multimodality tumor-directed therapy based on HSPs in combination with radio, chemo, and/or hyperthermia (86) therapy can be a treatment option for further clinical trials.

## SUMMARY

Heat shock proteins and especially members of the HSP70 and HSP90 families have been found to elicit protective antitumor immunity in preclinical models and in tumor patients either alone or in complex with tumor-derived peptides. HSPs and HSP–peptide complexes can act as typical tumor-specific foreign antigens, chaperokines, and adjuvants that facilitate uptake, processing, and presentation for tumor-specific antigens which are cross-presented by APCs to CD8+ cytotoxic T lymphocytes. Uptake of HSPs and HSP–peptide complexes is mediated by a large variety of different receptors. Depending on the dose of the HSP-based vaccine either immunosuppressive or immunostimulatory activities can be elicited.

## AUTHOR CONTRIBUTIONS

MS wrote the paragraph about HSPs as an adjuvant and produced the figure; GM wrote the MS.

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# Extracellular Release and Signaling by Heat Shock Protein 27: Role in Modifying Vascular Inflammation

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Heat shock protein 27 (HSP27) is traditionally viewed as an intracellular chaperone protein with anti-apoptotic properties. However, recent data indicate that a number of heat shock proteins, including HSP27, are also found in the extracellular space where they may signal *via* membrane receptors to alter gene transcription and cellular function. Therefore, there is increasing interest in better understanding how HSP27 is released from cells, its levels and composition in the extracellular space, and the cognate cell membrane receptors involved in effecting cell signaling. In this paper, the knowledge to date, as well as some emerging paradigms about the extracellular function of HSP27 is presented. Of particular interest is the role of HSP27 in attenuating atherogenesis by modifying lipid uptake and inflammation in the plaque. Moreover, the abundance of HSP27 in serum is an emerging new biomarker for ischemic events. Finally, HSP27 replacement therapy may represent a novel therapeutic opportunity for chronic inflammatory disorders, such as atherosclerosis.

**Keywords: heat shock protein 27, inflammation, atherosclerosis, coronary artery disease, HSPB1, non-classical secretion, extracellular HSP27**

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## INTRODUCTION

Since its first discovery in 1962 by Ritossa, the heat shock response has been established as the classical molecular mechanism underlying the cellular response to heat stress, involving the upregulation of a spectrum of protein families designated as "heat shock proteins" (HSPs) (1, 2). These proteins range in molecular weight from 10 to 110 kDa and primarily function as "chaperones" that facilitate proper protein folding (3, 4). Exposed hydrophobic residues in nascent or misfolded proteins act as the signal to recruit and activate HSPs, making this class of proteins powerful sensors of environmental (e.g., heat, oxidative) and physiological (e.g., infection, inflammation) stress (5, 6). Although initially characterized as *intracellular* chaperones, subsequent studies pointed to the presence of HSPs in the *extracellular* space (7, 8). Extracellular HSPs (eHSPs) have consequently been ascribed novel functions (such as "outside–in" signaling leading to cellular proliferation and immune response modulation), expanding their canonical role of maintaining homeostasis in the cell to that of the whole organism.

## INTRACELLULAR ROLE OF HSP27

Unlike the larger molecular weight HSPs, HSP27 was first identified as an estrogen-responsive protein (9–11), which was later confirmed to be heat-inducible and sharing sequence homology with other HSPs (12–16). It is a member of the small heat shock protein beta (HSPB) family, an HSP subgroup characterized by a lower molecular weight range (12–43 kDa) and a shared α-crystallin domain flanked by variable N- and C-terminal sequences (17, 18). The encoding gene, *HSPB1*, is located on chromosome 7 (7q11.23) (19), and its associated mutations have been linked to Charcot–Marie–Tooth disease type 2 (20, 21) and possibly Williams syndrome (22, 23). The intracellular chaperoning function of HSP27 is largely regulated by the phosphorylation/dephosphorylation of three key N-terminal serine residues at positions 15, 78, and 82 (24–26), which influences the assembly of HSP27 monomers into large oligomeric complexes (200–800 kDa). Such complexes create an ATP-independent chaperone network that effectively traps misfolded protein substrates, preventing their aggregation through a series of controlled binding and release reactions that, in conjunction with other larger HSPs (HSP70, HSP90), facilitate their correct folding (27–29). Thus, in concert with other chaperones, HSP27 maintains cellular homeostasis by holding misfolded proteins in a soluble, refolding-competent form. Intracellular HSP27 has also been implicated in (i) cytoskeletal architecture and dynamics (30–32), (ii) mRNA stabilization (33), (iii) antioxidant responses (34–36), and (iv) anti-apoptosis (37–40). Not surprisingly, its multiple, cytoprotective effects are associated with the amelioration of a range of pathological conditions, including degenerative, ischemic, neurological, and cardiovascular disease (41). For more comprehensive reviews on the biology of intracellular HSP27, several references are available (17, 42–44).

## EXTRACELLULAR ROLE OF HSP27

Heat shock proteins were first observed in the extracellular space nearly 30 years ago as proteins that were transferred from glia to neurons in the squid giant axon (45) and later confirmed to be released after heat shock in mammalian cells (46). Like other HSPs, HSP27 has been found extracellularly – initial evidence indicated its secretion from tumor cells (47–51). The presence of HSP27 has been detected in human serum, with elevations observed after extreme exercise (52, 53). Increases in serum HSP27 have also been measured in patients with chronic pancreatitis (54), gastric adenocarcinoma (55), insulin resistance (56), and during acute attacks in multiple sclerosis (57), while decreases have been associated with type 1 diabetes (58). HSP27 has also been found in the cerebrospinal fluid during brain and spinal cord ischemia (59).

In the context of cardiovascular disease, two independent groups utilizing a proteomic analysis of human atherogenic arterial tissue have shown that HSP27 is differentially secreted into the extracellular milieu (60, 61). Moreover, serum levels of HSP27 have been detected in patients with atherosclerosis (60, 62, 63), acute coronary syndromes (61), and reperfusion after ischemic clamping during heart bypass surgery (64). The presence of serum HSP27 was confirmed in an atherosclerosis mouse model ($ApoE^{-/-}$), overexpressing HSP27 ($HSP27^{o/e}$) using commercially available ELISA kits (65, 66). However, these experiments revealed the presence of HSP27-reactive autoantibodies (AAB) in the serum that effectively shield the detection of HSP27, thereby resulting in erroneously low levels of HSP27. Consequently, our laboratory has developed a mass spectrometric-based method that can more sensitively detect serum HSP27 at levels higher than those quantified using commercial ELISA kits (67). Even though the detection of serum HSP27 levels can only improve, results to date firmly establish the extracellular presence of HSP27. However, key questions remain (1) how is HSP27 released from cells; (2) what is its function outside the cell; and (3) how does it signal from outside to inside the cell?

## POSSIBLE MECHANISMS OF HSP27 EXPORT

Secreted proteins typically exit eukaryotic cells *via* the endoplasmic reticulum–Golgi network (known as the "classical secretory pathway"). Such proteins contain an N-terminal signal peptide that marks them for secretion – which interestingly is absent in other types of secreted proteins, including HSPs. Several groups have shown that despite this, HSPs can still be released – for example, early experiments that pharmacologically blocked the classical pathway still resulted in HSP secretion (in this case, HSP70) (46). Although HSP release was proposed to be a consequence of passive transport, i.e., necrosis (68), secretion can also occur independent of cell death, implying an active transport process that involves alternative, "non-classical" secretory mechanisms (46, 69–71). Indeed, some secretory proteins lacking the signal peptide for the classical pathway have since been identified, including interleukin (IL)-1a, IL-1b, and fibroblast growth factor (FGF)-2, and increasing evidence indicates that these proteins along with some HSPs are released by non-classical secretory pathways (7, 72, 73). Although there is still much mechanistic information to uncover regarding how HSP27 is secreted, this review will discuss a few key findings indicating that it may leave the cell *via* lysosomes and/or exosomes and bring up the possibility of direct protein translocation (**Figure 1**).

### Endolysosomal Pathway

Known as the recycling units of the cell, lysosomes are vesicular organelles containing enzymes that break down biomolecules (proteins, lipids, nucleic acids, carbohydrates) into their basic components. In addition to their role in the degradation of cellular waste, lysosomes can also serve as storage compartments for proteins targeted for secretion. These "secretory lysosomes" have been found in blood cells, among other cell types, and share similarities with conventional lysosomes, including an acidic pH interior (82). Utilization of this alternative secretion pathway has been observed in the case of HSP70. In one study using prostate carcinoma cell lines, lysosomal fractions contained more HSP70 following heat shock, and conversely, treatment with the lysosomal inhibitors, methylamine, and ammonium chloride prevented heat shock-mediated release of HSP70 into the culture

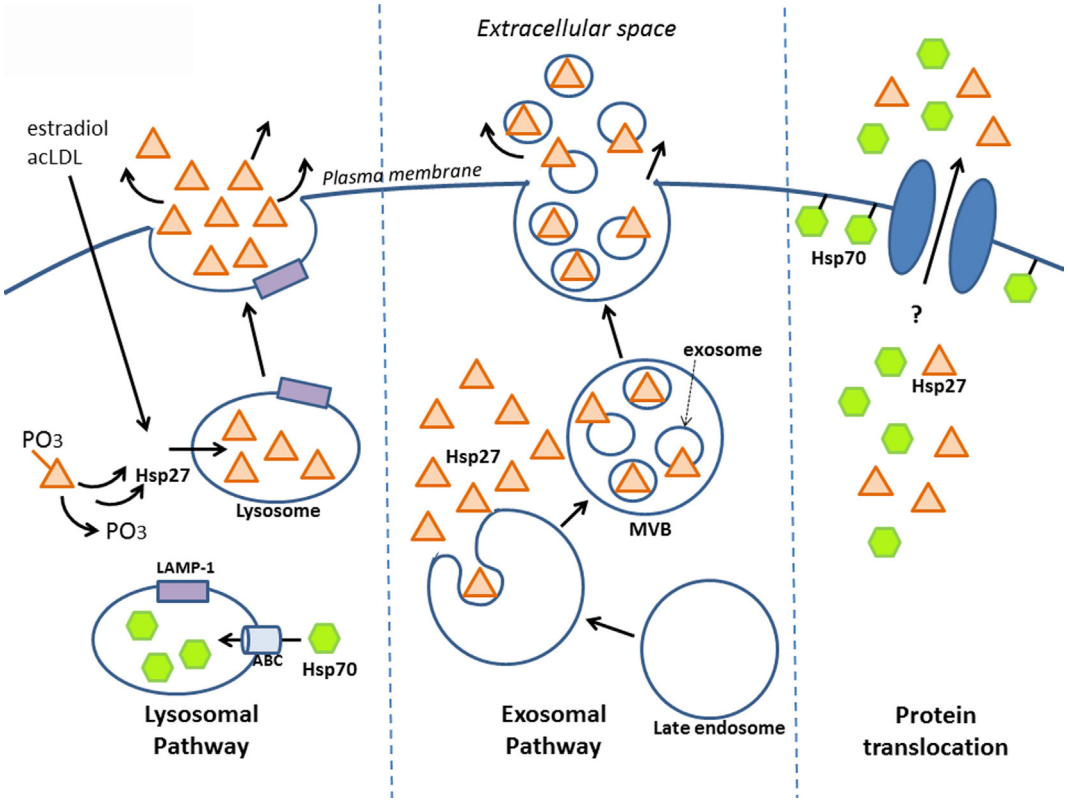


**FIGURE 1 | Release of HSP27 involves non-classical secretory mechanisms**. Experimental evidence suggests that HSP27 exits cells *via* the *endolysosomal pathway* and through *exosomes*. Localization of HSP27 to lysosomes (65) may involve the dephosphorylation of two serine residues at amino acids 15 and 82 (74); meanwhile, HSP70 appears to enter lysosomes with the aid of ABC transporters spanning the lysosomal membrane (75). Although direct protein translocation accounts for the unconventional secretion of FGF-2 (76, 77), it remains to be determined whether this mechanism plays a part in HSP27 extracellular release. Like FGF-2, HSP70 can interact with lipids as well as integrate into lipid membranes (7, 46, 78–81), and it also appears that HSP70 can integrate into artificial lipid bilayers forming channels.

media – which together suggest that the lysosome is involved in secretion (75). It is likely that HSP70 enters the lysosomal interior *via* ATP-binding cassette (ABC) transporters, since inhibition with glibenclamide, a general ABC transporter inhibitor, and DIDS, a specific ABCA1 inhibitor, reduced HSP70 release (75). As well, its release was associated with the relocalization of lysosomal markers, typically found in the lysosomal interior to the exterior cell membrane leaflet (75, 83). Taken together, these findings indicate that HSP70 may exit cells *via* secretory lysosomes, resulting in their fusion to the cell membrane. Similar findings were seen in cultured primary human peripheral blood mononuclear cells (PBMCs), whereby HSP70 secretion was significantly inhibited by the lysosomal inhibitor, methylamine, but not by brefeldin A (a blocker of transport from the ER to Golgi) (70). Furthermore, the presence of HSP70 in the lumen of lysosomes has been reported by another group (84).

What is the evidence that HSP27 uses the endolysosomal pathway? Findings from our laboratory have shown that upon treatment of macrophages with estradiol or acetylated low-density lipoprotein (ac-LDL) – conditions that stimulate HSP27 secretion – there was increased co-localization of HSP27 with the lysosomal markers, LAMP-1, and lysotracker (65). A later study yielded new insight into how HSP27 could be released through lysosomes (74). Their experiments utilized two types of HSP27 mutations, a constitutively phosphorylated mimic (S15D/S82D) and a non-phosphorylatable form (S15A/S82A), which were linked to GFP in order to visualize cellular localization in endothelial (HUVEC) cells. Interestingly, it was observed that the S15A/S82A mutation co-localized more frequently with the lysosomal marker, LAMP-1, using fluorescence microscopy. Furthermore, using His-tagged HSP27 mutants expressed in HUVEC cells, the non-phosphorylatable HSP27 was once more found at higher levels in cell culture media compared to S15D/S82D, as assessed by Western blotting for the histidine tag. These findings suggest that in order for HSP27 to localize to the lysosome (for subsequent secretion), these two serine residues must first be dephosphorylated (**Figure 1**).

## Exosomal Pathway

Exosomes are the most commonly accepted vehicle for HSP release (69). This type of secretory vesicle is derived from a multistep process that first involves the internalization of the plasma membrane to form endosomes, which then develop into late endosomes. Late endosomal membranes are invaginated within,

forming smaller vesicles (exosomes) that measure 40–100 nm in diameter and contain cytoplasmic components. These complex structures, referred to as "multivesicular bodies" (MVBs), fuse to the cell membrane, releasing exosomes into the extracellular space (**Figure 1**) (69, 85). Numerous findings have indicated the presence of several HSPs (HSP70, HSP60, HSP90) in exosomes, with localization observed at both the exosomal membrane (conceivably, facilitating interactions with cell surface receptors on target cells) and lumen (implying that for HSPs to have an effect on target cells, exosomes will have to burst to release their contents) (7, 86–90).

There is mounting evidence indicating that HSP27 is found in exosomes (**Figure 1**). HSP27 has been detected in exosomes derived from a variety of cell and tissue types, including keratinocytes, platelets, cancer cells, saliva, thymus, and urine, making the exosomal pathway a compelling system for HSP27 export (http://exocarta.org/gene_summary?gene_id=3315). Experiments in B-lymphoblastoid cells also demonstrate the presence of HSP27 in exosomes (90). Here, basal levels of HSP27, HSP70, and HSP90 localized to exosome fractions as assessed by Western blotting – an effect that increased with duration of heat shock treatment. When isolated exosomes were coupled to beads for flow cytometry analysis, positive staining for exosomal surface markers (CD81 and MHC class I), but not HSPs, were noted, implying that these HSPs were not expressed on the exosomal surface. However, when the exosome–bead complexes were solubilized by boiling in SDS (disrupting the exosomes) and total protein analyzed by Western blotting, elevated levels of HSP70 were detected in exosomes from heat-shocked cells compared to controls. These findings indicate that disruption of the exosomal membrane was required in order to detect HSP70, implying its localization within the exosome. Unfortunately, experiments detecting HSP27 after exosomal disruption were not presented in this study, thus it could not be confirmed whether the absence of HSP27 in the exosomal membrane points to a luminal presence. Results from our laboratory also support that exosomes may be one system involved in HSP27 release, since the treatment of human macrophages with an exosomal inhibitor, dimethyl amiloride (DMA), substantially decreased extracellular HSP27 levels (66). More recently, HSP27 was detected in exosomal fractions originating from primary cultures of rat astrocytes (91). Consensus has yet to be achieved with regards to the precise location of HSPs in exosomes. The suggestion of a luminal localization for HSP70 (90) is in contrast to results in macrophages infected with two different viruses (88, 92) and in blood cells after ischemic preconditioning (87), which indicate the presence of HSP70 in the exosomal membrane. Differences in exosomal localization is likely determined by the type of HSP, the cell from which exosomes originate, and the kind of stimuli triggering exosomal release (e.g., heat shock vs. viral infection). Another aspect of the exosomal pathway that is worth further exploration is determining the sorting signal that directs HSPs to populate exosomes. In the case of HSP60, for example, it appears to be ubiquitinated in exosomal fractions (86), suggesting that ubiquitinylation may signal sorting to exosomes. Whether this applies to HSP27 is currently unknown.

## Direct Protein Translocation

A final, intriguing possibility for HSP27 export may involve direct protein translocation across the plasma membrane, as has been proposed for FGF-2 (73, 93). Exit of HSPs from cells could be mediated by transmembrane proteins, like ABC transporters (also located on cell membranes), which, as already mentioned, are instrumental in transporting HSP70 in the lysosomes for secretion (75, 83). Another hypothetical way for HSPs to leave the cell is by directly associating with the cell membrane, which could then somehow facilitate their extracellular release. In fact, such lipid interactions have been observed with HSP70 (**Figure 1**) (46, 78–80), and it also appears that HSP70 can integrate into artificial lipid bilayers forming channels (7, 81). Despite the lack of evidence supporting the direct release of HSP70 (and other HSPs) after insertion or association into lipid membranes, it is still possible to consider direct membrane translocation of HSPs as a mechanism for exit from cells by looking to the cellular export paradigm utilized by FGF-2.

Over the years, the Nickel research group has systematically delineated the various steps involved in the export mechanism for FGF-2 (76, 77). According to their findings, FGF-2 localizes to the inner leaflet of the cell membrane *via* interactions with lipids, and after phosphorylation by Tec kinase, is then primed to oligomerize and integrate into the membrane, forming a transient pore structure, which itself is speculated to disassemble into FGF-2 monomers during extracellular release (76). Subsequent results highlight the importance of two surface cysteines unique to FGF-2 (notably absent in other FGF family members) that mediate disulfide bond formation with another FGF-2 monomer, forming dimers that then assemble into hexamers – the final FGF-2 structural complex that is inserted into the membrane (93). Since mutations of these two cysteines abrogate FGF-2 secretion, it appears that disulfide formation between cysteines facilitates the insertion of FGF-2 oligomers into the cell membrane and its subsequent release (93). Perhaps, similar mechanisms are involved with HSP export. FGF-2 and HSP70 have both been observed to interact with lipids and integrate into membranes. In addition, HSP27 also shares a key feature with FGF-2 – both contain unique cysteine (C) residues that happen to be missing in members of their respective protein families. In HSP27, this unique cysteine residue, C137, located in the α-crystallin domain, also mediates dimerization with other HSP27 monomers, however the consequence of dimerization differs in that disulfide formation between opposing cysteine residues modulates HSP27 function during oxidative stress (94). In reducing conditions, cysteines are directly opposite each other at the dimer interface, while under oxidative conditions, a disulfide bond forms, altering the structure of HSP27's α-crystallin domain, leading to increased exposure of hydrophobic residues and promoting chaperoning activity (95–97). Although C137 is thought to be critical in mediating HSP27's chaperoning activity during oxidative stress (i.e., disulfide bond can more easily bind to misfolded proteins that arise from oxidative stress), it will be interesting to see if mutation of this cysteine can alter secretion of HSP27, as has been observed with FGF-2. Another issue to address is whether HSP27 can interact with membrane lipids, like HSP70 (98–100). There is much

to be discovered before ruling out that HSP27 could, in part, be exported from cells *via* direct protein transport (**Figure 1**).

## SIGNALING OF EXTRACELLULAR HSP27

Once outside the cell, what does extracellular HSP27 do? Most evidence suggests that, like other HSPs, HSP27 serves as a signaling molecule released by cells, which bind to surface receptors on distant cellular targets, such as endothelial and immune cells (7, 101, 102). Thus far, it appears that HSP27 can function outside the cell as (i) an uncomplexed, extracellular protein, (ii) part of a larger protein complex, for example, *via* interactions with AAB in the serum, and (iii) molecular cargoes located on the surface or inside exosomes. This review will focus on how recombinant extracellular HSP27 modifies receptor-mediated signaling in target cells and will only briefly comment on its effects as part of antibody complexes and exosomes.

Several cell membrane receptors have been identified for eHSPs, including CD91, CD40, CD36, CD14, toll-like receptors (TLRs), and scavenger receptor-A (SR-A) (8, 102). HSP27 has also been shown to interact with TLRs, as well as other types of receptors, altering signaling mechanisms in a variety of cell types and conditions (**Figure 2**). For example, HSP27 treatment of *human macrophages* promoted NF-κB transcriptional activation as well as protein expression and secretion of the NF-κB-dependent cytokines, GM-CSF and IL-10 – the latter important in anti-inflammatory responses (103). NF-κB activation by HSP27 was also observed in *mouse coronary endothelial cells*, occurring *via* interactions with TLR-2 and TLR-4 and resulting in the upregulation of intercellular adhesion molecule-1 (ICAM-1) and monocyte chemoattractant protein-1 (MCP-1) (64). In *human coronary endothelial cells*, HSP27 also upregulated ICAM-1, MCP-1, as well as IL-6 and IL-8, although the receptors mediating the response in human cells were not identified (64). In contrast, TLR-3 was implicated as the receptor utilized by HSP27 in *human microvascular endothelial cells*, leading to NF-κB activation and secretion of IL-8 and vascular endothelial growth factor (VEGF) (104). The effect of HSP27 on VEGF was corroborated in *endothelial progenitor cells (EPCs)*, although this study did not address which receptors play a part in VEGF secretion (105). HSP27 increased secretion of IL-6 in *bone marrow-derived dendritic cells*, as well as TNF-α, IL-1β, IL-12p35, and IL-12p40, which partly occurred through TLR-4 signaling (106). In *primary human monocytes*, release of IL-10 by HSP27 involved phosphorylation of p38 and MAPKAPK-2, while increased TNF-α levels were attributed to the activation of both p38 and ERK1/2 signaling pathways (107). HSP27 also interacts with estrogen receptor-β (ER-β) (108, 109). Altogether, these findings implicate extracellular HSP27 in (i) immune signaling (*via* activation of TLR signaling pathways), leading to cytokine production and modulation of the immune response, (ii) cellular migration (due to the upregulation of ICAM-1 and VEGF), and (iii) cellular proliferation (through its interactions with ER-β).

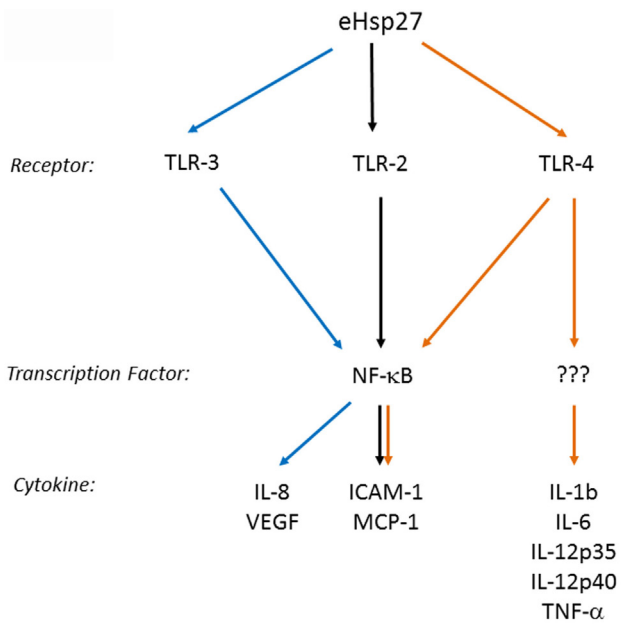


**FIGURE 2 | Extracellular HSP27 (eHSP27) can trigger TLR signaling, leading to NF-κB activation and cytokine upregulation (64, 103–106).**

Extracellular HSP27 has also been found as part of larger macromolecular complexes. For example, our laboratory's attempts to develop a more sensitive method for measuring HSP27 in serum samples led to the serendipitous discovery that this protein is complexed to AAB, which effectively shields the detection of serum HSP27. *In vitro* experiments later showed that AAB could potentiate the signaling effects of HSP27 (67). Another example of HSP27 being part of a larger complex is its presence in exosomes. Exosomes carrying HSP27 (either on the surface or in its interior) could then interact with target cells through receptor interactions leading to endocytosis, or by simple membrane fusion that is independent of receptors (69). Precisely how exosomes cargoing HSP27 affect target cells is unknown; however, it may instigate a variety of effects depending on the exosomal source and downstream cellular target. This has been observed with HSP70, whose expression on exosomal surfaces has been found to (i) trigger TLR-4 signaling leading to myocardial protection (87) and (ii) stimulate natural killer (NK) cells, increasing its cytolytic activity against HSP70-membrane positive tumors (110). Thus, by interacting with receptors on both immune and endothelial cells, leading to the differential release of cytokines and growth factors, extracellular HSP27 is a potentially important modulator of the immune response – an important process that contributes to atherosclerosis.

## HSP27 IN ATHEROSCLEROSIS

Atherosclerosis is a chronic cardiovascular condition that is marked by the progressive accumulation of inflammatory scar tissue containing lipid deposits, calcification, and necrotic debris within the walls of arteries that can lead to the formation of unstable plaques at risk of rupture. Plaque rupture is unpredictable and is the final phase that leads to a reduction in blood flow, ischemia in the distal vascular bed, and clinical symptoms or events (e.g., heart attack and/or death). Although the etiology of

atherosclerosis was primarily thought to originate from a lipid/cholesterol imbalance (be it genetic or environmental), it appears that atheroprogression is a complex, multifactorial process that is, in part, due to coordinated immune responses. Recently, several lines of evidence support the emerging role of HSP27 in modulating atheroprogression, by altering the inflammatory components of atherosclerotic plaques.

Although evidence of cardiovascular disease has been traced back to thousands of years, with various prehistoric populations displaying signs of arterial plaque (111), it is only within the past 100 years that this condition has emerged as the leading cause of mortality in the world. Epidemiological studies from the twentieth century have indicated that women are at lower risk for developing cardiovascular diseases compared to men; however, this bias toward the female sex disappears after menopause, implicating female hormones as protective factors against CVD (112). Consequently, various clinical trials conducted in the 1970s–2000s sought to test whether the replacement of ovarian hormones could protect menopausal women from CVD. Unfortunately, the conclusions of these trials indicate that hormone replacement therapy not only failed to protect women from CVD but also increased side effects as well as CVD risk (113, 114). Although hormone replacement therapies were ineffective against CVD, the question of how the presence of female hormones protect against CVD in premenopausal women remains. Given that HSP27 was initially identified as an estrogen-inducible gene (10, 11), it was hypothesized that HSP27 is one important downstream "foot soldier" of estrogen signaling that mediates cardiovascular protection (108).

There is a precedent in establishing a link between other HSPs and cardiovascular disease. For example, some studies correlate HSP60 with atherosclerosis severity and suggest that it could serve as a biomarker (115–117), while others associate HSP70 serum levels with low CVD risk (118, 119). HSP27 was first implicated in atherosclerosis more than 10 years ago, when a proteomic screen comparing levels of released proteins noted lower levels of HSP27 secretion from human atherosclerotic arteries compared to normal arteries (60). Indeed, HSP27 secretion and expression in atherosclerotic human coronary arteries (108, 120) decreases with increasing plaque progression, complexity, and instability. Conversely, areas of normal-appearing atherosclerotic arteries display increased HSP27 expression compared to plaque-filled areas from the same vessel (61). Three independent studies confirm the reduction in secreted HSP27 levels in atherosclerosis patients. The first study involving small patient cohorts (28 patients vs. 12 controls) noted significantly lower HSP27 serum levels in CVD patients compared to healthy controls (60). Two later studies in larger patient cohorts (76 patients vs. 53 controls; 80 patients vs. 80 controls) validated this finding, with CVD patients again displaying lower levels of serum HSP27 (62, 63). Moreover, our laboratory also demonstrated that in patients diagnosed with coronary atherosclerosis (>50% stenosis by angiography), lower levels of HSP27 was predictive of subsequent major clinical events (i.e., heart attack, stroke, cardiovascular death) over a 5-year period (63). However, in a prospective, nested, case–controlled study involving initially healthy female participants who later developed cardiovascular disease, baseline HSP27 plasma levels are inversely associated with age but neither significantly associated with other established cardiovascular risk factors nor with future cardiovascular events (121). These seemingly disparate findings regarding HSP27's potential as a marker in predicting cardiovascular events can be reconciled if one considers that all of the female subjects studied in the nested case–control series were, on average, 61 years of age at baseline and beyond menopause. Therefore, one would expect relatively low serum levels of HSP27 in both the controls and later CVD event patients at baseline. Moreover, at this age, coronary atherogenesis may have already reached a moderately advanced stage.

Hence, the results to date reveal that plasma HSP27 levels may indeed be a potential marker for atherosclerosis and predictive of adverse cardiovascular events. With further refinements in the HSP27 serum assay that will remove the confounding presence of HSP27 AAB, we are confident that this conclusion will be further validated. Additionally, diminished expression of HSP27 in atherosclerotic plaques compared to vessels free of disease further highlights the association between augmented HSP27 levels and protection against atherosclerosis.

Some investigators suggest that extracellular HSP27 may be subject to protease degradation that is more abundant in atherosclerotic compared to normal arteries. Indeed, it has been shown that plasmin is upregulated in atherosclerotic plaques and can cause the degradation and aggregation of recombinant HSP27 (rHSP27) as well as HSP27 endogenously secreted from arterial explants – findings reversed by aprotinin, a specific plasmin inhibitor (122). Other enzymes that are more abundant in vulnerable atherosclerotic plaques due to a proteolytic imbalance, like matrix metalloproteases (MMPs) (123), can also interact with (124, 125) and possibly degrade HSPs (126). Another idea to consider is that HSP27 (as well as other HSPs) could function as "danger signals" – highly abundant self-proteins normally found intracellularly in healthy tissues, which are released into the extracellular space when tissues are damaged, infected, or stressed. Such danger signals are capable of activating antigen-presenting cells (APCs) to initiate the adaptive immune response (127). Stress and damage associated with atheroprogression could thus release more HSP27 from cells into the circulation, resulting in the following possibilities: (i) less HSP27 expression in cells as plaque accumulates, (ii) proteolytic cleavage and degradation of extracellular HSP27, (iii) increased susceptibility to apoptosis of cells that have secreted HSP27, and (iv) activation/modulation of the immune response. Although the link between reduced HSP27 levels in the serum and atherosclerotic vessel walls is strong, it remains unclear whether HSP27 levels are a secondary feature of atherosclerosis or the stress response during atherogenesis, or if HSP27 plays a causal role in directly modulating vessel wall homeostasis.

## Immunological Contribution in Atherosclerosis

In order to understand how HSP27 is involved in atheroprogression and whether it could protect against development of CVD, the various stages and key cellular players involved in atherosclerosis must first be highlighted. Atherosclerosis is characterized

by the development in arteries of lesions or plaques containing lipids, inflammatory markers, and dead cells surrounded by a fibrotic cap structure. Some areas of the vasculature are more susceptible to plaque accumulation. For example, branch points in the circulatory tree experience altered laminar blood flow that could lead to atherosclerosis-promoting changes (128), including endothelial dysfunction, accumulation of oxidized low-density lipoproteins (ox-LDL), alterations in matrix protein composition, and accumulation of proteoglycans, which increase ox-LDL accumulation at the cellular level (129). Plaque accumulation can impair blood flow, causing health complications, and if left alone, can escalate to the grave consequence of plaque "rupture," exposing pro-thrombotic (pro-clotting) material from the plaque to the blood and causing occlusion of the artery. These local arterial blood clots deprive nearby cells of oxygen, leading to cell death and various adverse outcomes (depending on the rupture site), including heart failure, stroke, renal impairment, and/or hypertension (129).

The first stage of plaque formation involves the development of "fatty streaks." This gradual process originates in endothelial cells lining the inner arterial wall ("intima") and involves (1) the uptake and accumulation of circulating oxidized lipids and low-density lipoprotein (LDL), (2) expression of adhesion molecules, and (3) chemokine release, resulting in the recruitment and deposition of immune cells (mainly lipid-containing macrophages also known as foam cells and T-cells) along the intima (130). Fatty streaks do not give rise to overt symptoms and are reversible (129). It is interesting to note that the partial attenuation of HSP27 expression starts in this stage of atherogenesis (**Figure 3**) (108, 131).

Over time, fatty streaks become mature atherosclerotic plaques ("atheromas") distinguished by the following regions: (1) a core region, consisting of foam cells, lipids, immune cells, such as dendritic cells, mast cells, and B-cells (129), apoptotic cells, debris, cholesterol crystals (132–134), and proteases like plasmin (122); (2) a fibrous cap region surrounding the core, made up of smooth muscle cells (122) and a collagen-rich matrix; and (3) an interface between core and fibrous cap regions ("shoulder" region), housing mainly T-cells and macrophages (129) (**Figure 3**). Immunohistochemical analysis of atherosclerotic arteries obtained from patients undergoing cardiac surgery show HSP27 localization mainly in the fibrous cap and media (122); however, its abundance diminishes as the complexity and volume of the plaque increases (108).

The presence of immune cells early in plaque formation indicates the involvement of the immune response; however, it is still unclear whether this promotes or attenuates atherosclerosis. The canonical idea that atherosclerosis is primarily caused by dietary and lifestyle factors still carries weight in that the rise of cardiovascular diseases came about within the last century, coinciding with increasingly sedentary lifestyles and increased caloric and fat intake. Given that fatty streaks are found in young people, it appears to be a normal adaptive process to have an immune response generated against lipids in the arteries. However, for this response to promote atheroprogression likely involves an interplay between the presence of cholesterol (in plaques) derived from the diet and circulating immune cells that become resident in the arterial wall. Hence, the human body can be tipped into developing atherosclerosis if the source of cholesterol becomes dysregulated (dietary or genetic predisposition to the development of hypercholesterolemia) or if the immune system becomes abnormally sensitized to normal levels of lipid in the blood vessels, or both.

The immune cells primarily responsible for propagating the inflammatory response against lipids in atherosclerotic plaque are macrophages. Upon encountering lipids, particularly ox-LDL, macrophages engulf ox-LDL by binding to LDL membrane

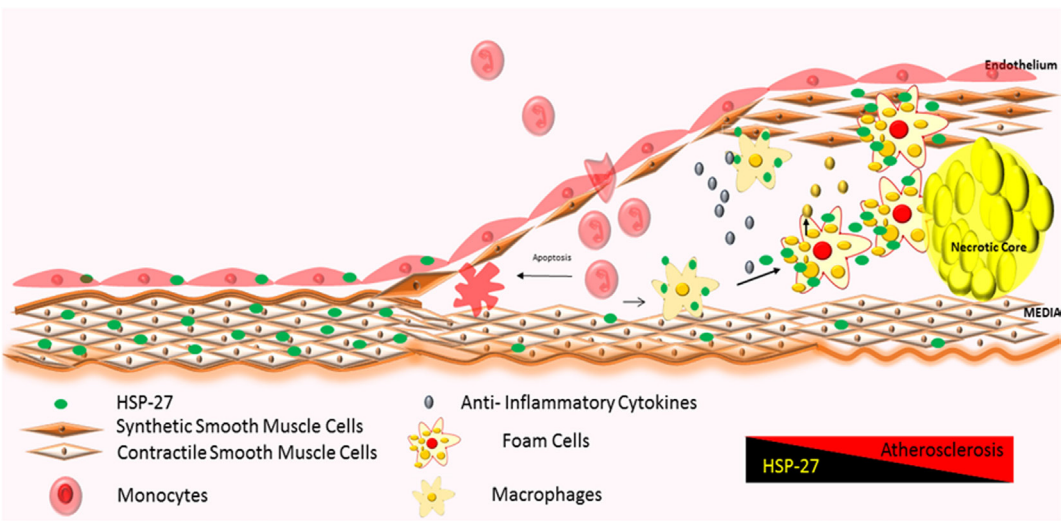


**FIGURE 3 | Atherosclerotic plaque progression: severity of atherosclerosis correlates with increased infiltration of monocytes and their local activation into macrophages and foam cells, further promoting the inflammatory process (128, 129).** HSP27 is expressed in normal areas of arteries, with reduction in expression in areas of advanced plaque (60, 61, 108, 120). Increases in HSP27 expression have also been noted in the fibrous cap and media (122).

receptors. Internalization of ox-LDL then triggers a cascade of events that transform macrophages into foam cells. Foam cells also secrete pro-inflammatory cytokines that result in the recruitment and activation of additional immune cells to the lesion site – further amplifying the immune response and promoting the development of complex, advanced plaques. Our previous results have shown that HSP27 competes with LDL binding to SR-A, known to take up atherogenic lipids, as well as downregulating SR-A expression (65, 135), thus attenuating foam cell formation. Furthermore, administration of recombinant HSP27 has been shown to block the differentiation of monocytes to dendritic cells, a cell type that can also promote the pro-inflammatory process (136). Another way that HSP27 can regulate the inflammatory response involved in atherosclerotic plaque progression is through the release of GM-CSF, which we have seen in cultured human macrophages, following rHSP27 treatment (103). Depending on the existing cytokine milieu, GM-CSF appears to promote the development of the M1 macrophage lineage (137), considered to play a role in the pro-inflammatory immune response (138). HSP27 may thus favor the preponderance of one macrophage phenotype over another, thereby affecting plaque progression.

Finally, another immune cell type involved in atheroprogression (albeit to a lesser degree) are T-regulatory cells, which mediate atheroprotection by amplifying the release of IL-10, thereby resulting in immune suppression (139–142). However, the role of HSP27 in T-regulatory function during atheroprogression is uncertain (143). Mast cells, a key cell type that populates atherosclerotic plaque, are classically known to react immediately to allergens *via* cross-linking of cell surface IgE resulting in degranulation, histamine release, and synthesis of arachidonic acid metabolites, which can activate stress-signaling and pro-apoptotic pathways (144). Mast cells have been shown to release proteases that can degrade HSP27 (145, 146).

## Potential Mechanisms Involved in HSP27-Mediated Atheroprotection

The key findings from our research group and others showing that (i) the loss of HSP27 serum levels correlates with plaque and disease progression and (ii) HSP27 is absent at sites of mature, complex plaque (while present in normal intima from the same patient with atherosclerosis), suggest that HSP27 could be atheroprotective. To address this possibility, mice prone to atherosclerosis ($ApoE^{-/-}$) (147) were crossed to transgenic mice overexpressing (human) HSP27 ($HSP27^{o/e}$) (65). Circulating HSP27 serum levels were higher in $HSP27^{o/e}$ $ApoE^{-/-}$ mice compared to $ApoE^{-/-}$ control mice and were inversely correlated with lesion area in both male and female mice (65, 148). More importantly, HSP27 overexpression protected mice from atherosclerosis, particularly in female mice (65) – a process that was abrogated by ovariectomy but rescued by administration of exogenous estrogens (66). Taken together, these data indicate that secretion of HSP27 into the circulation requires estrogens (66). Compared to $ApoE^{-/-}$ mice, $HSP27^{o/e}$ $ApoE^{-/-}$ mice exhibited reductions in cholesterol cleft areas, macrophage infiltration, apoptotic cell number in the plaques, and free serum cholesterol. Features indicative of favorable plaque remodeling (e.g., increased smooth muscle cell and collagen content and greater vessel stiffness) were also evident (148). Administering HSP27 to atheroprone mice *via* bone marrow transplant from $HSP27^{o/e}$ mice or recombinant HSP27 injections also reduced atherosclerotic lesions and increased plaque stability (63). These *in vivo* findings yielded several mechanistic possibilities underlying HSP27-mediated atheroprotection. First, the presence of estrogen appears to be important for HSP27's extracellular release. Second, HSP27 could modify various processes involved in atherosclerosis, including cholesterol homeostasis/trafficking, inflammation, immune cell infiltration into and migration out of plaques, activation of macrophages to foam cells, and plaque remodeling (**Figure 4**).

### Regulation of Cholesterol in Macrophages by HSP27

An interesting finding from the HSP27 overexpressing atherogenic mouse model is the reduction in cholesterol, in both plaque and serum (148), suggesting that HSP27 is involved in lipid homeostasis. One class of receptors known to bind to modified lipoproteins and facilitate their internalization in macrophages is SR-A (149, 150). It has been suggested that unregulated uptake of modified LDL in macrophages promotes the differentiation of monocyte-derived macrophages into pro-inflammatory foam cells, resulting in their accumulation in the lumen and, consequently, the narrowing and hardening of blood vessels (151, 152). *In vitro* experiments from our laboratory have shown that extracellular HSP27 affects SR-A activity *via* protein interactions on the surface of macrophages (65). This interaction reduces the binding of LDL to SR-A, and consequently, the formation of cholesterol-laden macrophages (65, 135). However, recent experimental data also support the role of HSP27 as a downregulator of SR-A protein expression (135). It has been shown that treatment of macrophages with extracellular recombinant HSP27 (rHSP27) is associated with reductions in both SR-A mRNA levels (−39%) and cell surface expression (−58%) as well as less foam cell formation – effects that occur *via* NF-κB signaling (135). Knockout of SR-A in $ApoE^{-/-}$ atheroprone mice abrogated the protective effects (reductions in atherosclerotic lesions) of HSP27, indicating HSP27 atheroprotection appears to depend, in part, on the presence of SR-A (135). Recent experiments in our laboratory indicate that HSP27 modulates cholesterol efflux in macrophages *via* ABCA1 and ABCG1 transporters (153). Thus, HSP27 has numerous, pleiotropic effects on cholesterol trafficking, modulating both import and export of cholesterol in macrophages (**Figure 4**).

### Modulation of the Inflammatory Response

Less atherosclerotic lesions in $HSP27^{o/e}$ $ApoE^{-/-}$ suggested that HSP27 can modulate the immune processes involved in atherosclerosis. One possibility lies in HSP27-mediated regulation of NF-κB activity in macrophages. NF-κB transcription factors regulate a vast number and diversity of gene targets, including those involved in cell proliferation, apoptosis, the cell stress response, inflammation, and both innate and adaptive immune responses (154, 155). Normally found in the cytoplasm as inactive dimers associated with IκB, NF-κB translocates to the nucleus once IκB is phosphorylated by the upstream IκB kinase complex (IKK), leading to the transactivation of numerous gene targets (154, 156).

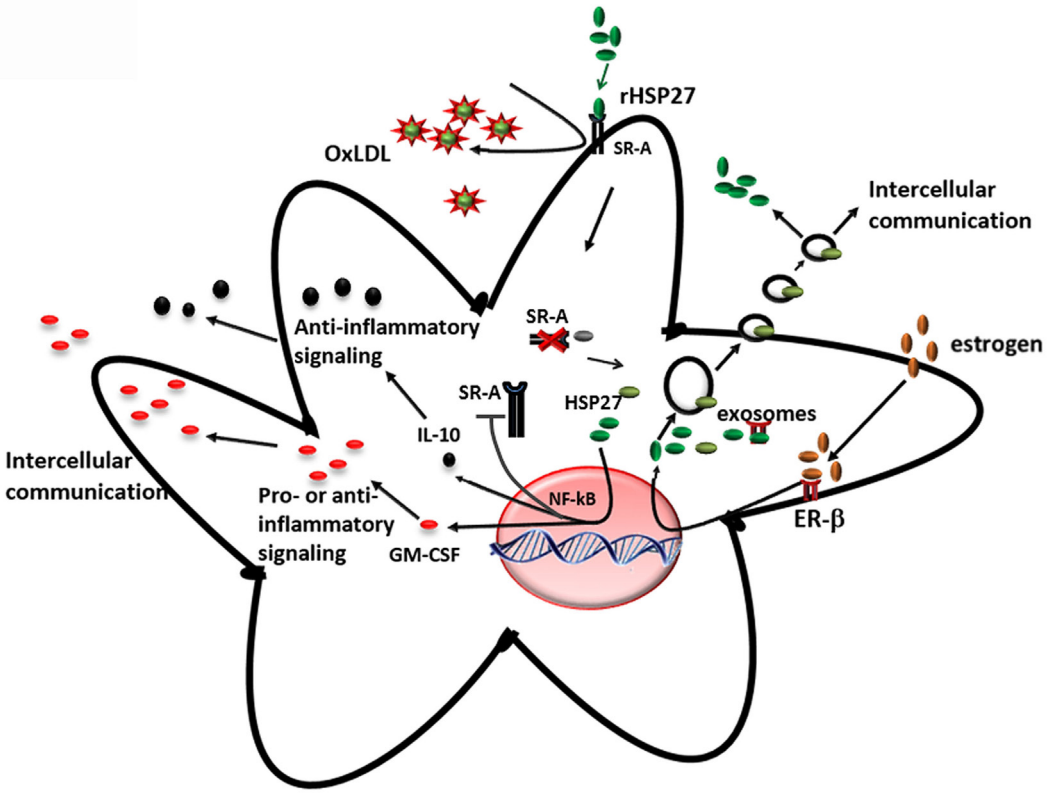


**FIGURE 4 | Extracellular HSP27, whose release is stimulated by estrogen, affects macrophages by (i) reducing ox-LDL uptake and (ii) activating NF-κB, which results in increased secretion of IL-10 and GM-CSF (65, 103, 135).**

NF-κB-dependent pathways may influence the inflammatory process in atherosclerosis. Various experimental models indicate that *intracellular* HSP27 attenuates NF-κB activation and signaling. In skeletal muscle disuse-mediated atrophy, the activation of NF-κB is reversed by HSP27 (157). Cardiac-specific overexpression of HSP27 was found to be protective against LPS-induced cardiac dysfunction in mice *via* inhibition of the NF-κB pathway (158). Also, heat shock treatment, which upregulated HSP27, was thought to protect against angiotensin II-induced inflammation *via* an inhibitory effect of HSP27 on the NF-κB pathway by reducing both phosphorylated and non-phosphorylated IKK-α (159, 160). However, several studies show the opposite effect of HSP27 on NF-κB. HSP27 overexpression enhances the 26S proteasome and augments the degradation of phosphorylated IκBα. As described previously, this leads to the activation and nuclear translocation of NF-κB, which the authors suggest is responsible for HSP27's downstream anti-apoptotic properties (161). More recently, Park and colleagues found that some pro-inflammatory cytokines, such as TNF-α, can enhance the binding of HSP27 to other NF-κB protein partners, such as IκB kinases (IKKs), and this interaction is promoted by the phosphorylation of HSP27 (162). While these studies all focused on *intracellular* actions of HSP27, whether *extracellular* HSP27 has an effect on the NF-κB pathway is starting to be explored.

Within atherosclerotic plaque, NF-κB is activated mainly in endothelial cells, smooth muscle cells, and macrophages. Based on the evidence supporting the role of HSP27 in the activation of NF-κB, and also the release of HSP27 from macrophages by estrogen (66, 135), it is apparent that extracellular HSP27 may also contribute to NF-κB signaling during atherosclerosis (**Figure 4**). Indeed, macrophages exposed to an extracellular recombinant HSP27 (rHSP27) consistently activated NF-κB through the degradation of IκBα. This effect was followed by a change in the transcriptional profile of some target genes coding for pro- and anti-inflammatory cytokines, such as IL-6, GM-CSF, TNF, or IL-10 (103), confirming the evidence that cytokines are implicated at various stages of atherosclerosis. Moreover, it was also shown that HSP27 attenuated ac-LDL-induced release of the pro-inflammatory cytokine, IL-1β, while increasing secretion of the anti-inflammatory cytokine, IL-10, in macrophages (65). Previous findings in primary human monocytes support this anti-inflammatory role, as extracellular rHSP27 significantly induced IL-10 production and only minimally upregulated TNF-α (107).

## Protection of the Endothelial Vasculature from Oxidative Stress and Apoptosis

Extracellular HSP27 may also protect the endothelium from various stress factors inherent in the vasculature. The vessel wall

and subendothelial space are continually exposed to stress from hemodynamic strain, as well as damaging factors within the blood itself (i.e., pro-inflammatory molecules, pathogens, carcinogens, and products of oxidative stress). Reactive oxygen species (ROS) can modify LDLs in the blood, turning it into ox-LDL, which has been shown to activate NADPH oxidase and xanthine oxidase, thereby inducing oxidative stress (163). Additionally, exposure of endothelial cells to LDL and ox-LDL increases the levels of the ROS species, $O^{2-}$, and NO (164, 165). During oxidative stress, HSP27 functions as an antioxidant in cells, lowering the levels of ROS by reducing the levels of intracellular iron and raising intracellular levels of glutathione (166). HSP27's presence in the circulation may therefore affect ox-LDL's contribution to atherogenesis on two levels – by competing with its uptake into macrophages (**Figure 4**) (65) and by lowering intracellular ROS production in endothelial cells, which would then curb the subsequent oxidative modification of LDL in the blood. An additional cardioprotective effect stems from the ability of HSP27 to protect the endothelium from ischemic insult by maintaining the integrity of cytoskeletal proteins (167–171). HSP27 can also preserve the endothelial barrier by functioning as a general anti-apoptotic protein, since downregulation of HSP27 in retinal capillary endothelial cells by both cytokines and siRNA increased apoptosis (172). Thus, HSP27-based treatment strategies – which could potentially prevent LDL oxidative modification as well as protect the endothelium/accelerate its repair – may be effective in reducing lesion formation. Although the majority of the above findings related to endothelium protection involve *intracellular* HSP27, recent results from our laboratory implicate *extracellular* HSP27 in the regeneration of the endothelial barrier (105). Using primary human EPCs treated with rHSP27 and mice overexpressing HSP27 ($HSP27^{o/e}$ mice), it was found that after vascular injury, re-endothelialization improved in both the experimental models, and neointima formation was decreased in $HSP27^{o/e}$ mice compared to wild type – effects partly mediated through the secretion of VEGF and its paracrine effect on EPCs (105).

## FUTURE DIRECTIONS

By mediating the immunological process of atherosclerosis (**Figures 3** and **4**) and attenuating disease symptoms and progression, HSP27 has considerable therapeutic potential in the treatment of cardiovascular diseases. Currently, our laboratory is considering several strategies to capitalize on the atheroprotective effect of HSP27 *via* its emerging role as an immune modulator.

The first and simplest approach is to administer HSP27 to patients. Indeed, as described above, in mice prone to developing atherosclerosis, subcutaneous injections of rHSP27 reduced lesion formation and total cholesterol levels (63). Although these preclinical findings in animal models are encouraging, several questions remain in the context of its application in the clinical setting – for example, what is the best route of administration (subcutaneous vs. intravenous injection vs. oral intake)? Also, what is the most effective formulation (the complete HSP27 protein vs. an HSP27 protein fragment)? An important caveat to keep in mind as well is that the administration of HSP27 may pose risks in the case of cancer, since *extracellular* HSP27 has been shown to promote (i) the transendothelial migration of primary tumor cells (49) and (ii) the differentiation of macrophages to phenotypes that tolerate human breast cancer cell progression (48). One must therefore carefully weigh the advantages and disadvantages concerning exogenous administration of HSP27 as a therapeutic strategy in atherosclerosis.

Another approach to consider is to harness the organism's inherent immunity when exposed to rHSP27. The discovery in our laboratory of HSP27 being complexed with AAB in the blood (thus confounding its detection through conventional ELISA kits) led to the development of a more sensitive, mass spectrometric-based detection assay, as well as mechanistic insight into how HSP27 exists/functions outside the cell. Our preliminary studies suggest that HSP27 AAB may, in fact, have salutary effects on HSP27 signaling and atheroprotection (67). The existence of AAB against HSPs in the context of atherosclerosis is not new – for example, antibodies generated against microbial HSP60 may cross-react with endogenous HSP60 expressed in the vessel lumen (molecular mimicry), enhancing inflammation and promoting atherosclerosis (173). HSP27 AAB are present in other disease states like cancer (174) and acute heart disease (175–177), although it is difficult to gage how levels correlate with disease. Higher antibody titers are associated with improved survival of breast cancer patients, but elevations also correlate with adverse conditions, such as metabolic syndrome (177) and the early phase of acute coronary syndromes (e.g., myocardial infarction and unstable angina) – although in acute coronary syndromes, HSP27 antibody levels rapidly fall after a brief spike (175). It is still unclear how HSP27 AAB are linked to CVD – one study correlated elevations with CVD (176), while other findings indicate the opposite, that HSP27 autoantibody titers (67, 178) are higher in healthy control subjects compared to CVD patients. Indeed, if the presence of HSP27 antibodies complexed to HSP27 promotes atheroprotection, immunization strategies, either active (vis-à-vis injection with an HSP27-derived antigenic peptide with or without adjuvant) or passive (administration of the antigenic peptide combined with already generated AAB), could provide exciting, new therapeutic possibilities.

Finally, there are alternative methods for boosting HSP27 function. For example, some microRNAs ("miRs," which are 20–22 nt-long non-coding RNA molecules that regulate gene expression by binding to nascent mRNA transcripts, targeting them for degradation), which downregulate HSP27 mRNA transcripts, can be targeted for degradation by its complimentary sequence, known as "miR inhibitor" or "anti-miR" (179, 180). Proof of this principle has been demonstrated in an insulin-resistance rat model, where decreased expression of miR-1 and miR-133 increased the level of HSP27, contributing to the loss of muscle adaptability (181). Our laboratory is currently investigating which miR species are specifically upregulated in atherosclerosis and whether these alter HSP27 levels; if so, there is a potential to target such miRs with anti-miR therapy in atherosclerosis patients. There are also endogenous methods of increasing levels of circulating HSPs – for example, a huge literature already exists correlating exercise with increased serum HSP levels (182). In light of a recent Finnish study linking frequency of sauna use with reduced risk of CVD (183), it appears that subjecting the body to periods of mild to

moderate heat stress may be very beneficial for the health. These sentiments – extolling the virtues of heat – are best expressed by a quote attributed to the ancient sage and physician Hippocrates: "That which drugs fail to cure, the scalpel can cure. That which the scalpel fails to cure, heat can cure. If the heat cannot cure, it must be determined to be incurable" (184, 185). By modulating atheroprogression through cross talk with the immune system, strategies that increase HSP27 levels are promising therapeutic approaches to consider.

## AUTHOR CONTRIBUTIONS

ZB – planning and organizing structure of the review; contributed to the sections (writing): introduction, background, extracellular HSP27, HSP27 in CVD, immunological contribution, mechanisms of atheroprotection, future directions; and planning and creation of figures. VV – contributed to the sections (writing): immunological contribution; planning and creation of figures. YL – planning and organizing structure of the review; contributed to the sections (writing): background, HSP27 in CVD, mechanisms of atheroprotection; planning and creation of figures. GK – planning and organizing structure of the review; contributed to the sections (writing): HSP27 in CVD, immunological contribution, mechanisms of atheroprotection; and planning and creation of figures. DA-O – contributed to the sections (writing): mechanisms of atheroprotection. CS – planning and organizing structure of the review; contributed to the sections: background, extracellular HSP27, HSP27 in CVD. EO – planning and organizing structure of the review; contributed to the sections writing/critically reviewing manuscript, planning the structure/outline; critical review of the manuscripts; and contributed to all sections (writing/editing).

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# Generation of the First TCR Transgenic Mouse with CD4+ T Cells Recognizing an Anti-Inflammatory Regulatory T Cell-Inducing Hsp70 Peptide

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Antigen-specific regulatory T cells (Tregs) directed at self-antigens are difficult to study since suitable specific tools to isolate and characterize these cells are lacking. A T cell receptor (TCR)-transgenic mouse would generate possibilities to study such antigen-specific T cells. As was shown previously, immunization with the mycobacterial heat shock protein (Hsp) 70-derived peptide B29 and its mouse homologs mB29a and mB29b induced anti-inflammatory responses. Furthermore, B29 induced antigen-specific Tregs *in vivo*. To study mB29b-specific Tregs, we isolated the TCR from T cell hybridomas generated against mB29b and produced a TCR transgenic mouse that expresses a MHC-class II restricted mB29b-specific TCR. These TCR transgenic CD4+ T cells were found to cross-react with the B29 epitope as identified with peptide-induced proliferation and IL-2 production. Thus, we have successfully generated a novel mouse model with antigen-specific CD4+ T cells that recognize self and bacterial Hsp 70-derived peptides. With this novel mouse model, it will be possible to study primary antigen-specific T cells with specificity for a regulatory Hsp70 T cell epitope. This will enable the isolation and characterization CD4+CD25+ Tregs with a proven specificity. This will provide useful knowledge of the induction, activation, and mode of action of Hsp70-specific Tregs, for instance, during experimental arthritis.

**Keywords: heat shock protein 70, transgenic mouse, autoimmunity, hybridoma, regulatory T cells**

## INTRODUCTION

Heat shock protein (Hsp) 70 is a ubiquitously expressed protein and plays a role as chaperone in protein folding, either after protein synthesis or under conditions of cellular stress (1, 2). Hsp70 is evolutionary conserved and is expressed by many species, including bacteria and vertebrates. This is reflected by a high degree of homology of Hsp between species. Interestingly, Hsps are also highly immunogenic, which might be explained by the fact that Hsps are found in bacteria that surround us. However, not only Hsp-derived peptides from bacteria are immunogenic but also peptides derived from self-Hsp can trigger immune responses (3, 4). Peptides derived from endogenous Hsp70 can not only be found in MHC class I molecules but are also known to be present in MHC

class II (5–8). This indicates that Hsp70-derived peptides can be recognized by immune cells in a MHC class II-dependent manner. This is supported by experiments in which CD4+ T cell responses against Hsp70-derived epitopes have been identified after immunization with bacterial Hsp70 (7). Apart from presentation during cellular homeostasis, endogenous Hsp can also be presented in MHC when it is upregulated during cellular stress such as heat shock (8, 9). Due to the high degree of homology of Hsp between species, cross-reactive responses occur in which foreign Hsp-peptide reactive T cells can recognize self-Hsp peptides (7, 8, 10, 11).

Interestingly, the administration of Hsp70 can result in anti-inflammatory responses, which has been shown by the suppression of disease in animal models for chronic inflammation, due to activation of Hsp70-specific regulatory T cells (Tregs) that are cross-reactive with self-epitopes of Hsp70 (4, 7). Tregs are a subset of specialized CD4+ T cells with high suppressive potential and are therefore important targets for immune modulation of inflammatory diseases (12). Therefore, activating these cells *via* Hsp peptides is a growing field of interest, especially in inflammatory diseases in which the disease-inducing antigens are unknown.

Previously, we have shown that mycobacterial Hsp70 peptide B29 is highly conserved and immunogenic. Immunization with, or intranasal administration of B29, activates B29-specific Treg *in vivo*, which are potent suppressors of experimental arthritis (7). Several tools, such as T cell lines and T cell hybridomas, have been used to study Hsp-specific T cell responses in the past. For instance, T cell lines generated from mycobacterial Hsp60 immunized rats specific for a highly conserved sequence of Hsp60 have been used to study the suppressive potential of Hsp-specific T cells (10). Similar results were obtained with T cell lines generated from mycobacterial Hsp70 immunized rats: Hsp70-specific T cells reduced the severity of arthritis in the experimental arthritis model (13). However, none of these systems allows the evaluation of primary T cell responses (*in vivo)*, the behavior of Hsp70-specific Tregs upon activation, or the induction of particular T cell subsets such as effector T cells and Tregs after administration of Hsp70. Especially, antigen-specific Tregs are difficult to study since these cells comprise only a small fraction of the total T cell population and are difficult to culture and maintain *in vitro* (14).

Therefore, we aimed to generate a mouse model to study primary and naive Hsp70-specific CD4+ T cells in more detail. For that reason, we isolated the T cell receptor (TCR)-α and TCR-β chain genes from a T cell hybridoma generated against peptide mB29b, a mammalian homolog of B29 (**Table 1**). This hybridoma was found to cross-react with B29 and another mammalian homolog: mB29a (9). With the TCR-α and TCR-β chain genes, we generated a TCR transgenic mouse with Hsp70 peptide-specific CD4+ T cells. We show that CD4+ T cells from the mB29b-TCR transgenic mouse undergo antigen-specific proliferation and produce IL-2 after restimulation with B29 or its mouse homologs. In future studies, primary CD4+ T cell responses directed against self and bacterial Hsp70 peptides can be investigated *in vitro* and *in vivo*. Particularly, the activation and differentiation of antigen-specific CD4+ Tregs can be studied with this model, which are not possible with long-term T cell lines or T cell hybridomas.

# MATERIALS AND METHODS

## Mice, Peptides, and Antibodies

Female Balb/c mice aged 8–12 weeks were purchased from Charles River and used as cell donors to create hybridomas and as source of APCs for coculture assays. Animals were kept under standard conditions at the animal facility, and all experiments were approved by the Animal Experiment Committee of Utrecht University. Mice strains used for the generation of the mB29b-TCR transgenic mouse were F1 of (CBA × C57BL/6) mice (Charles River). Hsp70-derived peptides (mB29a, mB29b, B29) were identified previously (7) and were obtained from GenScript Corporation. The amino acid sequences and origin of the peptides are shown in **Table 1**. Anti-MHC-II (I-Ad/I-Ed) antibody (clone M5/114) 5 μg/ml; gift from Louis Boon from Bioceros B.V., Utrecht, The Netherlands was used to block MHC-II-peptide TCR interactions in cocultures. To stain mB29b-specific cells, an APC-labeled murine – mB29b-specific tetramer that is composed of mB29b [VLRIINEPTAAAIAY linked to I-A(d)(BALB/c haplotype-matched MHC class II molecule)] was used for 90 min at 20 μg/ml. This tetramer is a gift from NIH Tetramer Core Facility (Emory University, Atlanta, GA, USA).

## Generation of mB29b-TCR Hybridoma (LHEPs)

CD4+ T cell hybridomas (named LHEPs) were generated against Hsp70 peptide mB29b in our laboratory, as described previously (9). Activation of specific hybridoma clones by mB29b was addressed by incubating hybridoma cells ($2 \times 10^4$/well) with irradiated (10,000 rad) A20 B lymphoma cells as APC ($2 \times 10^4$/well) loaded with Hsp70 peptides in 96 wells flat bottom plates for 48 h and pulsed with $^3$H-thymidine (0.4 μC/well) for an additional 16 h to measure activation-induced cell death (AICD).

As a positive control, hybridomas were stimulated with 2 μg/ ml ConA. After 48 h coculture, supernatants were harvested and frozen at −20°C. IL-2 production by hybridomas was studied by culturing the harvested supernatants with IL-2 responder CTLL-16 cells (cytotoxic T cell line; $5 \times 10^3$/well) for 24 h. The CTLL-16 cells were pulsed with $^3$H-thymidine (0.4 μC/ well) for another 16 h (Amersham Biosciences Europe GmbH, Roosendaal, The Netherlands). The 5/4E8 hybridoma specific for

**TABLE 1 | Amino acid sequences of Hsp70 peptides.**

| Peptide | Sequence | Protein | Origin |
|---|---|---|---|
| mB29a | VLRVINEPTAAALAY | Hspa9 (GRP75) | *Mus musculus* *Homo sapiens* |
| mB29b | VLRIINEPTAAAIAY | Hspa1a, Hspa8 | *Mus musculus* *Homo sapiens* |
| a1a-long29 | DAGVIAGLNVLRIINEPTAAAIAYGLDRTGK | Hspa1a (Hsp72) | *Mus musculus* *Homo sapiens* |
| a8-long29 | DAGTIAGLNVLRIINEPTAAAIAYGLDKK | Hspa8 (Hsp70) | *Mus musculus* *Homo sapiens* |
| B29 | VLRIVNEPTAAALAY | DnaK (Hsp70) | *Mycobacterium tuberculosis* |

*The amino acid sequence and origin of the Hsp70-derived peptides used in this study.*

proteoglycan (PG)-derived peptide PG70-84 (15) was used as a control. RNA from the mB29b-TCR hybridoma was sequenced to determine the TCR usage. Sequencing revealed that the isolated mB29b TCR consisted of Vα 7 (TRAV-7.01-J26.01 in the IMGT nomenclature) and Vβ 8.2. The Vβ 8.2 is according to the NCBI nomenclature, this correlates with TRBV-13-2.01-D2.01-J2-7.01 in the IMGT nomenclature.

## MHC Restriction of T Cell Hybridomas

The MHC restriction of the peptide mB29b-specific hybridomas was determined using mouse anti-I-Ad (BD PharMingen) mAb. Hybridoma cells were cultured with A20 APCs in the presence of 0.5 μg/ml peptide mB29b and anti-I-Ad mAb (MKD6) at a concentration of 20 μg/ml. The effect of mAb on IL-2 production was determined with the CTLL-16 bioassay, as described above.

## Cloning of the αβ TCR

Total RNA was isolated from the mB29b-TCR hybridoma cells by extraction with RNAzol (Invitrogen). The oligo(dT)$_{12–18}$ primer from the Superscript Reverse Transcription kit (Invitrogen) was used for reverse transcription of the isolated RNA. To express the TCR mB29b genes in the transgenic mice, we cloned the Vα and Vβ genes into pTα and pTβ cassettes obtained from Kouskoff et al. (16). Isolation of genomic DNA from the mB29b-TCR hybridoma was performed to obtain full length rearranged TCRα and TCRβ DNA, including leader and intron sequences. mB29b-TCR DNA was amplified by PCR using the primer for the Vα chain (Forward: TRAV7-1-Xmal: *TAAT*CCCGGG*AGAATGAAGTCCTTGTGTGTTTCAC*, Reverse; TRAJ26.01-NotI: *TAAT*GCGGCCGC*ACAGTAGACCTCAGGTCCCCCTCAC*) and the Vβ chain (Forward: TRBV13-2.-1-XhoI:*TAAT*CTCGAG*AAGATGGGCTCCAGGCTCTTC*;Reverse:TRBJ2-7.01-*Sac*II:*TAAT*CCGCGG*CCTGGTCTACTCCAAACTACTCC*). The PCR products of the two fragments were cloned using TA overhang into the pGEM-T easy vector (Promega). The constructs were subsequently introduced into *E. coli* DH5α. The *Xma*I and *Not*I released DNA fragment, containing the TCRα chain, was cloned into the pTα cassette. The *Xho*I and *Sac*II DNA fragment, containing the TCRβ chain, was cloned into the pTβ cassette. Both were transfected into XL10 gold cells (Stratagene) by electroporation.

## *In Vitro* Expression of the αβ TCR

The pTα cassette, the pTβ cassette, and the pcDNA3 plasmid (containing neomycin resistance gene) were electroporated into the mouse 58α$^-$β$^-$ T cell hybridoma that lacks functional TCR chains (17). Transfected cells were cloned using limiting dilution in 96 wells plates using the FACS Vantage (BD) and cell lines were cultured in the presence of Geneticin 418 (0.8 mg/ml). PCR was used to validate DNA incorporation and transfected cells were tested for antigen specificity in a similar manner as the hybridomas (described above).

## Generation of the mB29b-TCR Transgenic Mouse

T cell receptor transgenic mice were generated in our laboratory, as described previously (15, 17, 18). The pTα mB29b-TCR and the pTβ mB29b-TCR plasmids were linearized using *Sal*I (pTα) and *Knp*I (pTβ). *Via* pronuclear injection a mixture of the plasmids were introduced into fertilized eggs of F1 (CBA × C57BL/6) mice. Two mB29b-TCR transgenic founders were identified by PCR analysis of genomic DNA (same primers as described above). Founder 2 was mated with Balb/c mice (Balb/cBYJRj; Jackson laboratories), and offspring was tested for peptide specificity, as described below.

## Measurement of Antigen-Specific T Cell Responses from mB29b-TCR Mice

Blood was taken from founders and depleted from erythrocytes with ACK lysis buffer ($H_2O$ containing 150 mM $NH_4Cl$, 10 mM $KHCO_3$, and 0.1 mM EDTA, pH 7.2–7.4). Blood cells (founder 1: $1 \times 10^5$, founder 2: $5 \times 10^5$, depending on cell yield after blood collection) were cultured for 96 h with $1 \times 10^6$ irradiated A20 cells as APCs. Cells were stimulated with 2 or 20 μg/ml B29 or with 5 μg/ml ConA as a positive control. Peripheral blood lymphocytes (PBLs) from founders were tested for antigen-specific responses to 2 or 20 μg/ml mB29a, mB29b, or B29 peptides. Proliferation was determined by $^3$H-thymidine incorporation during the final 16 h of culture, and IL-2 production was determined by Luminex. Splenocytes from offspring were screened for the expression of TCRα and TCRβ chain. The mB29b-TCR positive splenocytes were also tested for antigen specificity.

## Flow Cytometric Analysis

Single cell suspension of splenocytes, lymph node cells, or thymocytes were made, and these were stained with antibodies CD3-APC (OKT-3, BD Biosciences), CD4-V450 (RM4-5, eBioscience), CD8-V500 (RPA-T8, BD Biosciences company), Vβ8-PE (F23.1, BD Biosciences) KI-67-PerCp-Cy5.5 (BD56, BD biosciences), CD25-PerCp-Cy5.5 (PC61.5, Ebioscience), IFN-γ-FITC (XMG1.2, BD biosciences), CD44-APC (IM7, ebioscience), CD62L-FITC (MEL-14, BD biosciences) or FoxP3-eFluor450 (FJK-16s, ebioscience) and incubated for 30 min at 4°C. Cells were washed three times with PBS containing 2% FCS. Cells were acquired on the FACS Canto II (BD) and analyzed with FlowJo 7 (Tree Star). For cell activation experiments, splenocytes from transgenic mice or littermates were cultured ($1 \times 10^5$ cells/well) for 24 h in the presence of 20 μg/ml mB29b, in which the last 4 h was in the presence of 1 μg/ml Brefeldin A.

## Histology

For histology, thymus, spleen, inguinal lymph nodes (iLN; representative draining LNs), and liver were isolated from mB29b-TCR positive mice, or negative littermates. Tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, and 5 μm saggital sections were stained with hematoxylin and eosin (H&E). Immunohistochemistry was performed to T cells and general proliferation in lymphoid tissues. Briefly, cryosections (5 μm) were fixed in ice-cold acetone and blocked against endogenous peroxidase with 0.3% hydrogen peroxide in methanol. Non-specific staining was blocked with a 1% BSA solution, and sections were incubated with primary antibodies against CD3 (BD Biosciences) or Ki-67 (BD Biosciences). Secondary staining was performed with an anti-rat HRP antibody (Millipore), and Peroxidase activity was developed using the DAB Peroxidase Substrate kit (Vector

Laboratories). Sections were counterstained with hematoxylin and mounted with Aquatex (Merck, Darmstadt, Germany). Pictures were taken using an Olympus BX41 microscope and analyzed with Cellsens entry 1.9 software (Olympus Corporation).

## Statistics

Unless stated otherwise, data are expressed as mean ± SD. Statistical analyses were carried out using Student's *t*-test or the two-way ANOVA test using Prism software (Version 6.05). $p \leq 0.05$ was considered significantly different.

# RESULTS

## MHC Class II Restricted Recognition of Hsp70 Peptides mB29a, mB29b, and B29 by mB29-TCR Hybridoma LHEP4

T cell hybridomas specific for Hsp70 peptide mB29b were generated by immunizing BALB/c mice with the peptide mB29b and fusing splenocytes from these mice with BW5147 cells followed by limiting dilution to obtain individual clones of the mB29b-TCR hybridomas. In order to test the TCR specificity of the generated hybridomas, which we named LHEPs, the cells were cultured in the presence of A20 cells as APCs and peptide mB29b, or its homologs mB29a and B29 (**Figure 1**). AICD and IL-2 production (9), two characteristics of an activated hybridoma, were measured for seven LHEPs. The AICD was determined by $^{3}$H thymidine incorporation, and the IL-2 production was measured by IL-2-dependent proliferation of the CTLL-16 cell line. Data of LHEP4 are depicted as a representative example in **Figure 1**. As seen in **Figure 1**, stimulation of LHEP4 with mB29a, mB29b, and to some extend with B29 resulted in cell death and IL-2 production. To determine whether recognition of Hsp70 peptides was MHC class II restricted, LHEPs were stimulated with peptide in the presence of an MHC class II blocking antibody. In this case, no AICD and a strongly reduced IL-2 production was seen upon stimulation with Hsp70 peptides, indicating that peptide recognition of mB29b-TCR hybridomas was indeed MHC class II restricted (**Figures 1A,B**). The mB29b peptide was presented in the context of I-Ad molecules, since a mAb against I-Ad (MKD6) completely abrogated the mB29b-specific *in vitro* proliferation of the LHEP4 hybridoma (data not shown).

Screening of the obtained selected mB29b-specific T cell hybridomas revealed seven LHEPs in total that were responsive to mB29b, of which five were cross-reactive to mB29a, and four that were cross-reactive to B29.

## LHEPs Respond to Several Length Variants of the Hsp70 Peptide

We next determined a dose response of LHEP4 to the Hsp70 peptides, as well as recognition of specific length extension variants of mB29b to assess the response to processed peptides. For this, LHEP4 was cocultured with irradiated splenocytes in the presence of the extended hspa8 (=Hsc70) or hspa1a (=Hsp72) (19) variants of the mB29b peptide, later referred to as a8-long29 and a1a-long29. CTLL-16 proliferation induced by IL-2 production from LHEP4 indicated that these length variants could be recognized (**Figure 2A**). Although IL-2 production decreased in cultures in which LHEP4 was stimulated with low amounts of mB29b or B29 (data not shown), CTLL-16 proliferation

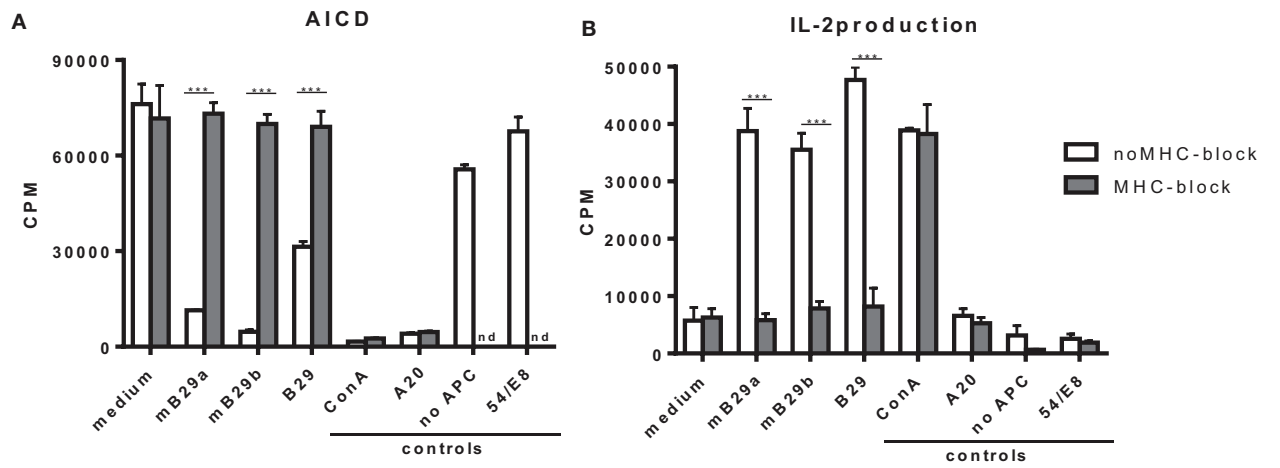
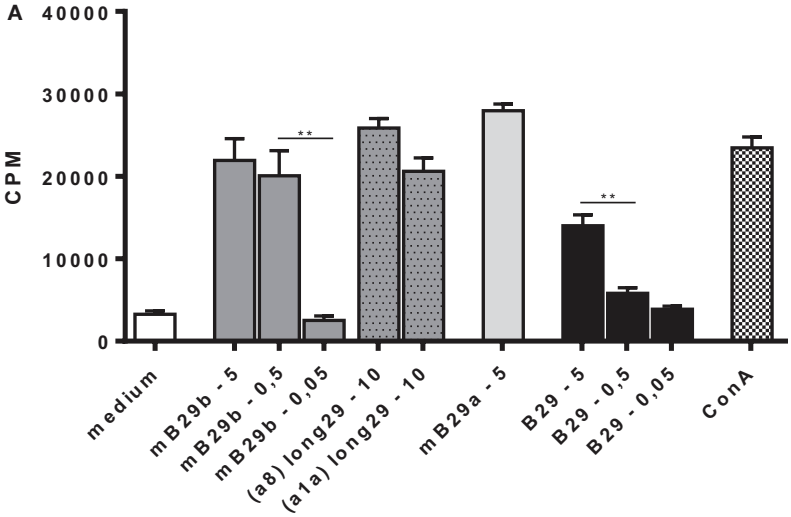



**FIGURE 1 | The hybridoma LHEP4 recognizes Hsp70 peptides mB29a, mB29b, and B29 in an MHC class II restricted way**. Splenocytes from BALB/c mice immunized with mB29b were fused with BW5147 cells, which resulted in mB29b-specific CD4$^{+}$ T cell hybridoma clones (LHEPs). **(A)** Activation-induced cell death (AICD) is measured by reduced thymidine incorporation upon activation with mB29a, mB29b, and B29. MHC-II blockage results abrogate AICD, which indicate that the mB29b-TCR hybridomas are MHC-II restricted. **(B)** IL-2-dependent CTLL-16 proliferation was measured to demonstrate that supernatant of activated hybridomas as depicted in **(A)** produce IL-2. As shown in **(B)**, IL-2 production is present after stimulation with mB29a, mB29b, and B29. MHC-II blockage results in a diminished IL-2 production. As a positive control, cells were stimulated with ConA. As a negative control, LHEP4 hybridoma cells were cultured in medium only, in the presence of mB29b (no APC). Additional controls included A20 cells cultured with mB29b, or the 5/4E8 hybridoma cultured in the presence of mB29b peptide. $^{3}$H-thymidine incorporation is shown as mean of triplicate samples/well ± SEM. Data shown are representative of three independent experiments.

A




B

| | mB29a | mB29b | (a8) long29 | (a1a) long29 | B29 |
|---|---|---|---|---|---|
| LHEP 4 | + | + | + | + | + |
| LHEP 7 | + | + | + | + | + |
| LHEP 8 | - | + | + | - | + |
| LHEP 9 | + | + | +/- | - | - |
| LHEP 11 | + | + | + | + | + |
| LHEP 14 | + | + | +/- | - | - |
| LHEP 21 | + | + | + | + | + |

**FIGURE 2** | **The mB29b-TCR hybridoma LHEP4 produces antigen-specific IL-2 upon coculture with primary antigen-presenting cells**. **(A)** LHEP4 was cocultured with irradiated splenocytes loaded with different concentrations [ranging from 5, 0.5, to 0.05 μg/ml, as well as 10 μg/ml length variants of mB29b (hspa8 = Hsc70 or hspa1a = Hsp72)] (20) of Hsp70 peptides as indicated, after which supernatants were collected. CTLL-16 cell cultures were supplemented with supernatants from these stimulations to determine IL-2-dependent proliferation of CTLL-16 cells. As a control, cocultured LHEP4 cells were unstimulated, or stimulated with ConA. $^{3}$H-thymidine incorporation is shown as mean of triplicate samples/well ± SEM. Data shown are representative of three independent experiments. **(B)** mB29-TCR hybridomas were stimulated with Hsp70 peptides mB29a, mB29b, or B29, as well as length variants of mB29b [hspa8 = Hsc70 or hspa1a = Hsp72 (20), as described in **(A)**]. The + symbol (cpm >15,000) indicates AICD and IL-2 production upon coculture with supernatants from peptide-stimulated LHEPs. Weak responses are depicted as ± (on average the cpm = 4000). Medium stimulated samples show on average a cpm of 1500. Data shown summarize three independent experiments.

remained detectable, indicating that LHEP4 responds sensitively to presented Hsp70 peptides (**Figure 2A**).

Apart from LHEP4, the previously selected T cell hybridomas were screened for the recognition of Hsp70 peptide (length variants) (**Figure 2B**). All LHEPs responded to the mB29b peptide when presented by different primary APC, indicating that the mB29b-TCR hybridomas recognize Hsp70 peptides presented by APCs from various sources (data not shown). As was previously seen (**Figure 2B**), not all LHEPs were cross-reactive to other peptides, including the length variants of mB29b (hspa8 or hspa1a). All mB29b-TCR hybridomas that recognize different length variants of mB29b provide a broad recognition spectrum.

## Cloning of the TCRα and TCRβ Chain from mB29b-TCR Hybridoma LHEP4 into TCR$^{-}$ Cells Results in Hsp70 Peptide-Specific Transfectants

Based on the specificity and the strong cross-reactive responses of LHEP4, the TCRα and TCRβ chain of this mB29b-TCR

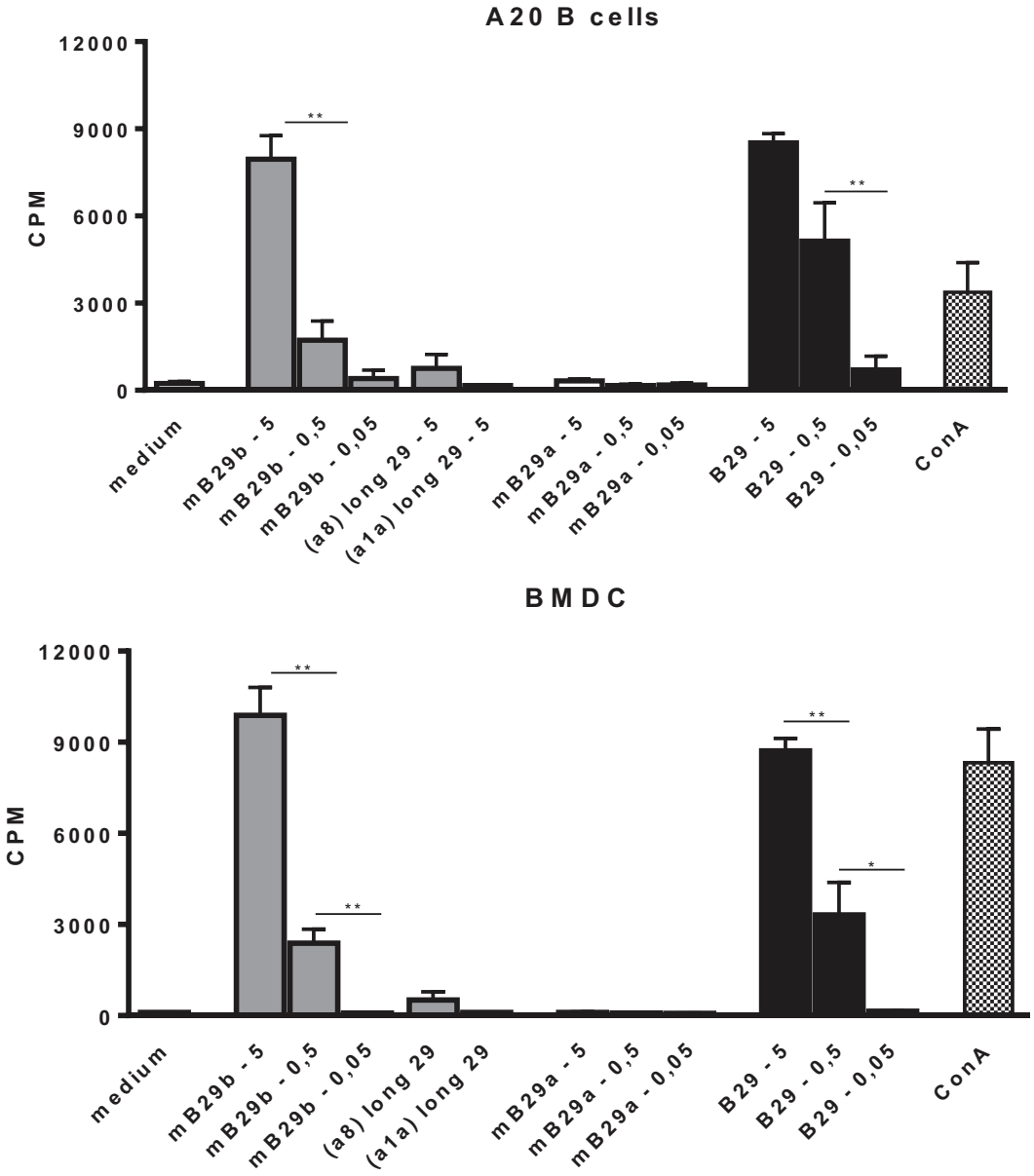



**FIGURE 3 | Cloning of the TCRα and TCRβ chain from mB29b-TCR hybridoma LHEP4 into TCR$^-$ cells results in Hsp70 peptide-specific transfectants**. Cloned transfected cells were cocultured with A20 B cells **(A)** or BMDCs **(B)**, which were loaded with 5, 0.5, or 0.05 μg/ml Hsp70 peptides. After stimulation with Hsp70 peptides mB29a, mB29b, or B29, as well as length variants of mB29b (a1a or a8), supernatants were added to CTLL-16 cells and IL-2-dependent proliferation was determined after 24 h. The graph shows more IL-2-dependent proliferation of CTLL-16 cells when stimulated with a high concentration Hsp70 peptide mB29b or B29. There is no response to stimulation with mB29a and to a minor extent to the length variants of mB29b (a8 or a1a). Similar results were obtained with the BMDC coculture **(B)**. As a control, cocultured LHEP4 cells were unstimulated or stimulated with ConA (data not shown). $^3$H-thymidine incorporation is shown as mean of triplicate samples/well ± SEM. Data shown are representative of two independent experiments.

hybridoma were cloned into TCR expression vectors, which were transfected into cells lacking the TCR (TCR$^-$). The transfected cell line was cocultured with BMDC or irradiated A20 B cells, which were loaded with Hsp70 peptides to confirm the antigen specificity of the transfectant. Results showed a dose-dependent response to mB29b and B29, but not to mB29a (**Figure 3**). Irradiated thymocytes and splenocytes were also used as APC and gave similar proliferative responses (data not shown). Furthermore, addition of shorter variants of the mB29b peptide to LHEP4 and the transfected cells, failed to stimulate the mB29b-specific hybridoma and cells indicating the specificity of the hybridoma and transfected cells (data not shown). Together, these data confirmed that the TCRα and TCRβ chain were successfully cloned into TCR expression vectors, and therefore we transferred the TCRα and TCRβ chain constructs to mouse oocytes *via* pronuclear injection.

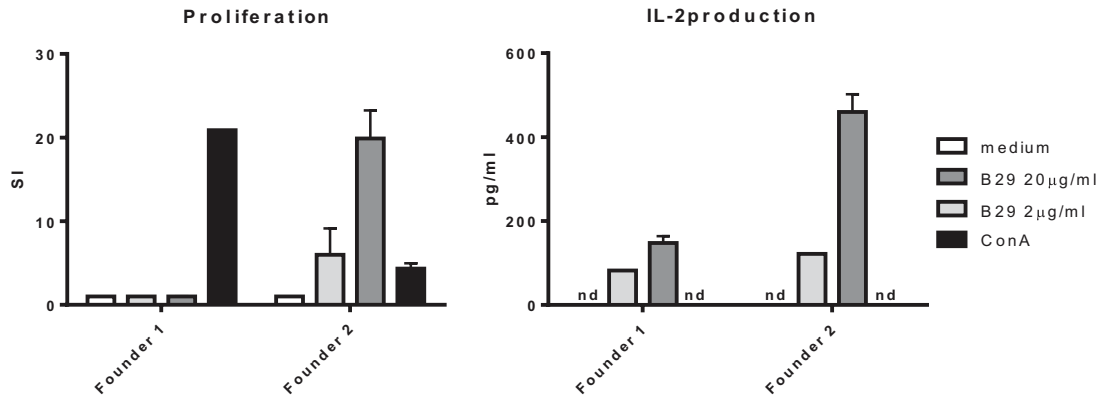



**FIGURE 4 | Antigen recognition by mB29b-TCR transgenic mouse**. PBLs from two founders, positive for the TCRα and TCRβ chain, were cultured for 96 h with irradiated A20 B cells and stimulated with 2 or 20 μg/ml B29, or 5 μg/ml ConA as a positive control. PBLs from founder 2 proliferated in a dose-dependent response. The PBLs from founder 1 did not show any proliferation after B29 stimulation. The PBLs from both founders produced IL-2 but the PBLs from founder 2 produced higher amounts. Proliferation was determined by $^{3}$H-thymidine incorporation during the final 16 h of culture and IL-2 production was determined by Luminex. Data are from one experiment.

## Antigen Recognition of Cells from mB29b-TCR Transgenic Mouse

After pronuclear injection of the DNA expressing TCRα and TCRβ into the donor zygote, the zygote was injected in a foster mouse from which several pups were born that had incorporated the constructs. From these mice, two founders were positive for both constructs with PCR (data not shown). PBLs from the two founders were stimulated with B29 in a coculture with irradiated splenocytes. PBLs from one founder proliferated and produced IL-2 in response to B29 stimulation (**Figure 4**). Next, the positive founder was mated with Balb/c mice and F1 mice were screened for the expression of the TCRα and TCRβ chain and splenocytes from mB29b-TCR positive offspring were tested for antigen specificity and compared to negative littermates. We observed responses to mB29b, B29, and mB29b length variant (a8) long29 (**Figure 2B**), while mB29b-TCR negative littermates showed no response to any of the peptides tested (data not shown). These data show that we successfully generated mB29b-TCR transgenic mice with cells with a functional TCR that recognized Hsp70 peptides.

## Flow Cytometric Analysis of mB29-TCR Transgenic Mouse Tissues

Next, we examined the presence of CD3$^+$, CD4$^+$, CD8$^+$ T cells in thymus of the mB29b-TCR transgenic mouse. Since the mB29b-TCR hybridoma was recognized by the antibody directed against Vβ8, we also screened the thymus for Vβ8$^+$ T cells. The transgenic mice and littermates had a similar frequency of CD4$^+$ cells, whereas the transgenic mouse had an increased percentage of Vβ8$^+$ T cells in the thymus and a decreased amount of CD8$^+$ cells (**Figure 5A**). In the spleen, the same differences in CD4$^+$ T cell and CD8$^+$ T cell distribution (more CD4$^+$ T cells compared to a wild type mouse) were observed, as well as an increased number of Vβ8$^+$ T cells were detected (**Figure 5B**). This resulted in changes in the CD4:CD8 ratio in both the thymus and spleen. The increased CD4:CD8 ratio became more evident in later generations. We observed a similar distribution and percentage of T cells in the LN (data not shown). To test the specificity of the CD4$^+$ T cells in the mB29b-TCR Tg mouse, we stained splenocytes with an APC-labeled murine – mB29b-specific tetramer that is composed of mB29b [VLRIINEPTAAAIAY linked to I-A(d)] and also cultured splenocytes in the presence of mB29b for 24 h. As shown in the lower part of **Figure 5B**, the mB29b TCR Tg mouse contains more tetramer-specific CD4$^+$ T cells than the WT Balb/c and the negative littermate. **Figure 5D** demonstrates that after 24 h of culture in the presence of mB29b, more CD4$^+$ T cells are activated (IFN-γ and CD25 expression) in the spleen of the transgenic mouse, in comparison with the negative littermate. Furthermore, also the CD4$^+$CD25$^+$FoxP3$^+$ population is more pronounced in the mB29b TCR Tg mouse compared to the negative littermate, while in naive mice, FoxP3 expression is lower in mB29b Tg mice (**Figure 5C**).

## mB29b-TCR Transgenic Mice Show an Increase in Naive Cells

To investigate cell distribution and activation, both histology and flow cytometry were used. Tissue sections from thymus, spleen, iLN, and liver were made and stained for H&E or CD3 (**Figure 6**). Based on the H&E stained tissue slides, no apparent changes are observed in iLN, spleen, thymic, or liver tissue architecture (data not shown). In addition, the distribution of CD3$^+$ T cells in the different lymphoid tissues was comparable between the TCR transgenic mouse and the littermate (**Figure 6A**). However, we did find a difference in proliferative activity, as based on the Ki-67 expression in splenocytes (**Figure 6B**). The KI-67 positive population is reduced in the mB29b TCR Tg mouse when compared to the wild type mice and negative littermates. This correlates with the enhanced non-proliferative naive cell population (defined as CD62L$^{hi}$CD44$^{low}$) in naive mB29b TCR Tg mice (**Figure 6B**). Overall, histological analysis revealed no major differences in

T cell distribution or activation between the founder and control littermates in lymphoid organs, suggesting no development of gross pathology.

## DISCUSSION AND CONCLUSION

Investigating Hsp-specific T cell responses *in vivo* and *in vitro* is of great interest to examine the effect of Hsp administration on T cell activation and differentiation. Although there are Hsp-specific cell lines that have been generated after long *in vitro* culture (10, 20), as well as Hsp-specific T cell hybridomas (9), these cells lack the properties of primary T cells. A huge disadvantage of these cells is that they lack the ability to differentiate from naive cell to effector or Treg and can therefore only be used for qualitative analysis of Hsp recognition. Several studies have shown that the immunomodulatory effect of Hsp administration (being immunization, intranasal administration, or oral administration) is due to the activation of Hsp-specific Tregs (7, 21–23). However, little is still known about the function of these cells. For instance, it is difficult to study Tregs *in vitro*, since these cells require more than peptide stimulation alone (e.g., growth factors like IL-2 and/or TGF-β) for their expansion and differentiation (24, 25), in comparison to immortalized T cell lines. Eventually, one would like to study primary antigen-specific T cells; however, these are only a minor population within the total population of T cells. Therefore, a TCR transgenic mouse is a valuable tool to obtain larger quantities of antigen-specific T cells. As a result, we set out to generate a TCR transgenic mouse with Hsp70 peptide-specific T cells to establish a tool to study the activation, differentiation, and suppressive capacity of Hsp-specific T cells.

In this study, we have successfully cloned an mB29b-TCR hybridoma with specificity for the Hsp70 peptide mB29b. Due to sequence homologies of Hsp70 family members, this hybridoma can recognize self-Hsp peptides mB29a and mB29b, and the mycobacterial Hsp70 peptide B29. Recognition was MHC class II dependent, and all APCs tested were capable of activating the mB29b-TCR hybridoma (**Figures 1** and **2**). Next, the TCRα and TCRβ chain were isolated and cloned into TCR expression vectors, which were electroporated into TCR$^-$ T cells, which showed peptide-specific activation upon electroporation of the construct

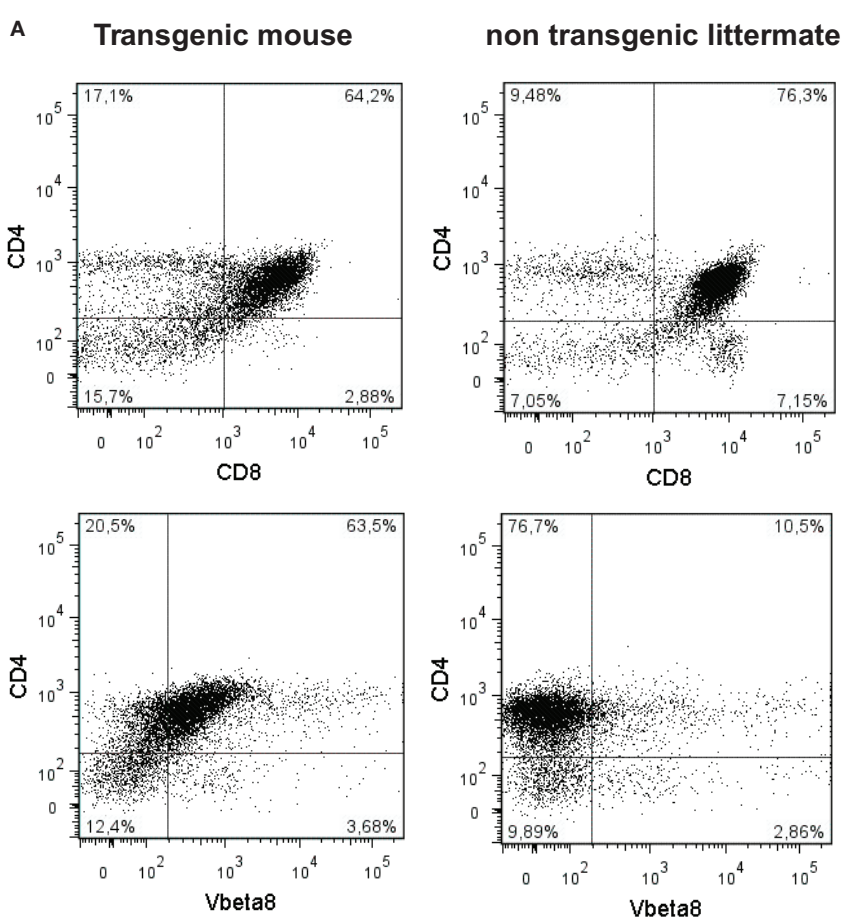



**FIGURE 5** | **Continued**

(**Figure 3**). Although the hybridomas did show a response to mB29a (**Figures 1** and **2**), the TCR$^-$ cells in which the expression vectors from hybridoma LHEP4 were transfected showed little response to this peptide as measured by IL-2 production. This can be explained by the fact that hybridomas produce much larger amounts of IL-2 in response to antigen-specific stimulation compared to the TCR$^-$ cells. In addition, the mB29a peptide was found to be a less strong agonist than the founding peptide mB29b when used to stimulate splenocytes from the mB29b TCR Tg mouse, most likely due to the two amino acids difference (**Table 1**).

After linearization of the mB29b-TCR plasmids, the TCRα and TCRβ chain were injected pronuclear into mouse oocytes.

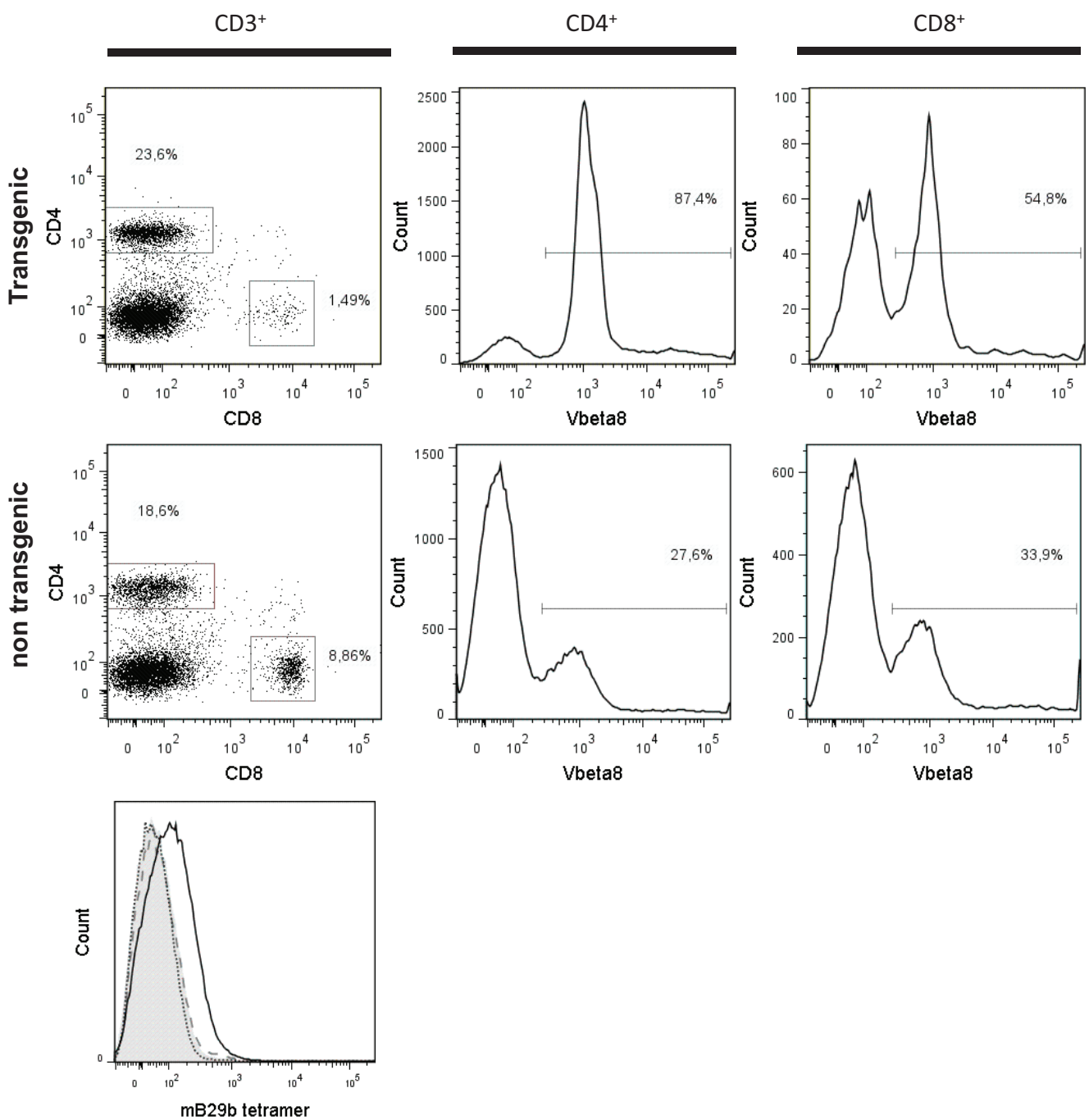



**FIGURE 5** | **Continued**

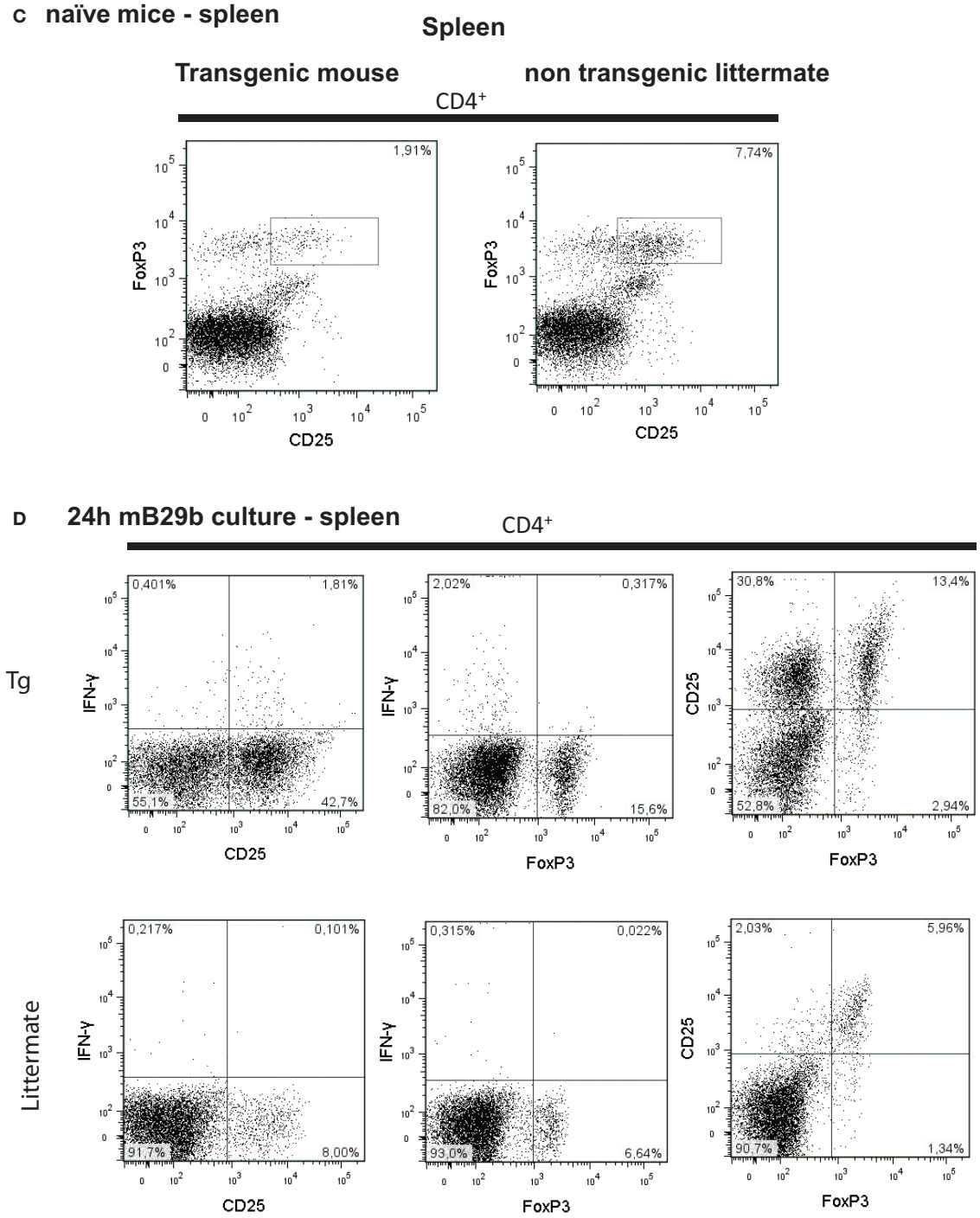



**FIGURE 5 | CD4⁺ and CD8⁺ T cell distribution in tissues of mB29-TCR transgenic mouse**. **(A)** Single cell suspensions were made from thymus of mB29b-TCR transgenic mice or negative littermates. Cells were stained for the expression of CD3, CD4, CD8, and Vβ8. Live cells were gated on the forward scatter (FSC) and side scatter (SSC) and the percentage of CD4⁺ and CD8⁺ cells of the live cells are depicted in the upper panel of **(A)**. In the lower panel of **(A)**, the percentage of CD4⁺Vβ8⁺ of all live cells is shown. The different CD4⁺ and CD8⁺ T cell distribution of the mB29b-TCR transgenic mouse compared to non-transgenic littermates is due to the transgenic background, in which formation of T cells is changed. Plots shown are representatives of three independent experiments. **(B)** Distribution of CD4⁺ and CD8⁺ cells in the CD3⁺ cells (left row of graphs), histograms of CD4⁺ cells (middle row of graphs) and CD8⁺ cells (right row of graphs) of total CD3⁺ cells are depicted. The transgenic mouse shows an increase in CD8⁺Vβ8⁺ and CD4⁺ Vβ8⁺ T cells compared to the non-transgenic littermate. The overlay histogram at the bottom of **(B)** represents the amount of mB29b-specific CD4⁺ T cells in a WT Balb/c (filled gray), a negative littermate (dashed line), and an mB29b TCR Tg mouse (black line). The dotted line shows splenocytes stained with a CLIP tetramer as negative control. Each line represents three different mice. **(C)** CD25 and FoxP3 expression measured within de CD4⁺ population of splenocytes derived from naive mice. **(D)** CD25, IFN-γ, and FoxP3 markers are measured by flow cytometry upon 24 h culture of splenocytes from both negative littermates as transgenic mouse in the presence of mB29b.

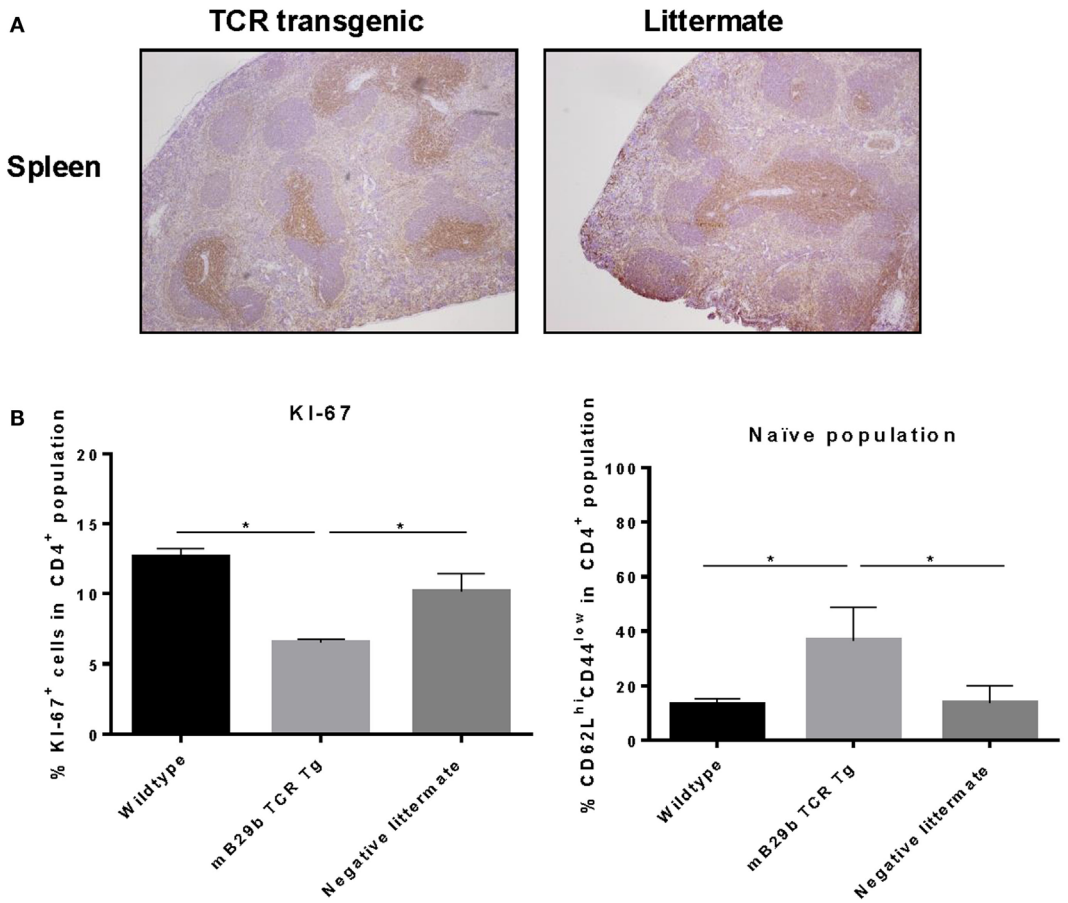



**FIGURE 6 | mB29b-TCR transgenic mouse shows no pathological changes in histology**. **(A)** Thymus, inguinal lymph nodes (iLN), and liver (data not shown) were isolated from mB29b-TCR positive mice, or negative littermates. **(A)** shows sections of the spleen in which no structural changes were observed between the mB29b-TCR transgenic mouse and the non-transgenic littermate. Immunohistochemistry was performed to T cells (αCD3) and general proliferation in lymphoid tissues. The distribution of $CD3^+$ T cells in the mB29b-TCR transgenic mouse does not deviate from the non-transgenic littermate. Pictures show the spleen in a 4× magnification. **(B)** Splenocytes were isolated from naive wild type Balb/c, mB29b TCR Tg mice and negative littermates and $CD4^+$ cells were stained for KI-67 or CD44 and CD62L. Data are obtained by flow cytometry. Data are shown as mean of triplicate samples/well ± SEM. Data shown are representative of two independent experiments.

Two founders were born that carried both vectors. PBLs from one of the mB29b-TCR transgenic founders showed mB29b-specific activation, as well as cross-recognition to the Hsp70 peptide B29 (**Figure 4**). This founder (founder 2) was mated with Balb/c mice and offspring was screened for bearing the mB29b-TCR. Splenocytes were stimulated with Hsp peptides and showed similar peptide recognition as the founder did. FACS analysis revealed that the distribution of $CD4^+$ and $CD8^+$ T cells in thymus and spleen of the mB29b-TCR transgenic were slightly different to that of littermates (**Figure 5**). Differences in $CD4^+$ and $CD8^+$ T cell distribution in TCR transgenic mice compared to non-transgenic littermates is due to the transgenic background in which formation of T cells is altered. This is considered normal for a TCR transgenic mouse (26, 27), since these mice will have selective development of αβTCR T cells in the thymus. The distribution of TCR transgenic cells could be seen in histology of the thymus (**Figure 6**). Although, in general, the T cell distribution in the tissue sections was comparable between the mice, a decreased $CD4^+CD25^+FoxP3^+$ population was observed in naive mB29b TCR transgenic mice by flow cytometry, comparable to several other TCR transgenic mice (28, 29). Furthermore, the amount of proliferated cells was altered in lymphoid tissues from the mB29b-TCR transgenic mouse compared to littermates. The mB29b-TCR Tg mice showed a decrease in $KI\text{-}67^+$ cells, which might be due to the fact that mB29b is a self-peptide which causes self-regulation. The data do indicate that tissue morphology is normal in the mB29b-TCR transgenic mouse. Furthermore, no spontaneous autoimmune disease was observed in these young TCR transgenic mice.

With this new TCR transgenic mouse, we are now able to study the properties of naive T cells differentiating and proliferating into effector and Tregs. Furthermore, we now hope to elucidate the mechanism of Hsp70-mediated T cell regulation without prior Hsp70 immunization and its associated non-specific immune activation.

## AUTHOR CONTRIBUTIONS

MJ and MH designed and performed experiments, analyzed data, wrote the paper, and approved the submitted version. PK designed and performed experiments, analyzed data, and approved the submitted version; AH performed experiments, analyzed data, and approved the submitted version; RZ designed experiments, analyzed data, commented on the manuscript at all stages, and approved the submitted version; WE commented on the manuscript at all stages and approved the submitted version; FB designed experiments, commented on the manuscript at all stages, and approved the submitted version.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Modulation of Adjuvant Arthritis by Cellular and Humoral Immunity to Hsp65

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Heat shock proteins (Hsps) are highly conserved, and their expression is upregulated in cells by heat and other stressful stimuli. These proteins play a vital role in preserving the structural and functional integrity of cells under stress. Despite the ubiquitous expression of Hsps in an individual, the immune system is not fully tolerant to them. In fact, Hsps are highly immunogenic in nature, and immune response to these proteins is observed in various inflammatory and autoimmune diseases. Studies on the immunopathogenesis of autoimmune arthritis in the rat adjuvant arthritis (AA) model of human rheumatoid arthritis (RA) as well as observations in patients with RA and juvenile idiopathic arthritis (JIA) have unraveled immunoregulatory attributes of self-Hsp65-directed immunity. Notable features of Hsp65 immunity in AA include protection rather than disease induction following immunization of Lewis rats with self (rat)-Hsp65; the diversification of T cell response to mycobacterial Hsp65 during the course of AA and its association with spontaneous induction of response to self-Hsp65; the cross-reactive T cells recognizing foreign and self homologs of Hsp65 and their role in disease suppression in rats; the suppressive effect of antibodies to Hsp65 in AA; and the use of Hsp65, its peptides, or altered peptide ligands in controlling autoimmune pathology. The results of studies in the AA model have relevance to RA and JIA. We believe that these insights into Hsp65 immunity would not only advance our understanding of the disease process in RA/JIA, but also lead to the development of novel therapeutic approaches for autoimmune arthritis.

**Keywords: heat shock proteins, arthritis, T cells, antibodies, autoimmunity, Hsp60, Hsp65**

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Adjuvant arthritis (AA) is a well-established model of human rheumatoid arthritis (RA) (1–3), and it has extensively been used both for studying arthritis pathogenesis and for testing new anti-arthritic drugs. AA can be induced in inbred Lewis (RT.1[l]) rats by immunization with heat-killed *Mycobacterium tuberculosis* H37Ra (Mtb) (1). AA manifests as a polyarthritis after about 8–10 days of Mtb immunization. The disease rapidly progresses to reach a peak phase by about days 14–16, followed by spontaneous regression of inflammation over the next 10–12 days. Following recovery, AA generally does not relapse or exhibit flares that are typically seen in many RA patients. In this regard, AA differs from RA. Mycobacterial heat shock protein 65 (Bhsp65) is one of the disease-related target antigens in AA (1–3). Studies on immune responses to Hsp65 have offered critical insights into both induction and regulation of autoimmune arthritis (3–9). Arthritic rats raise T cell response to Bhsp65, and the T cells directed against the epitope region 180–188 of Bhsp65 (B180) can adoptively

transfer AA to naive rats (2, 10). However, the T cells reactive against certain other epitopes of Bhsp65 are disease-regulating in nature. In our studies in AA summarized here, we have addressed several important questions. For example, are Lewis rats tolerant to self-Hsp65?; how does activation of self-Hsp65-reactive T cells affect the development of arthritis?; how does immune response to foreign Hsp65 evolve during the course of AA?; what is the significance of the T cell repertoire against foreign Hsp65 that is cross-reactive with self-Hsp65?; what role do antibodies to Hsp65 play in AA?; and does Hsp65 treatment influence arthritis induced by a non-antigenic compound? These aspects of cellular and humoral immunity to Hsp65 are elaborated below, based on results of studies by others and us. (Mammalian Hsp60 is a mitochondrial protein of 61 kDa, whereas Hsp65 is a mycobacterial protein of 65 kDa. Hsp60 is used as a synonym for Hsp65, and *vice versa*, therefore, for simplicity, we have used Hsp60 and Hsp65 interchangeably in this article.)

## THE ROLE OF SELF (RAT)-HSP65 IN ARTHRITIS PATHOGENESIS: IMMUNE REACTIVITY TO SELF-HSP65 IS PROTECTIVE RATHER THAN PATHOGENIC

As autoimmune reactivity is generally driven by an endogenous self-antigen, we directed our study on AA pathogenesis to rat Hsp65 (Rhsp65) (9, 11). Ours was the first study to examine the state of immune tolerance to Rhsp65 in the Lewis rat. Surprisingly, Lewis rats were not fully tolerant to Rhsp65 (11). The rats challenged with Rhsp65 raised a potent response to this self-antigen, and the pre-treatment of naive rats with Rhsp65 afforded protection against subsequent induction of AA by Mtb injection (11). These results showed that immune response to systemically administered self-Hsp65 is protective against arthritis instead of being pathogenic (**Figure 1**). Our studies further revealed that Rhsp65 C-terminal determinants (RCTD) are displayed as dominant epitopes following the processing and presentation of native Rhsp65 (11). Furthermore, peptides comprising RCTD, when administered into Lewis rats in a synthetic adjuvant, also induced a T cell response that is protective against AA (11). Thus, anti-self-Hsp65 immune response is protective against AA.

## IMMUNE RESPONSE TO HUMAN HSP60 INDUCES PROTECTION AGAINST ARTHRITIS IN RATS

Studies using DNA vaccination approach have revealed the disease-regulating activity of human Hsp60 in AA (**Figure 1**) (12). Human Hsp60 was more effective than Bhsp65 in suppressing AA. This protective effect was associated with increased interferon-γ (IFN-γ) and transforming growth factor-β (TGF-β) response to Hsp60, but reduced IFN-γ response to B180 (12). Subsequent experiments identified Hu3 as a regulatory epitope within human Hsp60 (13). Immunization with human Hu3, but

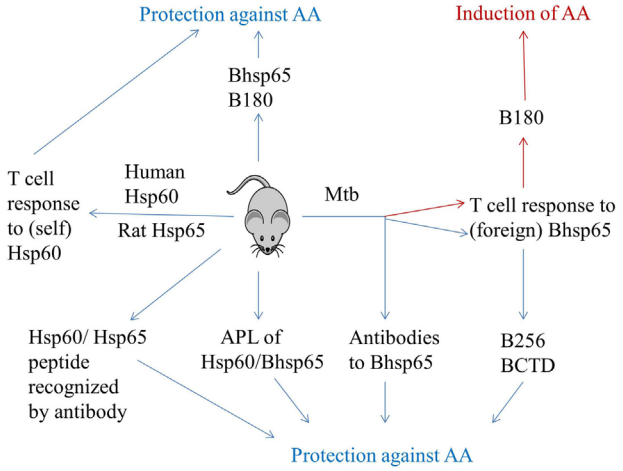


**FIGURE 1 | Induction and regulation of adjuvant arthritis by immune response to Hsp65**. AA can be induced in Lewis rats by subcutaneous immunization with heat-killed *M. tuberculosis* H37Ra (Mtb). Mycobacterial hsp65 (Bhsp5) is one of the targets of immune response in arthritic rats. The T cells against epitope 180–188 of Bhsp65 (B180) are pathogenic, whereas those against other epitopes of Bhsp65 [e.g., 256–270 (B256) and Bhsp65 C-terminal determinants (BCTD)] are regulatory in AA. Tolerization of rats with soluble Bhsp65 or B180 renders these rats relatively resistant to induction of AA by subsequent Mtb injection. Arthritic rats also raise antibody response to Bhsp65, and antibodies have been shown to be protective against AA. Intriguingly, immunization of Lewis rats with self-Hsp65 [e.g., human Hsp60 or rat Hsp65 (Rhsp65)] is protective rather than pathogenic. Similarly, induction of antibody response by a peptide of self-Hsp60 also affords protection against AA. Finally, challenge with altered peptide ligands (APLs) of Bhsp65 and self-Hsp60 can induce a disease-protective response in arthritic rats. Thus, immunity to Hsp65 has dual attributes: pathogenic as well as protective. (This figure summarizes information from multiple studies described in the text.)

not its mycobacterial homolog, was protective against AA, and the protective response involved both Th1 and Th2/3 cells (13). A cross-reactive T cell response to human Hsp60 was also invoked in the protective effect of DNA vaccination using Hsp70/Hsp90 (14). Another set of studies have highlighted the role of human Hsp60 in activation of B cells (15), T cells (16), $CD4^+CD25^+$ regulatory T cells (Treg) (17), and maturation of dendritic cells (18), in part *via* Toll-like receptor 4 (TLR4) (B cells)/TLR2 (T cells and Treg) signaling. Furthermore, acting in a different manner, Hsp60 expressed on activated T cells can render them targets of regulatory T cells (19). In an entirely different approach based on $CD8^+$ regulatory T cells instead of $CD4^+$ regulatory T cells, it was shown that Hsp60(p216) peptide-specific $CD8^+$ T cells restricted to a class I-like MHC molecule Qa-1 in mice (HLA-E in humans) effectively suppressed CIA (20). Thus, besides self-Hsp60 epitopes recognized by $CD4^+$ T cells, others recognized by $CD8^+$ T cells may also contribute to regulation of autoimmune arthritis. Thus, Hsp60 can modulate both innate and adaptive immune response through multiple modes of action, and as for rat Hsp65, immunity to human Hsp60 also induced protection against AA. Further insights into the T cell repertoire against self-Hsp60 and maintenance of tolerance would help advance understanding of the pathogenesis of arthritis and its control (21).

## IMMUNOMODULATORY IMMUNE REACTIVITY TO HUMAN HSP60 IN JIA AND RA PATIENTS

In a study on patients with JIA, the response to Hsp60 of peripheral blood mononuclear cells (PBMC) from oligoarticular JIA was compared with that of polyarticular JIA. The former group revealed increased expression of CD30 on T cells along with increased interleukin-10 (IL-10)/IFN-γ ratio, whereas the latter group showed no expression of CD30 and low IL-10/IFN-γ ratio (22). Furthermore, remission from acute oligoarticular JIA was attributed to Hsp60-reactive T cells. In another study, it was reported that serum human Hsp60 predicts remission in JIA (23). In a study on PBMC of RA patients, it was observed that pan-DR-binding human Hsp60 peptides induced 5- to 10-fold higher IL-10/tumor necrosis factor-α (TNF-α) ratio than that by microbial peptides, indicating immunomodulatory activity of self-Hsp60 peptides (24). Thus, self (human)-Hsp60 not only induced protection against AA but also contributed to remission in JIA.

## FOREIGN–SELF-HSP65 CROSS-REACTIVITY AND SPREADING CONTROL OF ARTHRITIS IN RATS

Lewis rats treated with Bhsp65 prior to disease induction are protected from subsequent AA (25). Unlike Mtb-immunized rats, which raise T cell response predominantly to epitope B180, Bhsp65-treated rats raised response to multiple additional epitopes besides B180 (25, 26). Epitope 256–270 was among the nine epitopes identified upon Bhsp65 pre-treatment. The T cells reactive against mycobacterial 256–270 were found to be both cross-reactive with self counterpart (Rhsp65 256–270) as well as disease-regulating in nature (26). The T cells against this peptide could be restimulated by stressed antigen-presenting cells (APCs), indicating that the self-ligand recognized was processed and presented from endogenous Hsp60. Furthermore, the treatment of rats either with this peptide or with the T cells primed with it resulted in protection against AA (**Figure 1**). This cross-reactivity between homologous foreign and self-Hsp60 has been implicated in a spreading regulatory control during the course of arthritis (6). Self-Hsp60-reactive T cells can contribute to immune regulation in different ways. For example, such cells may be anergized, and these anergic cells mediate suppressive effects. Alternatively, self-Hsp60 epitopes may serve as altered peptide ligands (APLs) and induce a regulatory response, while their corresponding microbial Hsp60 ligands may act as potent agonists (6). Thus, T cells activated by Bhsp65, but cross-reactive with self-Hsp65, displayed anti-arthritic activity against AA.

## THE DIVERSIFICATION OF T CELL RESPONSE TO BHSP65 DURING THE COURSE OF AA

Rats with AA undergo spontaneous regression of inflammation. However, the immunological basis of this phenomenon remains to be fully explained. Our study revealed the diversification of T cell response to Bhsp65 during the course of AA (3). Arthritic rats in the late phase of AA displayed new T cell reactivity against BCTD compared to rats in early phase of the disease. Furthermore, synthetic peptides containing the sequences of BCTD, when administered into Lewis rats with a synthetic adjuvant, induced protection against subsequent AA (3). Similarly, the adoptive transfer of BCTD-primed T cells reduced the severity of AA in recipient arthritic rats (27). Thus, deliberate priming and expansion of the T cells against BCTD led to protection against AA (**Figure 1**). This was the first report on regulatory epitope spreading in autoimmunity because an earlier report on epitope spreading in the EAE model described it as pathogenic (3, 28). Thus, our study expanded the scope of the impact of epitope spreading in autoimmunity and presented a framework to explain natural regression of inflammation in AA.

## DEFINING THE MECHANISM OF SPONTANEOUS REGRESSION OF AA

We observed that BCTD represent cryptic epitopes of Bhsp65, meaning that these epitopes are potentially immunogenic as peptides, but are not efficiently revealed upon processing of the native foreign antigen (Bhsp65) injected into Lewis rats (27). On the contrary, RCTD represent the dominant epitopes of Rhsp65, implying that these epitopes are efficiently displayed following the processing and presentation of the native self-antigen administered into Lewis rats (11). Yet, BCTD can prime T cells that are cross-reactive with the self counterpart. We therefore explained regulatory epitope spreading by suggesting as well as providing evidence that inflammation accompanying acute arthritis upregulates Rhsp65 expression; its RCTD are then displayed to induce a T cell response; and these T cells can then suppress the progression of arthritis, as evident from adoptive transfer experiments using Rhsp65- or RCTD-primed T cells (3, 11, 27, 28). We further suggested that inflammation also upregulates the display of cryptic BCTD, which then can induce T cells that cooperate with RCTD-primed T cells in suppressing pathogenic T cell response, leading to natural recovery from acute AA (**Figure 1**). Responses to BCTD are also involved in environmental modulation of AA (29).

## UNEXPECTED CROSS-REACTIVITY BETWEEN THE PATHOGENIC AND THE PROTECTIVE T CELL EPITOPES OF HOMOLOGOUS HSP65 IN AA

Peptide 177–191 of Bhsp65 (B177) contains the arthritogenic epitope B180 (2), whereas peptide 465–479 of Rhsp65 (R465) represents a regulatory epitope for AA (30). Immunization of rats with B177 in incomplete Freund's adjuvant or CpG s.c. prior to Mtb injection showed marked suppression of arthritis instead of disease induction (30). We reasoned that the T cell subset activated by B177 injected in IFA/CpG is distinct from that activated by Mtb challenge; the latter subset mediates induction of arthritis, and that there might be some connection between B177

and one of the regulatory C-terminal determinants of Hsp65, including R465 described above. In fact, surprisingly, there was cross-reactivity between B177 and R465, and it involved a subset of T cells shared by the two epitopes (**Figure 2**) (30). This cross-reactivity was further validated by tolerance induction to R465, which compromised B177-induced protection against AA (30). This is a fine example of fortuitous mimicry between a pathogenic Bhsp65 epitope and a regulatory Rhsp65 epitope, and it might represent an inbuilt mechanism of a crosstalk between such pairs of epitopes to mediate regression of inflammation during the course of autoimmunity. In retrospect, a similar mechanism might offer additional explanation to the observations that a T cell clone (A2c) recognizing B180 was protective against AA (10) and that soluble B180 administered intraperitoneally (i.p.) prior to arthritis induction can suppress the development of AA (31).

## ANTIBODIES TO HSP65 ARE PROTECTIVE RATHER THAN PATHOGENIC IN AA

Adjuvant arthritis is believed to be a T cell-mediated disease. Unlike T cells, antibodies of arthritic rats fail to induce AA in naive recipients. Instead, anti-Hsp65 antibodies can mediate protection against AA. It was first shown that AA-resistant rat strains possess antibodies that are protective against AA, whereas susceptible rat strains, such as Lewis, lack these antibodies (32). Accordingly, the transfer of antibodies of resistant strain into susceptible strain induced protection against AA. Subsequent studies by others (8) and us (33) showed that such protective antibodies also develop in arthritic rats after recovery from AA. During the course of AA, eventually the antibody response became focused on few defined epitopes of Bhsp65 and Rhsp65 (8, 33). Furthermore, immunization of naive rats with specific peptide epitopes or anti-peptide antibodies, or sera from recovered rats, leads to protection against AA (**Figure 1**) (8, 33). The precise mechanism of protection is not yet fully defined, but one proposition is based on antibody-induced IL-10 production by PBMC (8). In a recent study (34), a humanized monoclonal antibody against Hsp60 (Prozumab) was reported to display disease-suppressive activity against AA and CIA. This antibody also can induce IL-10 in PBMC. Thus, antibodies against Hsp65 in AA are disease-regulating in nature.

## RESPONSES TO C-TERMINAL DETERMINANTS OF HSP65 ARE INVOLVED IN ENVIRONMENTAL MODULATION OF AA

In a study on Fisher (F344) rats, whose susceptibility to arthritis is markedly modified by their housing environment, we showed that F344 rats raised in a barrier facility (BF-F344) are susceptible, whereas those raised in a conventional facility (CV-F344) were relatively resistant to AA (29). Similarly, F344 rats transferred from a barrier facility into a conventional facility (BF-CV-F344)

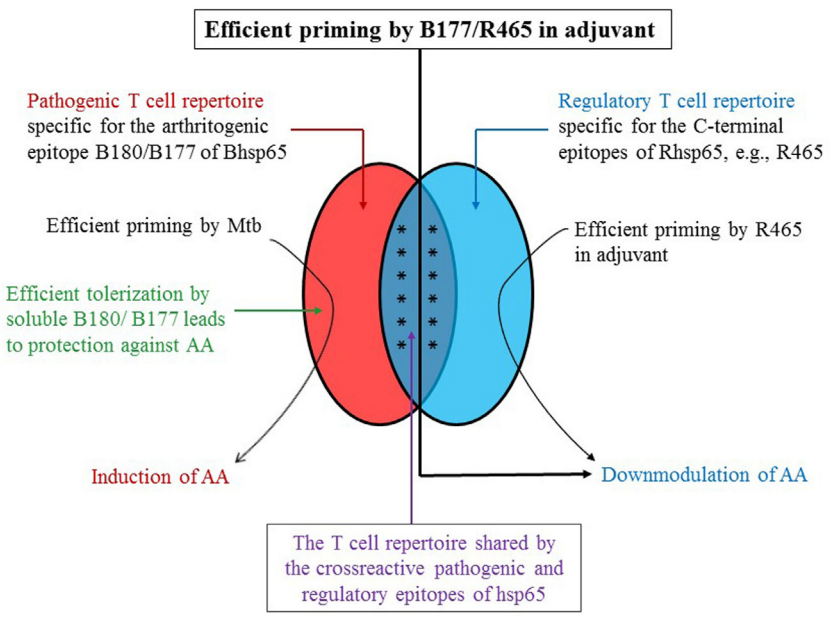


**FIGURE 2 | A schematic representation of the role of cross-reactive T cells directed against B180/B177 and R465 in regulation of AA**. The T cells potentially reactive against B180/B177 of Bhsp65 (pathogenic epitope) and R465 of Rhsp65 (regulatory epitope) comprised a cross-reactive T cell subset besides the corresponding epitope-specific subset. The priming and expansion of these cross-reactive T cells by immunization of Lewis rats either with peptide (B180/B177 or with R465) in adjuvant would induce protection against subsequent AA ("vaccination"). However, injection of Mtb, which contains Bhsp65, instead activates predominantly the B180/B177-specific pathogenic repertoire, resulting in the induction of AA. Furthermore, this pathogenic repertoire may be inactivated, or immune-deviated, by soluble antigen (B180/B177) administered i.p. or nasally, leading to protection against AA. This schematic figure is based on our results published elsewhere (3, 11, 27, 30).

acquired resistance to AA in a few weeks. In simple terms, BF and CV are also referred to as "clean" and "dirty" housing facilities, with the latter providing more opportunities for animals to be exposed to environmental microbes. We reasoned that Hsp65 of the environmental microbes might induce T cell response in CV-F344 that is cross-reactive with Bhsp65, and that these T cells in turn afford protection against AA. A similar situation might be expected for BF-F344 transferred into CV, whereas BF-344 will be spared as they are kept in strict controlled conditions. To test this idea, we examined the level of T cell response to BCTD and other peptides of Bhsp65 in these rats without any immunization with Mtb or Bhsp65 (29). Interestingly, CV-F344 spontaneously developed much higher level of T cell response to BCTD compared to BF-F344, whereas BF-CV-F344 were in between the two groups. Furthermore, adoptive transfer of spleen cells of CV-F344 rats, but not BF-F344 rats, reduced the severity of AA in recipients. Thus, BCTD-reactive T cells contribute not only to spontaneous regression of inflammation in Lewis rats (3) but also to environmental modulation of AA in F344 rats (29). However, the role of environmental factors on gut flora and its impact on AA remains to be defined.

## USE OF ALTERED PEPTIDE LIGANDS OF SELF-HSP60 TO CONTROL ARTHRITIC INFLAMMATION

We have described above the use of native Hsp65 and its peptide epitopes in inhibition of arthritis. APLs, with specific amino acid residues of the original peptide modified to affect its binding to the major histocompatibility complex or the T cell receptor leading to altered T cell response, have also been explored for arthritis therapy (**Figure 1**). Initial work involved an APL of B180 containing alanine 183, which was shown to inhibit AA (35). It involved the generation of regulatory T cells and production of anti-inflammatory/immunomodulatory cytokines IL-4, IL-10, and TGF-β. Subsequently, APLs of human Hsp60 (e.g., APL-2 and APL-1) have been shown to suppress AA/CIA (36, 37). In addition, APL2 increased IL-10 production, but reduced IL-17 production by PBMC of RA patients (36), and it induced IL-10 production in PBMC of JIA patients (37). APL-1 was reported to induce $Foxp3^+$ Treg coupled with apoptosis in activated $CD4^+$ T cells in PBMC of active RA patients (38).

## CONCLUDING REMARKS

As elaborated above, studies on immune response to Hsp65 have offered several novel insights into both the induction and regulation of autoimmunity (3–9). Studies in the AA model have revealed Bhsp65 as the target of arthritogenic T cells. However, Bhsp65-reactive T cells also can be disease-regulating in nature, and such T cells demonstrate cross-reactivity with self-Hsp65 (3, 26). In addition, the T cells induced by self-Hsp65 also possess immunoregulatory activity against AA (11, 13) and dimethyl dioctadecyl ammonium bromide-induced arthritis (DIA) in Lewis rats (39). The regulatory role of self-Hsp65 is evident not only in the AA and DIA models but also in patients with JIA (22). On the contrary, in other disease conditions, such as diabetes (40) and atherosclerosis (41), pathogenic immune response has been shown to be directed against self-Hsp65. Nevertheless, Hsp65, its peptides, and APL can also be employed as immunomodulatory agents to attenuate these diseases. Thus, the dual role of Hsp65 has been reported not only in arthritis but also in diabetes, atherosclerosis, tumors, and transplantation (42–44). Further understanding of the cellular and molecular conditions that facilitate pathogenic versus regulatory immune responses to Hsp65 would pave the way for harnessing the immunomodulatory attributes of Hsp65 for therapeutic purposes in human diseases, as exemplified by the use of p277 of self-Hsp60 in human type 1 diabetes (45). This in turn would add novel agents to the therapeutic arsenal to control a variety of immune-mediated diseases.

In brief, the major new conceptual developments emerging from studies on immunity to Hsp65 in AA can be summarized as follows: (a) foreign–self antigen cross-reactivity, also referred to as "molecular mimicry," has generally been viewed as a mechanism of induction of autoimmunity; however, studies in AA have shown that such a cross-reactivity (e.g., B256 and BCTD) can be immunoregulatory in nature; (b) most times, foreign–self cross-reactivity is limited to the corresponding homologous regions of the two proteins; in AA, a pathogenic epitope (B180/B177) can recruit a subset of T cells potentially reactive against a protective self-epitope (R465), thus elaborating a novel aspect of such cross-reactivity; (c) immune response to a self-antigen, which signifies a break in self-tolerance, has been the cornerstone of mechanisms to explain the initiation of autoimmunity; studies in AA have revealed that deliberate priming of self-reactive T cells using self-Hsp65 (human Hsp60 or rat Hsp65) as the immunogen, can effectively suppress arthritis; furthermore, the spontaneous emergence of anti-self T cell response during the course of AA can contribute to natural regression of autoimmune inflammation; (d) antibodies to foreign/self antigens are known to serve as mediators of immune pathology in a variety of autoimmune diseases; in AA, antibodies to Hsp65 have been shown to be protective against arthritis; and (e) previous work on peptide-based therapy of AA involved APL of peptides of foreign Hsp65 (Bhsp65); recent studies highlight that APL of self-Hsp60 also can serve as potent immunomodulatory agents.

## AUTHOR CONTRIBUTIONS

EK, MD, YM, HK, and KM participated in writing of this article; EK and KM edited the article; and EK, MD, and KM prepared the figures.

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# Modulation of Alloimmunity by Heat Shock Proteins

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The immunological mechanisms that evolved for host defense against pathogens and injury are also responsible for transplant rejection. Host rejection of foreign tissue was originally thought to be mediated mainly by T cell recognition of foreign MHC alleles. Management of solid organ transplant rejection has thus focused mainly on inhibition of T cell function and matching MHC alleles between donor and host. Recently, however, it has been demonstrated that the magnitude of the initial innate immune responses upon transplantation has a decisive impact on rejection. The exact mechanisms underlying this phenomenon have yet to be characterized. Ischemic cell death and inflammation that occur upon transplantation are synonymous with extracellular release of various heat shock proteins (Hsps), many of which have been shown to have immune-modulatory properties. Here, we review the impact of Hsps upon alloimmunity and discuss the potential use of Hsps as accessory agents to improve solid organ transplant outcomes.

**Keywords: Hsps, transplantation, alloimmunity, dendritic cells, immune regulation**

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## INTRODUCTION

During organ transplantation, tissues are injured as a consequence of ischemia and reperfusion. Upon organ harvest, ischemia ensues and continues during organ preservation. Reperfusion happens upon anastomosis of graft vessels. Ischemia–reperfusion injury (IRI) induces cell death by necrosis and apoptosis leading to production of molecules denominated damage-associated molecular pattern (DAMPs) or alarmins (1). These are self, intracellular molecules, which are released by injured or necrotic cells under pathological conditions. In the extracellular environment, they can interact with and activate innate immune cells, acting as "danger signals" (2, 3). Resident macrophages and dendritic cells (DCs) are sensitive to DAMPs signals. These cells sense initial ischemic insults through pattern recognition receptors (PRRs). The engagement PRRs, including toll-like receptors (TLRs) and scavenger receptors (SRs), trigger intracellular signaling cascades that culminate in activation of transcription factors, which coordinate production of inflammatory cytokines and chemokines, upregulation of MHC and co-stimulatory molecules. DCs are the major antigen-presenting cells (APCs) and once activated in tissues, migrate to draining lymph nodes, and stimulate alloreactive T cell responses. DCs have to deliver three signals for optimal activation of T cells: (i) the expression of peptide:MHC complexes that will be recognized by the T cell receptor (TCR); (ii) co-stimulatory molecules; and (iii) cytokines that will shape T cell-mediated responses. Macrophages are also important contributors to IRI-induced inflammation and produce immune-stimulatory cytokines TNF-α, IL-1β, IL-6, and MCP-1, and chemokines which facilitate recruitment of alloreactive T-cells to the graft site (4, 5). While alloantigen-specific T cells are responsible for subsequent organ rejection and destruction, this outcome is very much shaped

by the local inflammatory state of the graft site (6). Thus, alloreactive immune responses result from activation of cellular components of both innate and adaptive immunity (7).

To improve the longevity of solid organ transplants, it is desirable to develop methods to limit IRI-induced inflammation and the ensuing alloreactive immune response. This review will discuss how heat shock proteins (Hsps) can modulate inflammatory and allogeneic immune responses, and how this can be harnessed to manage graft rejection in solid organ transplantation.

## THE HEAT SHOCK RESPONSE AND CYTOPROTECTIVE PROPERTIES OF Hsps

The heat shock response is universal and conserved from bacteria to mammals. It can be triggered by a range of protein-damaging conditions that affect organisms such as heat, anaerobiosis/hypoxia, oxidative stress, inflammation, fever, and infection. Activation of the heat shock response is marked by upregulation of various Hsps. Under stress conditions, intracellular Hsps prevent protein aggregation, refold damaged proteins, and target damaged proteins for degradation. Under non-stress conditions, Hsps assist the folding of recently synthetized proteins, the translocation of proteins between organelles, and also regulate the cell cycle. These homeostatic functions of intracellular Hsps support proteome integrity and thereby promote cell viability. In addition, Hsps, such as Hsp70 and Hsp27, have been shown to negatively regulate multiple apoptotic signaling events including mitochondrial cytochrome *c* release (Hsp27), mitochondrial release of Smac (Hsp27), nuclear translocation of apoptosis-inducing factor (AIF) (Hsp70), and cleavage of procaspase 3 (Hsp70) (8–12). Hsps are conventionally grouped into families according to their molecular weight (e.g., Hsp40, Hsp70, and Hsp100) (13, 14). Functional cooperation exists between family members; however, individual Hsp species perform distinct functions that can also be context dependent. In the context of IRI and organ transplantation, increased Hsp levels have been associated with cytoprotection, improvement of organ viability, and function after ischemia–reperfusion (15).

## INTRACELLULAR Hsps PROTECT ALLOGRAFTS FROM ISCHEMIA–REPERFUSION INJURY AND IMPROVE GRAFT SURVIVAL

Increased levels of Hsps in transplant organ cells either by treatment or genetic manipulation have been demonstrated to be beneficial for transplant longevity (16). Hsps promote refolding of proteins denatured due to IRI, protecting cells from IRI-induced death. Hsp70 has been proposed to be the most potent anti-apoptotic mediator inside the cell (17). Heat pre-conditioning of organs prior to transplant upregulates the expression of Hsps and prevents tissue damage from IRI by different mechanisms (18). Hsps' cytoprotective capacity was also demonstrated in organs that were genetically modified to overexpress these proteins. Hearts from mice overexpressing Hsp27 induction correlated with increased survival when transplanted in fully MHC-mismatched hosts (16). These hearts presented reduced caspase activation after subjection of ischemic/reperfusion conditions. In addition to heat, hypoxic pre-conditioning seems to reduce ischemic renal failure through a HIF-α/Hsp70 signaling pathway (19). This literature has been extensively reviewed in previous works (15, 20, 21).

## Hsps CAN PROTECT FROM IRI BY MODULATING INFLAMMATION

Recently, several studies have highlighted a previously overlooked importance of innate cells in shaping T cell-mediated responses to alloantigens (5). Indeed, IRI and the subsequent intra-organ activation of innate cells have been shown to markedly enhance alloimmunity, contributing to poorer long-term outcomes and graft function. For example, delayed graft function (DRF) is a complication that occurs very early after the transplant procedure and results from a previous intense ischemic injury. Kidney transplant patients with DRF have a higher risk to graft loss (22). Thus, strategies and treatments that prevent or decrease the activation of APCs by the released of ischemic-derived DAMPs could result in diminished alloimmunity and improve both early and late graft function (23).

During IRI, an important DAMP released by injured cells is the nucleotide adenosine triphosphate (ATP). Extracellular ATP (eATP) is recognized by purinergic receptors expressed by immune cells. Once eATP engages such receptors, it can trigger innate inflammatory responses and activation and proliferation of T cells. This can lead to further inflammation and cell damage, contributing to rejection [extensively reviewed in Ref. (24, 25)]. Additionally, high-mobility group box 1 (HMGB1) can also be released from dying cells. HMGB1 has been reported to be involved in IRI. HMGB-1 can activate APCs through TLR2 and TLR4 (26), as well as the receptor for advanced glycation end products (RAGE) (27), triggering anti-donor T cell responses (28).

Heat shock proteins have been suggested to act as DAMPs (29). Initial observations demonstrated that Hsps are elevated in transplanted organs, and Hsp-reactive T cells do infiltrate organs undergoing rejection (30). This raised the initial idea that such proteins play a crucial role as immunogenic antigens during alloimmune responses (15). Hsps are among many intracellular proteins that are released to the extracellular environment as a consequence of cell death during IRI. This is one reason why many consider Hsps to be DAMPS. Another reason is that extracellular isoforms of Hsps were reported by some studies to interact with TLRs and SRs and trigger inflammatory responses (31).

Aside from being passively released, Hsps can reach the extracellular milieu through different active pathways. Hsp70 can be exported by an active non-classical secretory pathway, which cannot be blocked by inhibitors of the ER–Golgi pathway (32). Also, Hsp70 can be released by a lysosome–endosome mechanism, similar to IL-1β secretion (33), and a pathway involving secretory-like granules (34). Finally, Hsp70 can be secreted by a mechanism involving the insertion into exosome membranes (35).

Thus, a scenario in which Hsps are found extracellularly during transplantation is likely, independently of passive or active release. More recent studies suggested that extracellular Hsps in transplants play additional immune roles – triggering anti-inflammatory responses and acting as immune modulators. In contrast to the DAMPs hypothesis, it was proposed that Hsps could belong to a group of molecules denominated resolution-associated molecular patterns (RAMPs) (36). RAMPs are released from necrotic and damaged cells, and when they reach the extracellular environment, will exert anti-inflammatory and regulatory effects over immune cells.

Resolution-associated molecular patterns are proposed to counterbalance acute inflammation and restore immune homeostasis by modulating innate cells. After tissue damage, they can modulate acute inflammation by inducing the production of IL-10 (36). IL-10 has powerful anti-inflammatory and immune suppressive properties. It can modulate DCs activation and differentiation, inhibits the release of inflammatory cytokines by T cells, APCs, and NK cells, and impairs cytotoxic ability of CD8 T cells (37). The alpha B-crystallin (αBC) protein is considered a RAMP. Mice deficient for this protein have an exacerbated form of experimental autoimmune encephalomyelitis (EAE). Administration of αBC to mice with EAE reduces severity disease scores (38). Interestingly, and maybe not coincidentally, αBC is a chaperone, and a member of the small Hsps group. The induction of IL-10 by other Hsps, such as Hsp70 (39), Hsp60 (40), and BiP (41), is well documented and has been reviewed elsewhere.

## TRANSPLANT OF ORGANS GENETICALLY MODIFIED TO OVEREXPRESS Hsps GENERATE LESS INFLAMMATION

A member of the Hsp70 family that has immunomodulatory effects and acts as a RAMP is the endoplasmic reticulum (ER) protein GRP78 or BiP (38). When islet cells overexpressing GRP78 were transplanted in fully MHC-mismatched hosts, they presented decreased cell death, prolonged survival, and were less immunogenic compared with controls (42). In a murine cardiac transplant model, transgenic Hsp27 overexpression increased allograft survival. Hsp27tg-derived hearts exhibited reduced IRI-induced apoptosis *ex vivo* and stimulated a reduced allogeneic inflammatory response compared with hearts transplanted from littermate controls (16). Markers for infiltrating T cells were reduced within transplanted hearts from Hsp27tg mice, and this was coupled with less production of IFN-γ day 5 post-transplant and increased IL-4 at day 2. In other inflammatory models, overexpression of Hsps has also been shown to be beneficial not only due to their pro-survival roles but also by dampening inflammation. Overexpression of Hsp70 in transgenic (Tg) mice has been shown to protect animals from neuroinflammation (43). Hsp70 overexpression can also induce neuroprotection from stroke and traumatic brain injury (44, 45). Consistent with Hsp70 possessing protective properties against tissue injury, Hsp70 Tg mice were found to have protection from inflammatory colitis and pulmonary fibrosis in respective models compared with their wild-type counterparts (46, 47).

In summary, increased levels of Hsps can be beneficial through the prevention of cell death, precluding the release of DAMPs. It is also possible that in HspTg mice, after tissue damage is induced by transplantation, the concentration of extracellular Hsps released from damaged cells is higher, resulting in induction of tolerogenic responses and the dampening of inflammation.

## Hsp PEPTIDES AS ANTIGENS FOR Treg CELLS

Regulatory T cells (Tregs) can suppress excessive effector immune responses that are harmful to the host (48). Tregs can actively suppress innate and adaptive inflammatory immune responses through the production of the anti-inflammatory cytokines IL-10 and/or TGF-β (49). Induction or administration of Tregs during transplantation is a promising approach for the management of allograft rejection (50). Although the peptide ligands for Tregs have not extensively been characterized, several studies have reported that they recognize self-peptides bound to MHC class II molecules (51). Tregs originate in the thymus (tTregs) but can also be induced at peripheral sites (pTregs) (52). For example, pTregs can be generated in a tolerogenic microenvironment by interacting with DCs producing anti-inflammatory cytokines and low levels of MHC II and co-stimulatory molecules (53).

Recently, it was shown that heat pre-conditioning of the organ had protective effects in acute kidney injury induced by IRI, and that protection was mediated by a direct immunomodulatory response of Hsp70-specific Tregs (54).

## MODULATION OF ALLOGRAFT REJECTION BY EXTRACELLULAR Hsps

In addition to benefits for transplant organs conferred by increased intracellular Hsp levels, a number of studies have now demonstrated various extracellular Hsps to also extend graft survival. For example, subcutaneous treatment of recipients prior to transplant with a single dose of full-length murine Hsp60 or with two of its peptides (p12 and p277) was found to prolong skin graft acceptance (55). Interestingly, no improvement in skin allograft survival was observed when recipients were treated with an Hsp60 peptide from *Mycobacterium tuberculosis* (55). The authors suggested the differential effect between these Hsp60 species was likely due to a shift from an IFN-γ- to IL-10-producing phenotype in self-Hsp60-specific T cells, a shift which was not induced by treatment with mycobacterial Hsp60 (56). Consistent with skin graft protection conferred by mouse Hsp60 peptide administration, intranasal pre-treatment with encapsulated human Hsp60-derived peptide (p277) increased skin graft survival in two minor mismatched mice models (57). In this study, treatment with human Hsp60 also induced production of the anti-inflammatory cytokine Il-10. Together the findings from these studies indicate that Hsp-mediated extension of graft survival may be closely related to IL-10 induction.

Heat shock protein 10 is an Hsp60 co-chaperone and was first described as early pregnancy factor (EPF) (58). This protein is

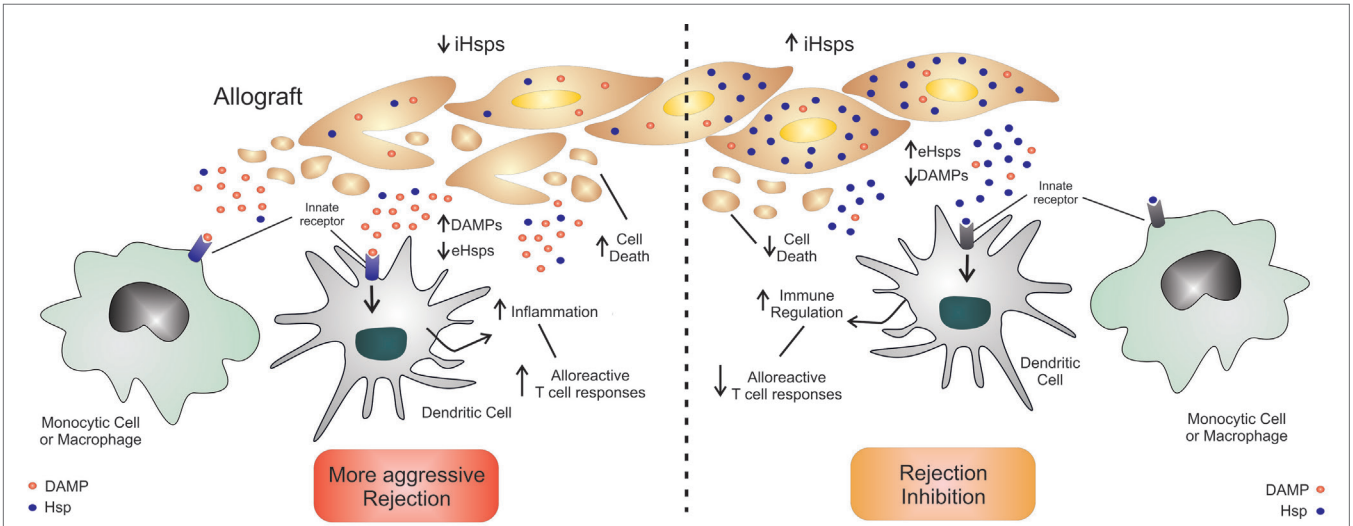


**FIGURE 1** | **Beneficial effects of Hsps during transplantation**. The upregulation of intracellular Hsps (iHsps) can protect cells from injury with a diminished release of DAMPs, decreased innate inflammation and less alloreactive T cell activation. The presence (in high concentrations) or administration of extracellular Hsps (eHsps) can also trigger immune-regulatory pathways on innate cells, favoring the generation of Tregs.

found in pregnant women's sera, and was described to be immunosuppressive, involved in fetus tolerance (58). The subcutaneous *in situ* delivery of recombinant Hsp10 improved skin allograft survival in rats (59). The authors suggested that Hsp10 would inhibit Th1 responses through donor DCs modulation (59).

Heat shock protein 70 (DnaK) from *M. tuberculosis* can improve graft survival in two different models of skin allografts. First, when the allogeneic B16F10 melanoma cells (H-$2^b$/I-$A^b$) are subcutaneously injected in BALB/c hosts (H-$2^d$/I-$A^d$), they are rejected due to MHC disparity. However, when those cells were injected in the presence of DnaK, they could form tumors in the hosts (60). *In situ* analysis demonstrated a tolerogenic environment with an increased infiltration of Tregs in DnaK-treated tumors, and depletion of Tregs abrogated DnaK-mediated tumor protection. Extracellular DnaK treatment of bone marrow-derived macrophages (BMMs) was also found to promote the immunosuppressive M2-like macrophage phenotype and favor tumor growth in a murine melanoma model (61). Together, these studies demonstrated DnaK to have immunosuppressive effects upon multiple cell types. To exclude that the extended graft acceptance observed upon DnaK pre-treatment was due to other tumor mechanisms of immune evasion, we tested whether DnaK pre-treatment *in situ* impacted upon alloreactive responses in a fully MHC-mismatched skin graft model. We observed that DnaK-treated allografts had a significant increase in survival when compared with controls and in addition, this effect was dependent on Tregs (60, 62).

In addition to Hsp60 and Hsp70 family members, Hsp90 proteins have also been shown to have protective properties. For example, subcutaneous treatment with mouse gp96 was shown to delay skin allograft rejection in minor and major mismatch models (63). In another study, intradermal treatment of heart transplanted rats with high doses of liver-purified gp96 from the donor strain prolonged graft survival. Treatment with gp96 appeared to improve cardiac graft function immediately post-transplantation. Interestingly, this treatment did not have an effect on graft survival if gp96 was derived from the host strain (64). The author's proposed gp96 acted upon innate cells such as APCs, which led to a reduced T cell response and delayed rejection. As the delayed rejection effect was only observed when donor strain-derived gp96 was used, we would suggest that it was donor cells that were subject to the immune-suppressive properties of gp96. The author did not exclude the gp96 effects upon graft longevity could be due to its wound-healing properties (64).

## CONCLUSION AND PERSPECTIVES

There is now substantial evidence to demonstrate the immunosuppressive potential of Hsps. These studies indicate that the anti-inflammatory properties of Hsps warrant further investigation into Hsp-based treatments for contexts in which repression of immune responses is desirable. As discussed here, Hsp treatments have been effective agents to inhibit alloimmunity and extend solid organ transplant survival in mice. One could hypothesize Hsp treatments that promote a tolerogenic environment to also have therapeutic applications for various autoimmune and inflammatory diseases.

It remains to be seen how universal the application of immune-regulatory properties of Hsps can be applied to transplants of different cells and tissues. It is also important to note that Hsps were reported to amplify inflammatory (31) and immune responses to tumor antigens (65). Thus, the impact of Hsps upon the resulting immune and inflammatory response currently appears to be very much context dependent (31).

In solid organ transplantation contexts, however, most studies have indicated higher levels of intracellular and extracellular Hsps extend graft survival. This is likely due to a combination of the cytoprotective properties of Hsps enabling better survival

following IRI and subsequent reduced DAMP release and inflammation as well as the immunomodulatory effect extracellular Hsps have upon multiple cell types including macrophages, T cells, and DCs. The resulting Hsp-induced immuno-biology described has included modulation of APCs to induce tolerogenic responses and regulatory T cells and decreased alloreactive T cell generation (**Figure 1**).

Thus, *ex vivo* manipulation of organs before the transplant in order to increase Hsps levels could constitute a promising approach in decreasing initial ischemic damage and inflammation, improving organ survival. Administration of Hsp-derived peptides or *ex vivo* expansion of Treg with Hsp-derived peptides could be an alternative strategy to improve solid organ outcome. Elucidation of the innate cell subsets and the receptors by which Hsps can specifically interact with will be extremely important to optimize Hsp-based therapy design.

## AUTHOR CONTRIBUTIONS

TB, BL, RL, and CB wrote the paper. TB did the cartoon.

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REVIEW
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# The Scavenger Receptor SREC-I Cooperates with Toll-Like Receptors to Trigger Inflammatory Innate Immune Responses

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Scavenger receptor expressed by endothelial cell-I (SREC-I) is a class F scavenger receptor expressed by immune cells with a significant role in $CD8^+$- and $CD4^+$-mediated T cell immunity. This receptor can also modulate the function of toll-like receptors (TLRs), which play essential roles in innate immunity. Earlier, it was found that human monocyte/macrophage THP1 cells and bone marrow-derived macrophages from mice exhibited increased responses to polyinosine–polycytidylic acid (poly I:C, PIC) and CpG (unmethylated) DNA and enhanced production of inflammatory cytokines with overexpressed SREC-I. Our data also showed that intracellular/endocytic TLR3 and TLR9 could directly interact with SREC-I in the presence of their respective ligands. We also observed that the internalized ligand along with TLR3/TLR9 colocalized in the endosome in macrophages and THP-1 cells overexpressing these receptors. In the absence of these ligands, there was no detectable colocalization between the SREC-I and endocytic TLRs. Earlier, it was shown that SREC-I stimulated double-stranded RNA/CpGDNA-mediated TLR3/TLR9 activation of the innate immune response by triggering signaling through the NF-κB, IRF3, and MAP kinase pathways leading to transcription of cytokine genes. We also established that SREC-I can associate with plasma membrane TLRs, such as TLR2 and TLR4. We demonstrated that SREC-I–TLR4 signals more efficiently from lipid microdomain in which lipopolysaccharide (LPS) can associate with SREC-I–TLR4 complex. We also proved that SREC-I is an alternate receptor for LPS capable of internalizing the complex and for endocytic TLR ligands as well. This binding activated endocytic TLR-mediated downstream cytokine production in THP1 cells and macrophages. Finally, SREC-I could also form complexes with TLR2 and induce the release of cytokines in the presence of bacterial, viral, and fungal ligands.

**Keywords: SREC-I, TLR3, TLR4, innate immunity, adaptive immunity**

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## INTRODUCTION

Scavenger receptors constitute a large family of protein molecules, which were identified by Brown and Goldstein in the year 1979 (1, 2). Their function was first characterized as the receptors capable of scavenging oxidized low-density lipoprotein (ox-LDL) (3). While initially identified to recognize modified self-molecules, SRs have since been shown to also recognize numerous pathogen-derived molecules and regulate the ensuing immune response. SRs are categorized into

10 class types designated A–J, although with very little sequence conservation between these groups. Nevertheless, this apparent lack of homology between SR classes is not reflected by the number of ligands recognized by multiple SR members as many of the structurally distinct SRs recognize common ligands (4–7). The ability of SRs to transmit ligand-specific biological signals combined with various ligands bound by some individual SR members is both remarkable and yet to be fully understood at the molecular level.

Here, we discuss how a member of scavenger receptor family F, scavenger receptor expressed by endothelial cell-I (SREC-I), cooperates with toll-like receptors (TLRs) and modulates its downstream signal activation in response to specific ligand stimulation. Also known as SCARF-I, SREC-I is an 86-kDa protein with an extended extracellular domain, which is composed of epidermal growth factor (EGF)-like cysteine rich motifs, characteristic of the class F group of SRs (8–10). Known SREC-1 ligands include modified LDL (including oxidized, acetylated, and carbamylated forms), lipopolysaccharide (LPS), apoptotic bodies, Hsp70, Hsp90, calreticulin, gp96, and zymogen granule protein 2 (GP2) (11). In the case of apoptotic bodies, heat shock protein (Hsp)70, Hsp90, calreticulin, gp96, and GP2, SREC-I recognition leads to engulfment and/or endocytosis. In the absence of known ligands, SREC-1 was shown to promote cell–cell homophilic interactions between murine fibroblast cells, an effect that was amplified upon coculture with SREC-II-expressing cells and negated by the SREC-I ligands AcLDL and ox-LDL (9). These findings identified a potential ligand-independent role for SREC-I in cell–cell interactions, a function possessed by other SRs, such as LOX-1, which was shown to facilitate leukocyte–endothelium adhesion (12). SREC-I receptor has also been shown to induce morphological changes in neurons *via* its intracellular domain (4).

SREC-I was found to be a key receptor for HSPs and also to play a key role in immune response (13–15). It was demonstrated that HSP–tumor antigen complexes could be internalized after interacting with this receptor expressed in antigen-presenting cells such as dendritic cells. Antigens internalized in this way could later be processed and presented to T cells, thereby activating adaptive immunity. It was shown that HSP-chaperoned tumor antigen could be presented to both $CD8^+$ and $CD4^+$ T cells to activate immune response, which is also known as T cell priming (14, 15). However, further investigation is required to understand basic mechanisms and selectivity of SREC-I involved in T cell priming *via* MHCI and MHCII molecules. SREC-I, a potent antigen cross-presenting HSP receptor, is also known to induce inflammatory responses through interaction and cross-talk with another group of receptors – TLRs. One method by which microbes are detected by innate cells is through engagement of these pattern-recognition receptors (PRRs). TLRs recognize the presence of pathogen-associated molecular pattern (PAMP) molecules, leading to downstream signal transduction triggering and the activation of innate cells (16, 17). While some TLR members, such as TLR1, 2, 4, 5, and 6, can be detected on the plasma membrane and recognize components of microbial membranes, others, such as TLR3, 7, and 9, are intracellular proteins are characterized as "endosomal TLRs" and recognize nucleic acids (16, 18). In some cases, SREC-I can cooperate with TLRs in signal transduction, and this molecule was demonstrated to be important as a scavenger receptor in the control of infections (19–22). SREC-I has been shown to cooperate with multiple TLRs to transmit ligand-specific signals and to function as a potent antigen-presenting receptor for HSP-associated antigens.

## SREC-I COOPERATES WITH TLR4 TO ACTIVATE LPS-INDUCED INFLAMMATION

To date, TLR4 is the most studied TLR member. TLR4 initiates inflammatory responses upon binding to LPS of Gram-negative bacteria. In this context, transduction of the TLR4 activation signal is facilitated by adaptor molecules, such as CD14 and MD-2. CD14 is termed as a recognizing receptor for TLR4. Once activated, TLR4 recruits the TIR domain-containing adaptors (TIRAP) and MyD88 and activates transcription factors, such as NF-κB, IRF3, NF-IL6, and AP-1, that will lead to the production of pro-inflammatory cytokines (23). In some circumstances, SREC-I can recognize LPS in the absence of CD14 (24) (**Figure 1**). Upon LPS binding, SREC-I and TLR4 activate NF-κB and MAP kinases and the subsequent production of inflammatory cytokines, such as IL-6, TNF-α, and IFN-β (25). Interestingly, IL-1α, IL-1β, and TNF-α were shown to inhibit SREC-I promoter activity, potentially representing a negative feedback loop to limit inflammatory signaling during contexts where levels of inflammatory cytokines are high (26). In the presence of LPS, SREC-I was shown to cause the translocation of TLR4 to lipid microdomains on cell surface (25) where signaling event initiates. Then, this complex (LPS–TLR4–SREC-I) appeared to become internalized to intracellular endosomes (25). In the presence of LPS, SREC-I and TLR4 translocated into lipid microdomains on plasma membrane and initiated pro-inflammatory signaling events from this location (**Figure 1**). We observed their presence in lipid microdomain when isolated using Optiprep density gradient centrifugation. One could hypothesize that the localization of SREC-I to lipid microdomains in the presence of some ligands promotes SREC-I interaction with certain TLRs by concentrating their proximity in these membrane substructures. Lipid microdomains are important signaling nexi for many surface molecules (27).

## SREC-I AND ITS COLLABORATIVE FUNCTION WITH TLR2

TLR2 can recognize a broad range of bacterial-, parasitic-, viral-, and fungal-derived PAMPs (28). This receptor can form heterodimers with other cell surface TLRs, such as TLR1 and TLR6, depending on its ligand (28). TLR2 also cooperates with SREC-I in the recognition of certain pathogens. For example, SREC-I and the other c-type lectin, scavenger receptor LOX-1 induced the production of IL-6 and IL-8 in the presence of outer membrane protein A (OmpA) from *Klebsiella pneumonia*, a response dependent on TLR2 (19). SREC-I also recognized β-glucans present on

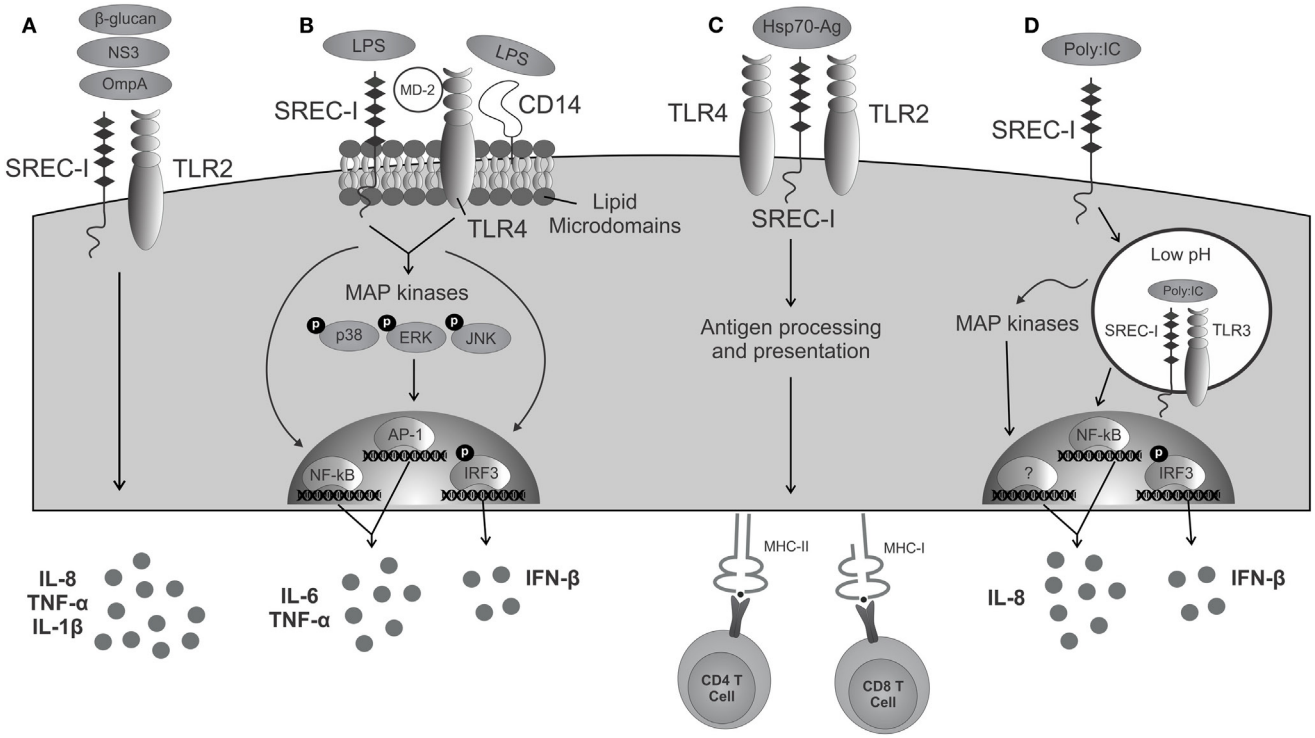


**FIGURE 1** | **SREC-I triggered immunity in cooperation with TLRs**. **(A)** Molecules derived from pathogens are recognized by both SREC-I and TLR2, activating signaling, and triggering the release of cytokines. **(B)** The complex LPS/SREC-I/MD-2/TLR4/CD14 launches an inflammatory cascade mediated by the activation of MAP kinases, transcription factors, and the presence of inflammatory cytokines. **(C)** Hsp70–Ag interacts with SREC-I/TLR complexes present on antigen-presenting cells and then becomes internalized. Acquired antigens are processed and presented to MHC class I and class II molecules activating $CD8^+$ and $CD4^+$ T cells, respectively. **(D)** SREC-I can recognize viral dsRNA such as PIC. SREC-I engagement leads to the recruitment of the complex SREC-I/TLR3/PIC to endosomes with low pH. TLR3 is then able to trigger the activation of MAP kinases, transcription factors, and the production of inflammatory cytokines.

the cell surface of fungi species *Cryptococcus neoformans* and *Candida albicans* and triggered the production of IL-1β and the chemokines, CXCL2 and CXCL1 in association with TLR2 (20). After hepatitis C virus stimulation, dendritic cells could recognize and lead to endocytosis of the non-structural protein 3 (NS3) through SREC-I and produce IL-6 in a TLR2-dependent manner (21), although there was no evidence of their direct interaction or binding.

## ENDOCYTIC TLR3 REQUIRES SREC-I FOR LIGAND RECOGNITION AND INTERNALIZATION IN SOME MONOCYTIC CELLS

During viral infections, viral double-stranded RNA (dsRNA) can be recognized by immune cell as a PAMP, indicating viral infection (29, 30). TLR3 is an intracellular member of the TLR family, present in the endosomes, and it has a unique capacity of recognizing and activation by viral dsRNA (31, 32). Once engaged by viral dsRNA, TLR3 signals through a molecular pathway that requires adaptor protein TRIF to activate transcription factors, IRF3 and NF-κB, triggering the production of type I interferon (IFN-1) and inflammatory cytokines, such as IL-8 and IL-6 (33). The EGF, ErbB1 and Btk, can phosphorylate two tyrosine residues in the cytoplasmic domain of TLR3 in order to facilitate interaction with TRIF (34, 35).

Despite most commonly being reported to reside in the intracellular endosomes, TLR3 has also been observed on the cell surface in some endothelial, epithelial, and fibroblastic cells in the presence of dsRNA and UNC93B1 (an accessory TLR protein) (36–38). The TLR3 ectodomain is required for its translocation to the plasma membrane (39). Recently, it was shown that TLR3 can interact with SREC-I in the presence of TLR3 ligand. Upon PIC (poly I:C, dsRNA) treatment, TLR3 and SREC-I can colocalize to the endosomes in THP-1 monocytes (40). The formation of the SREC-I–TLR3–PIC complex led to higher rates of NF-κB pathway activity and greater expression of phosphorylated (activated) MAP kinases p38 and c-jun kinase (JNK), along with secretion of pro-inflammatory cytokines, such as IL-8 and IL-6 (40) (**Figure 1**). Thus, TLR3 occupies SREC-I as a coreceptor and enhances its PIC-mediated activation (40).

## SREC-I IN IMMUNITY AND DISEASE: WHAT REMAINS UNKNOWN AND FUTURE DIRECTIONS

SREC-I has been characterized as a receptor for extracellular HSPs, gp96, and modified LDL. This promiscuous receptor is a

key component of innate immunity and is capable of recognizing TLR ligands, such as LPS, unmethylated DNA, or dsRNA. However, it has recently been shown to participate both in innate and adaptive immunity in endothelial cells, fibroblasts, and immune cells. In addition to internalizing HSPs, Means' group demonstrated that SREC-I can endocytose apoptotic cells by recognizing phosphatidylserine exposed on the outer leaflet of the plasma membrane and with the help of complement factor C1q (41). They also strongly reported that failure of this removal *in vivo* resulted in spontaneous development of autoimmune disease (41). It would be interesting to find out more how SREC-I can protect against autoimmunity and to understand the molecular basis for this property of SREC-I. Such work may ultimately enable modulation of SREC-I activity in contexts of autoimmunity for therapeutic benefit. Indeed, it has been shown that defective clearance of apoptotic cells by the scavenger receptors increases susceptibility to lupus (41). In addition to contexts of autoimmunity, understanding SREC-I function in apoptotic cell uptake and identifying its interacting partners will also open up the field to understanding infection-driven immune responses regulated by SREC-I. For its functional versatility and versatility in ligand, recognition makes it the scavenging Jack of all Trades.

## AUTHOR CONTRIBUTIONS

AM, TJB and BJL wrote the manuscript. SKC oversaw the study and provided intellectual input. All authors approved the manuscript for publication.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
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# Spatiotemporal Regulation of Hsp90–Ligand Complex Leads to Immune Activation

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Although heat shock proteins (HSPs) primarily play a pivotal role in the maintenance of cellular homeostasis while reducing extracellular as well as intracellular stresses, their role in immunologically relevant scenarios, including activation of innate immunity as danger signals, antitumor immunity, and autoimmune diseases, is now gaining much attention. The most prominent feature of HSPs is that they function both in their own and as an HSP–ligand complex. We here show as a unique feature of extracellular HSPs that they target chaperoned molecules into a particular endosomal compartment of dendritic cells, thereby inducing innate and adaptive immune responses via spatiotemporal regulation.

**Keywords: heat shock protein, danger signal, cross-presentation, dendritic cell, toll-like receptor, autoimmune disease**

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## INTRODUCTION

Heat shock proteins (HSPs) are known to act as molecular chaperones within cells. They are primarily considered to be intracellular proteins that have protective functions under cellular stress conditions. Recently, the existence of extracellular HSPs has been shown, and much attention has been paid to their role in stimulation of innate and adaptive immunity. Extracellular HSPs have been shown to activate innate immune responses through toll-like receptors (TLRs) and scavenger receptors (SRs) expressed on antigen-presenting cells (APCs), such as dendritic cells (DCs) and macrophages (1). Moreover, it has been demonstrated that extracellular HSPs augmented the ability of their associated molecules to activate immune responses by efficient targeting to antigen-presenting cells (2).

It is well known that immunization with an HSP–peptide complex is able to elicit peptide-specific T cell responses (2–6). However, the behavior of an HSP–peptide complex after uptake by APCs has not been completely elucidated. Presentation of an exogenous antigen to $CD8^+$ T cells is called cross-presentation (7). Cross-presentation is a process by which APCs, including DCs, uptake pathogens and dying cellular fragments and present proteolytic fragments derived from these antigens in the context of MHC class I molecules (8). This process is a fundamental mechanism of the induction of antitumor immune responses. However, it is not known how antigens are taken up and where are they destined to go and encounter MHC class I molecules. We have uncovered intracellular pathways that link the antigen internalization pathways and their processing as well as loading on MHC class I molecules (9–12). Antigenic peptides chaperoned by extracellular Hsp90 or the Hsp70 family member ORP150 are targeted to static early endosomes and processed by endosomal peptidases, followed by loading onto MHC class I. By contrast, HSP-chaperoned proteins that are required for proteasomal degradation enter both the endosomal pathway and proteasome–TAP-dependent pathway (11). Moreover, it is thought that HSP receptor-expressing APCs play a key

role in the targeting of an HSP–antigen complex into these cross-presentation pathways (13, 14). To begin with, we will describe the history and development of HSP in tumor immunology. Then, we will discuss the emerging roles of extracellular HSPs in the regulation of innate immunity and adaptive immunity with focus on how spatiotemporal regulation of HSP–ligand complexes within antigen-presenting cells affects immune responses.

## HSPs IN CANCER IMMUNOBIOLOGY: ORCHESTRATION OF INNATE IMMUNITY AND ADAPTIVE IMMUNITY

### HSPs as Tumor Antigens

Heat shock proteins are generally considered to be intracellular chaperones that are essential for maintaining cellular homeostasis. From an immunological point of view, much attention has been paid to emerging roles of extracellular HSPs as endogenous immunomodulators for innate and adaptive immune responses. Pioneering studies by Srivastava and colleagues first demonstrated tumor-specific antigenicity to Gp96, Hsp70, and Hsp90, a function associated with their ability to chaperone antigenic peptides and to activate antitumor cytotoxic T lymphocyte (CTL) responses (3, 4, 15). Immunization with tumor-derived HSPs initiates antitumor CTL responses via cross-presentation of their chaperoned peptides to MHC class I molecules (16, 17). By contrast, HSPs isolated from normal tissues are not effective, indicating that HSP-chaperoned peptides but not HSPs *per se* represent the tumor antigens recognized by antitumor CTLs (5). Immunization with high molecular weight stress proteins, such as Hsp110 and Grp170 (ORP150), also induced tumor-specific immune responses (10, 18, 19). Importantly, since HSP–peptide complexes act as exogenous antigens, they must be cross-presented after internalization by APCs to induce CTL responses. Therefore, focus was placed on elucidation of mechanisms including the pathway for cross-presentation as described later and identification of HSP-specific receptors.

### Role of HSPs and Their Receptors in Activation of Innate Immunity

Binder et al. first identified LDL receptor-related protein 1 (LRP1), also known as CD91, as a receptor responsible for cross-presentation of Gp96 expressed on APCs (20). Further examination revealed that CD91 was the common receptor for extracellular HSPs, including Gp96, Hsp70, Hsp90, and calreticulin (21). Some other receptors for HSP were subsequently identified. In summary, HSP receptors are divided into two groups: TLRs and SRs (1). TLR2 and TLR4 have been shown to function as receptors for Hsp60, Hsp70, and Gp96, leading to NF-κB activation. On the other hand, it has been demonstrated that Hsp70 can interact with at least four SRs: LRP1/CD91 (20), lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) (22), SR expressed by endothelial cells-1 (SREC-1) (23), faciclin, EGF-like, laminin-type EGF-like and link domain-containing SR-1 (FEEL-1) (23). Hsp70 can be bound at high affinity by these receptors and internalized, resulting in cross-presentation of the chaperoned antigen. Both Hsp90 and Hsp60 can also bind to LOX-1. Gp96, Hsp90, and calreticulin show significant binding affinity to SREC-1 and are internalized by these receptors (24).

### Role of HSPs in Activation of Innate Immunity

Matzinger proposed that the host releases endogenous signals (danger signals) that are derived from stressed or damaged cells, leading to the stimulation of immunity (the so-called danger theory) (25). Rock's group expanded this research area (26). As danger signals, danger-associated molecule patterns (DAMPs), such as uric acid (27, 28) and pathogen-associated molecular patterns (PAMPs), such as LPS (26), have been identified.

During the course of identification of HSP receptors, in addition to their role in adaptive immunity, it has been reported that extracellular HSPs act as potent activators of innate immunity, indicating HSPs act as danger signals. Hsp60, Hsp70, Hsp90, and Gp96 have been demonstrated to stimulate TLR4 to produce inflammatory cytokines, including TNF-α and IL-12 (29). As described previously, many HSPs bind to TLR4 and stimulate production of TNF-α, IL-1β, and IL-6 via the NF-κB pathway.

### Immunogenicity of Secreted HSP

How do intracellular HSPs act as extracellular proteins? Various mechanisms have been proposed for the release of HSPs into extracellular milieu, including passive release such as that by cell necrosis caused by exposure to hypoxia, severe trauma and lytic virus infection, and active release mechanisms, Asea et al. demonstrated that IFN-γ and IL-10 induce the active release of constitutively expressed Hsc73 as well as Hsp72 from tumors (30). Moreover, Asea et al. showed that Hsp72 is also secreted in the form of exoxomes (31, 32). Mambula et al. showed that a prostatic cancer cell line secreted Hsp72 via an endolysosomal pathway (33). It would be interesting to know whether these secreted HSPs show antitumor immunogenicity.

Taking advantage of the ability of an HSP to target a chaperoned antigen peptide to APCs and elicit cross-presentation, immunotherapy using secretable forms of HSP has been developed. Yamazaki et al. demonstrated that Gp96 secreted from tumor cells carries an antigenic peptide and induces peptide-specific CTL responses (34). We also showed that tumor-derived secretable BiP elicits antigen-specific tumor immunity (35). This secreted BiP is taken up by DCs and a BiP-chaperoned antigenic peptide is cross-presented in association with MHC class I molecules, leading to CTL responses. Thus, this strategy allows tumor cells to produce their own cellular vaccine. Moreover, this strategy may be superior to a peptide vaccine strategy because single peptide-based cancer vaccines have a disadvantage. Namely, vaccination with a single peptide induces a certain HLA-restricted CTL response, thereby allowing tumor cells to escape from CTL recognition. By contrast, since a broad-spectrum antigenic peptide repertoire is associated with HSPs, induction of CTLs against multiple antigens is expected. Furthermore, a secreted HSP-based cancer vaccine is applicable for all patients, regardless of HLA restriction. Thus, gene modification of HSPs for secretion may provide a unique therapeutic approach for cancer immunotherapy.

## HSPs NAVIGATE THE ASSOCIATED ANTIGEN INTO STATIC EARLY ENDOSOMES IN ANTIGEN-PRESENTING CELLS FOR CROSS-PRESENTATION

As described above, immunization with purified tumor-derived HSPs or HSPs complexed with an antigen peptide/protein *in vitro* elicits tumor- or antigen-specific $CD8^+$ T cell responses (4–6, 17, 35, 36). Importantly, Hsp70- and Gp96-antigenic peptide complexes facilitate antigen presentation in association with MHC class I molecules through a cross-presentation pathway (37–39). Cross-presentation is a prerequisite antigen-presentation pathway for the induction of CTL responses against viral infection and tumors. However, the precise mechanism for introduction of an exogenous antigen into a cross-presentation pathway remains unclear. Exogenous antigens can be processed through at least two distinct pathways (10, 11): one is a transporter-associated antigen-presenting (TAP)-dependent pathway, which is a classical MHC class I loading pathway, and the other is post-Golgi loading of MHC class I in endocytic compartments (endosome-recycling pathway). Cytosolic Hsp90 has been shown to translocate extracellular antigens from endosome to cytosol for TAP-dependent cross-presentation (40). In the latter pathway, internalized exogenous antigens are processed by endosomal peptidases, such as a cathepsin S, and thereafter are loaded in endocytic compartments onto MHC class I molecules that are recycled from plasma membranes (8, 41, 42).

Recently, Calderwood's group and we showed that Hsp90 also acted as an excellent navigator for chaperoned antigens to enter the cross-presentation pathway in a murine system (9, 11, 43). Furthermore, we showed that Hsp90–peptide complex-mediated and Hsp70 family member ORP150–peptide complex-mediated cross-presentation was independent of TAP and was sensitive to membrane recycling inhibitor primaquine, indicating that sorting of peptides onto MHC class I occurs via an endosome-recycling pathway (10). We further demonstrated that the Hsp90–cancer antigen peptide complex was efficiently cross-presented by human monocyte-derived dendritic cells (Mo-DCs) and stimulated peptide-specific CTLs (12). More importantly, we showed that translocation of Hsp90–Ag complex into the "static" early endosome after endocytosis was crucial for efficient cross-presentation. Lakadamyali et al. (44) demonstrated that early endosomes are comprised of two distinct populations: one is a population of dynamic early endosome that are highly mobile on microtubules and mature rapidly toward the late endosome, and the other is a population of static early endosomes that mature much more slowly. Cargos destined for degradation, including LDL, EGF, and influenza virus, are internalized and targeted to $Rab5^+$, $EEA1^-$ dynamic early endosomes, followed by trafficking to $Rab7^+$-late endosomes. By contrast, the recycling ligand transferrin is delivered to $Rab5^+$, $EEA1^+$-static early endosomes, followed by translocation to $Rab11^+$-recycling endosomes. Burgdorf et al. clearly showed that a mannose receptor translocated OVA specifically into an $EEA1^+$, $Rab5^+$-static early endosomal compartment for subsequent cross-presentation (45). By contrast, pinocytosis conveyed OVA to lysosomes for MHC class II presentation. In addition, OVA endocytosed by a SR did not colocalize with EEA1 but colocalized with LAMP-1 in lysosomes, resulting in a presentation in the context of MHC class II molecules. We showed that the Hsp90/ORP150–peptide complex is targeted into $Rab5^+$, $EEA1^+$-early endosomes after internalization by human Mo-DCs, suggesting that preferential delivery to the "static" endosome is required for cross-presentation of Hsp90/ORP150–peptide complexes (10, 12). By contrast, LDL protein was targeted to the $EEA1^-$, $Rab5^+$, and $LAMP\text{-}1^+$-dynamic early endosome–late endosome/lysosome pathway, leading to degradation. These findings suggested that Hsp90/ORP150 navigated the chaperoned antigen peptide into the static early endosome-recycling pathway, preventing extensive degradation of the peptide, followed by transfer of the peptide onto recycling MHC class I molecules within the recycling endosome. Taken together, our findings indicate that the role of Hsp90/ORP150 in cross-presentation is to shuttle the associated antigen into static early endosomes within DCs. Thus, Hsp90/ORP150 is a promising natural immunoactivator for a cancer vaccine due to its excellent ability to target human DCs and to induce specific CTLs (**Figure 1**).

## Hsp90-MEDIATED SPATIOTEMPORAL REGULATION IN INNATE IMMUNITY

In contrast to the idea that HSP itself acts as an endogenous danger signal, we have shown that HSP empowers the chaperoned innate ligands to activate an innate immune response via spatiotemporal regulation (46). Unmethylated single-stranded DNA containing a cytosine–phosphate–guanine (CpG) motif is recognized by TLR9, which is expressed primarily by plasmacytoid DCs (pDCs) and B cells, resulting in a large amount of IFN-α production (47, 48). Two classes of synthetic CpG-DNA have been classified: CpG-A, which stimulates to produce IFN-α by pDCs, and CpG-B, which does not. Instead, CpG-B stimulates IL-6 and TNF-α production by pDCs. It has been shown that the manner of internalization and the retention time in endosomes of these CpG-DNAs were

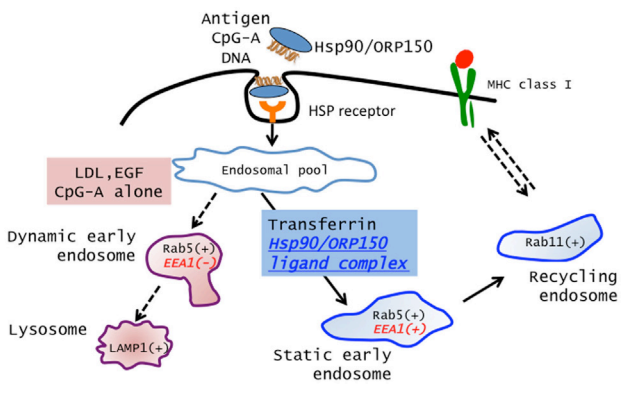


**FIGURE 1 | Extracellular Hsp90/ORP150 targets chaperoned molecules into static early endosome**. Hsp90/ORP150–ligand complexes are preferentially targeted and retained in static early endosome ($Rab5^+$ and $EEA1^+$) but not in dynamic early endosome ($Rab5^+$ and $EEA1^-$).

different. CpG-A forms large multimeric aggregates with a diameter ~50 μm. By contrast, CpG-B is monomeric and does not form such high order structure. The retention of the CpG/TLR9 complex in endosomes is the primary determinant of TLR9 signaling. The multimeric CpG-A retains for longer periods of time in the early endosomes, whereas monomeric CpG-B rapidly translocates from early endosomes to late endosomes or lysosomes of pDCs (49, 50). The prolonged retention of CpG-A provides extended activation of TLR9 signaling, which leads to robust IFN-α production by pDCs.

Based on the finding that Hsp90 can target and retain chaperoned ligands within static early endosomes, we showed that human pDCs pulsed with an Hsp90–CpG-A DNA complex produce a larger amount of IFN-α than that in the case of CpG-A alone (46). Moreover, unlike human DCs, since murine conventional DCs (cDCs) express both TLR7 and TLR9 (49–51), the Hsp90-chaperoned CpG-A was retained within static early endosomes for longer periods (more than 2 h) in murine cDCs, thereby leading to sustained activation of murine cDCs and eliciting TLR9 signaling for IFN-α production. The observed IFN-α production was TLR9-dependent because cDCs isolated from TLR9 knockout mice did not produce IFN-α. By contrast, CpG-A alone was trafficked to late endosomes and lysosomes within cDCs. Interestingly, not only CpG-A but also CpG-B when chaperoned by Hsp90 could stimulate TLR9 signaling via targeting and longer retention of CpG-B within static early endosomes, resulting in the production of IFN-α. Thus, extracellular Hsp90 has the extraordinary ability to directly associated CpGs into static early endosomes, leading to IFN-α production (52). Thus, the use of extracellular HSPs may augment the effect of a cancer vaccine in combination with CpG. More importantly, extracellular HSPs might play a pivotal role in the pathogenesis of nucleic acid-mediated autoimmune diseases, such as systemic lupus erythematosus (SLE).

## EXTRACELLULAR Hsp90–SELF-DNA COMPLEX BREAKS INNATE TOLERANCE VIA SPATIOTEMPORAL REGULATION

Viral/bacterial DNA sequences contain multiple CpG nucleotides that bind and activate TLR9. By contrast, pDCs normally do not respond to self-DNA because mammalian self-DNA contains fewer such motifs, which are most likely masked by methylation (47, 48, 53). Moreover, it has been suggested that self-DNA fails to access the TLR9-containing endolysosomal compartments and is thereby unable to stimulate TLR9 (54). One of the mechanisms is that DNase easily and rapidly breaks down extracellular DNA, thereby preventing self-DNA localization into endocytic compartments. The importance of this mechanism for inhibiting autoimmune responses has been shown by the fact that mice deficient in DNase II develop SLE-like syndrome (55). Recent evidence, however, suggests that self-DNA has the potential to trigger TLR9 when self-DNA is engaged TLR9 appropriately (56, 57). Mammalian DNA in the form of chromatin-containing immune complexes could stimulate TLR9 in association with B-cell receptors in an *in vitro* study (58, 59). Breakdown of innate tolerance to self-nucleic acids occurs when tissue injury or necrosis causes the release of some endogenous molecules, including antimicrobial peptides, such as LL37 (60) and nuclear protein HMGB-1, which help to promote stabilization and delivery of associated innate ligands, including nucleic acids, into early endosomes (61). These molecules have been shown to play a critical role in the initiation of autoimmune diseases through the production of IFN-α.

As described above, since Hsp90 can bind DNA (62), we examined whether Hsp90 targets self-DNA into static early endosomes, resulting in IFN-α production by human pDCs (46). Upon Hsp90-mediated enforced endosomal translocation as well as longer retention, human self-DNA could activate DCs via TLR9 to produce IFN-α. Thus, Hsp90 regulates TLR9-mediated responses through spatiotemporal regulation of Hsp90-chaperoned ligand complexes (**Figure 2**). Therefore, targeting TLR9 and modulating TLR9 signaling using Hs90 inhibitors or siRNA to Hsp90 have emerged as important strategies for the treatment of self-nucleic acid-related autoimmune diseases, including SLE. In the following section, we will discuss the emerging topic for the role of extracellular and intracellular Hsp90 in the pathogenesis of SLE.

## ROLE OF Hsp90 IN SLE

### Extracellular Hsp90 Plays a Pivotal Role in the Pathological Condition of SLE

Systemic lupus erythematosus is a prototype of autoimmune disease characterized by the production of autoantibodies specific for self-nuclear antigens, such as dsDNA and RNA-containing antigens, including Sm and RNP (63, 64). Recently, it has been demonstrated that a prominent feature of SLE is increased expression of type I IFN-regulated genes in blood cells, which is often associated with increase of IFN-α in the serum (65–67).

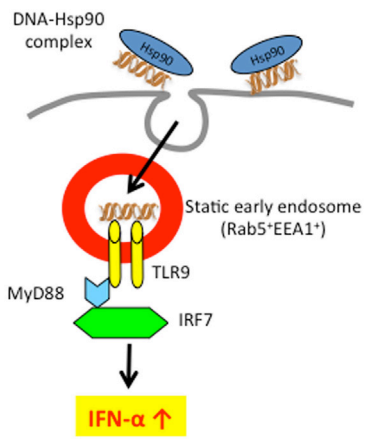


**FIGURE 2 | Extracellular self-DNA stimulates pDC to produce IFN-α when chaperoned by Hsp90 via spatiotemporal regulation.** Hsp90–self-DNA complex found in SLE patient's serum is targeted to pDCs and retained for longer periods within early static endosome via action of Hsp90, leading to production of IFN-α.

Moreover, it has been shown that elevated IFN-α in SLE patient's serum accelerates the disease severity of SLE and is associated with disease activity (68–71).

Plasmacytoid DCs are major producers of IFN-α (72), and are decreased in number in the blood but are abundant in skin and lymph nodes (67). In SLE patients, immune complexes consisting of autoantibodies bound to self-DNA and RNA can stimulate production of IFN-α through TLR9 and TLR7 after uptake via Fc receptors (73). The pathogenic role of IFN-α in SLE is mediated partly by its ability to induce immune activation, including a positive feedback loop that induces plasma cell maturation and increases autoantibody production (74). The role of IFN-α in this disease has now been confirmed in lupus-prone mouse models (75, 76).

Previous studies have demonstrated the presence of autoantibodies to Hsp90 (77, 78) and showed enhanced expression of Hsp90 in peripheral blood mononuclear cells of patients with active SLE (79, 80), suggesting a role of Hsp90 in SLE. In addition, Hsp90 has been shown to localize both in the cytoplasm and nucleus (81). Under stressful conditions, cytosolic Hsp90 translocates to the nucleus (82). This suggests that Hsp90 may bind self-DNA within the nucleus. When cells undergo necrosis, self-DNA associated with endogenous Hsp90 can be released into the extracellular milieu and might trigger IFN-α production by pDCs. In fact, we found that serum levels of Hsp90 were significantly increased in patients with active SLE compared with the levels in patients with inactive SLE or other autoimmune diseases (83). Serum Hsp90α levels increased with increase in SLEDAI score. Moreover, serum Hsp90α in patients with SLE was significantly decreased after treatment. Of interest, extracellular self-Hsp90 found in SLE sera could stimulate IFN-α production by pDCs. We also found that extracellular self-Hsp90 associated with self-DNA or an anti-DNA antibody–self-DNA complex (**Figure 2**). This Hsp90–self-DNA complex or Hsp90–anti-DNA autoantibody–self-DNA complex might be efficiently endocytosed and targeted to early endosomes via the action of Hsp90, leading to the robust IFN-α production observed in SLE sera (46). Moreover, immunodepletion of extracellular Hsp90 from SLE serum decreased IFN-α production by pDCs (83), indicating that depletion of Hsp90 might induce remission and prevent end-organ damage. Thus, control of deregulated pDC activation and IFN-α production provides an alternative treatment strategy for SLE.

Collectively, extracellular Hsp90 plays a crucial role in the disease activity of SLE and that Hsp90 inhibitors have promise for the treatment of IFN-α-mediated autoimmune diseases including SLE.

## Hsp90 Chaperones TLR7/9 to Sense Self-Nucleic Acids

The localization of TLR7/9 is unique, because they reside in the ER at the quiescent stage and traffic to endosomes only upon ligand recognition (60). The underlying mechanism for adequate transportation of these TLRs has been investigated. Recently, Unc93B1, a multitransmembrane ER-resident protein, has been shown to associate with and deliver TLR7/9 from the ER to endosomes, where TLR7/9 recognize their ligands (84). In addition, gp96, an ER-resident HSP, has been shown to be a master immune chaperone for both cell surface and intracellular TLRs, including TLR1, 2, 4, 5, 7, and 9 (85). Furthermore, ER luminal protein PRAT4A (also known as CNPY3) also translocates TLR1, 2, 4, 5, 7, and 9 to either cell surfaces or endolysosomes (86). Recently, it has been shown that gp96 cooperates with PRAT4A for the trafficking of theses TLRs (87).

We showed that endogenous Hsp90 also had a crucial role on the production of IFN-α in response to CpG-A by human pDCs using the Hsp90 inhibitor 17-AAG (83). The 17-AAG treatment dramatically inhibited the production of IFN-α. Further studies revealed that Hsp90 interacted with TLR7/9 and, more

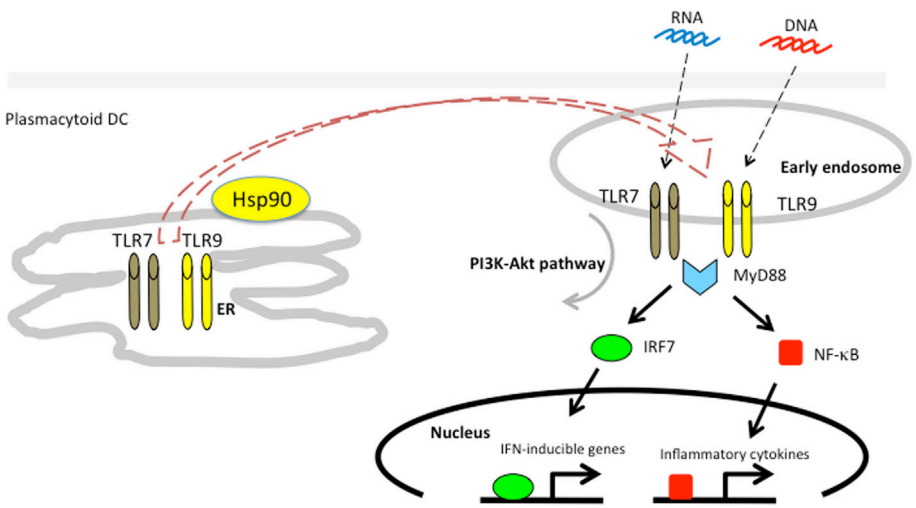


**FIGURE 3 | Hsp90 associates with toll-like receptors 7/9 and mediates self-nucleic acid recognition in plasmacytoid DCs**. Hsp90 has a spatial interaction with TLR7/9 and chaperones them from the ER to the early static endosome.

importantly, that Hsp90 chaperoned TLR7/9 from the ER to the early endosome (**Figure 3**). Inhibition of Hsp90 by 17-AAG resulted in disruption of the interaction of Hsp90 with TLR7/9, leading to inhibition of IRF7 phosphorylation and nuclear localization, which impaired the production of IFN-α. Thus, Hsp90 is a cytosolic chaperone for TLR7 and TLR9 and is essential for TLR7/9-mediated innate immune responses (83).

### Possible Therapeutic Use of an Hs90 Inhibitor in SLE

The involvement of IFN-α in the pathogenesis of SLE indicates the possibility of therapeutically targeting either IFN-α or the mechanisms leading to IFN-α production. We examined the effect of Hsp90 inhibitor in SLE-prone MRL/lpr mice (83). Treatment with the Hsp90 inhibitor 17-DMAG significantly ameliorated disease activity as well as histopathological findings in diseased mice. The dramatic decrease in severity of SLE seemed to be due to simultaneous blocking of TLR7 and TLR9 signaling by the Hsp90 inhibitor. Shimp et al. also showed that administration of 17-DMAG ameliorated SLE symptoms (88). Interestingly, similar to patients with SLE, serum Hsp90 in SLE-developed mice was clearly decreased in the 17-DMAG-treated group compared with that in the untreated group. Thus, our results indicate that both intracellular Hsp90 and extracellular Hsp90 play a crucial role in the pathogenesis of SLE and that Hsp90 inhibitors have promise for the treatment of IFN-α-mediated autoimmune diseases including SLE.

## FUTURE PROSPECTS OF HSPs IN IMMUNOLOGY

Our understanding of the mechanisms by which extracellular HSPs play pivotal roles in the regulation of innate immunity and adaptive immunity has been progressing at a very fast pace. We here focus on the extraordinary ability of Hsp90/ORP150 to target chaperoned molecules into static early endosomes after being taken up by DCs. Several receptors specific for HSPs expressed on DCs have been identified (21, 43). However, it has been unclear whether these receptors contribute to the sorting of HSP-chaperoned molecules into static endosomes. Moreover, it should be clarified whether HSPs other than Hsp90/ORP150 act in a fashion similar to Hsp90/ORP150. By clarifying these issues, HSPs will open innovative therapeutic opportunities in cancer and autoimmune diseases.

## AUTHOR CONTRIBUTIONS

YT wrote the paper. AY, NT, and KS conducted experiments and analyzed data.

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REVIEW
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# Unfolding the Role of Large Heat Shock Proteins: New Insights and Therapeutic Implications

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Heat shock proteins (HSPs) of eukaryotes are evolutionarily conserved molecules present in all the major intracellular organelles. They mainly function as molecular chaperones and participate in maintenance of protein homeostasis in physiological state and under stressful conditions. Despite their relative abundance, the large HSPs, i.e., Hsp110 and glucose-regulated protein 170 (Grp170), have received less attention compared to other conventional HSPs. These proteins are distantly related to the Hsp70 and belong to Hsp70 superfamily. Increased sizes of Hsp110 and Grp170, due to the presence of a loop structure, result in their exceptional capability in binding to polypeptide substrates or non-protein ligands, such as pathogen-associated molecules. These interactions that occur in the extracellular environment during tissue injury or microbial infection may lead to amplification of an immune response engaging both innate and adaptive immune components. Here, we review the current advances in understanding these large HSPs as molecular chaperones in proteostasis control and immune modulation as well as their therapeutic implications in treatment of cancer and neurodegeneration. Given their unique immunoregulatory activities, we also discuss the emerging evidence of their potential involvement in inflammatory and immune-related diseases.

**Keywords: heat shock protein 110, glucose-regulated protein 170, cytoprotection, innate immunity, antigen cross-presentation, inflammatory disease**

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## INTRODUCTION

Heat shock proteins (HSPs) are constitutively expressed in all living organisms. The HSP nomenclature, originated from the observation of their induction by thermal stress, may also be used to describe other stress-inducible proteins, such as glucose-regulated proteins or GRPs (1–3). In contrast to cytoplasm-resident HSPs, GRPs mainly reside in the endoplasmic reticulum (ER). The mammalian HSPs are classified into several families of proteins based on their molecular size. Hsp110 and Grp170 are high molecular weight HSPs, which have been less studied compared to other conventional HSPs, e.g., Hsp70 and Hsp90 (4, 5). The transcription and subsequent protein expression of Hsp110 or Grp170 are stimulated by cytotoxic or proteotoxic stresses, such as heat

shock, oxidative stress, chemical agents, hypoxia, viral infections, and inflammation. In such situations, these large HSPs work in concert with other chaperone molecules (e.g., Hsp70, Grp78) to restore protein folding and cellular function to counter proteotoxic stresses and promote cell survival (6–8). This intrinsic chaperoning feature of the large HSPs has significant implications in several diseases including neurodegeneration and cancer.

Given the superior protein-holding activity of these large HSPs and their unique ability to interact with the immune system, Hsp110 and Grp170 have been used to induce antigen/tumor-reactive immune response for cancer eradication (5, 9, 10). These large HSPs can broadly chaperone antigens for presentation to T lymphocytes and amplify inflammatory signals in the extracellular environment, suggesting a potential endogenous immunostimulatory effects of these large HSPs during tissue stress or injury (e.g., microbial infection). Although the mechanism of their intracellular or extracellular action still needs to be elucidated, emerging evidence indicates that these large HSPs may be involved in regulation of an inflammatory response and bridging innate and adaptive immunity, which may contribute to the pathogenesis of inflammatory and immune-related diseases. Here, we review the accumulating evidence of multifaceted functions of these large HSPs under physiological or pathological conditions and discuss the potential applications that exploit the distinct activity of the large HSPs to achieve therapeutic benefits.

## THE LARGE HSPs: DISTRIBUTION, INDUCTION, AND REGULATION

Hsp110, also referred to as Hsp105, is one of the major eukaryotic HSPs and a member of conservative Hsp70 superfamily (4, 5). The studies of Hsp110 have been largely ignored until the cloning of its cDNA in the early 1990s (11–13). Hsp110 has been well characterized in mammals (i.e., human, mouse, and hamster) (11, 14–19). There appears to be two forms of Hsp110, 105α, and 105β (43 fewer amino acids than 105α) (14). Hsp105α is expressed constitutively in the cytoplasm of cells and can be further induced by heat shock and other stressors, whereas Hsp105β, an alternatively spliced form of Hsp105α specifically localized in the nucleus, is strictly heat-inducible (20). However, the differential roles potentially played by these two versions of Hsp110 remain not well studied given their distinct compartmentalization. As one of the major HSPs, Hsp110 is easily detectable in most mammalian cell lines and tissues (11, 21). Hsp110 is constitutively expressed in the various tissues, e.g., brain, liver, ovary, spleen, heart, lung, small intestine, and muscles, but most abundant in the brain (13, 14, 22). Interestingly, mammalian cerebellum expresses little Hsp110 compared to other brain regions (13). It is not clear if the lack of Hsp110 expression contributes to the high sensitivity of the cerebellum to heat stroke or alcohol-associated toxicity (23, 24).

Hsp110 along with other HSPs could be induced in a specific set of stress conditions including hyperthermia, ethanol, oxidative reagents, recovery from anoxia (i.e., reperfusion injury), and inflammation (25, 26). Studies of heat shock factor (HSF) knockout mice indicated that the transcription factor HSF1 is required for induction of Hsp110, Hsp70, and Hsp25/27, by thermal stress (27). Exposure of BALB/c mice to fever-range whole body hyperthermia (39.5–40°C for 6 h) enhanced Hsp110 expression in the lung, lymph nodes, and thymus (28). However, thermal stress did not appear to influence the Hsp110 levels in the rat nervous system (29, 30). Peptide mapping analysis and use of various deletion or substitution mutants revealed that Hsp110 is phosphorylated at Serine (509) in the β-sheet domain by casein kinase II (31). An early study reported that Hsp110 suppressed 70-kDa heat-shock cognate protein (Hsc70)-mediated protein folding, while the phosphorylation of Hsp110 diminished its inhibitory activity *in vitro* (32). Intriguingly, phosphorylated Hsp110 is especially prominent in the brain compared to other tissues of mice or rats, suggesting that the phosphorylation of Hsp110 may be physiologically significant (33).

Like other HSPs reported to affect the morphologic development of cells and organisms (34), Hsp110 is also important for reproductive and embryonic development. Fujita and coworkers cloned two Hsp110 cognate cDNAs from testis, designated apg-1 and apg-2 (17, 35, 36). Apg-1 is developmentally expressed in human testicular germ cells and sperm, suggesting its potential role in spermatogenesis and fertilization (17, 35, 37). Injection of antisense oligodeoxynucleotides targeting Hsp110 into the rat uterine horn on day 3 of pregnancy substantially reduced the number of the implanted embryos (38), implicating a physiological role for Hsp110 in regulation of reproduction. Indeed, Hsp110 displayed a differential expression pattern associated with the development of palate in mouse embryo (39). A recent study showed that Hsp110 plays an essential role in embryonic development of mouse hindlimbs (40). Strikingly, the integrity of Hsp110 in β-catenin degradation complex is required for Wnt signaling pathway, which can lead to embryonic defects upon abnormal activation (41, 42).

Grp170, also known as oxygen-regulated protein 150 (Orp150) or hypoxia-upregulated protein 1 (HYOU1), was first described as a 170 kDa molecule inducible by glucose starvation (43). Hypoxia was soon found as a physiological inducer of Grp170 (44–48). Cloning and sequence analysis of Grp170 indicated that Grp170 represented a new stress protein family (12). Although they have a somewhat greater degree of sequence similarity to the Hsp110 family sequences than to members of the Hsp70 family, the Grp170s of mammals are essentially as diverged from the Hsp110 family of proteins as they are from the Hsp70s (4, 12, 49, 50).

Similar to other ER-resident GRPs (e.g., Grp78, Grp94/gp96), Grp170 possesses a C-terminal ER retention sequence, and therefore localizes in the ER after protein synthesis. Grp170 is inducible by glucose starvation, chronic anoxia/hypoxia, calcium depletion, low pH, a variety of reducing conditions, stress-induced activation of the hypothalamic–pituitary–adrenal (HPA) axis, and viral infection that perturb the ER function (44, 51, 52), suggesting that Grp170 participates in quality control of protein folding in the ER. Grp170 is co-regulated with Grp78 and Grp94/gp96 by the unfolded protein response (UPR) (53). Transcription of these UPR-inducible genes is regulated by ER stress–response elements (ERSE) and key transcription factors such as the X-box DNA binding protein 1 (XBP-1) and ATF6 (54–57). In addition,

Grp170 was also found to localize in the mitochondria and could be upregulated by CHOP/GADD153 in response to mitochondrial and ER stress (58).

Although HSPs are generally considered to be intracellular proteins, they can be mobilized to the plasma membrane or released into the extracellular environment (59). HSPs do not seem to be secreted from cells *via* the classical pathway since they do not possess a signal peptide. Nonetheless, HSPs may be released from cells through either a passive (e.g., cell injury) or an active (translocation to the membrane for secretion) process. Hightower et al. first reported that rat embryo cells upon heat-shock rapidly released Hsp70 and Hsp110 (60). The release of Hsp110 from human intestinal epithelial cells (IECs) was also observed in the physiological process of epithelial renewal, and this release was further increased upon pathological cell death (61). It was shown that Hsp110 preferentially associated with and stabilized misfolded protein in both the ER and cell periphery (62), suggesting Hsp110-based chaperoning activity may be involved in protein homeostasis at different locations. Recently, presence of Hsp110 on the plasma membrane of human B-cell non-Hodgkin lymphoma (B-NHL) cell lines was reported to correlate to the aggressiveness of lymphoma (63). Interestingly, Grp170 was identified on the surface of human sperm, implicating its potential physiological role in reproduction and fertilization (64). Additionally, Grp170 was found to be released from human cancer cells to the circulating system in patients with a range of neoplasms (65). Our study showed that both Hsp110 and Grp170 can gain access to the extracellular space upon cell necrosis and exhibit immunoregulatory activity (66). Although the biological significance of these surface or extracellular Hsp110 and Grp170 remain to be determined, these large HSPs are likely to exhibit multifaceted effects at cellular sites under physical and pathological conditions.

The constitutively expressed Hsc70, also known as HSPA8, and stress-inducible Hsp70 are central players in proteostasis control, including *de novo* folding, refolding of stress-denatured proteins, oligomeric assembly, protein trafficking, and proteolytic degradation (67). It has long been recognized that Hsp110 exists in multi-protein complexes containing Hsc70/Hsp70 (68–70) and Grp170 associates with other major GRPs (e.g., Grp78, Grp94/gp96) in the ER (51, 71), suggesting coordinated activities of chaperones in the maintenance of protein homeostasis. Our studies demonstrate that Hsp110 and Grp170 are highly effective in inhibiting the aggregation of heat-denatured protein substrates (50, 68, 72–74). The genetic evidence also came from the observation that the Hsp110 deficiency impaired the refolding of heat-denatured luciferase in mammalian cells (75). Not surprisingly, Hsp110, Hsp70, and Hsp40 constitute a disaggregase machinery with capacity to efficiently disaggregate and refold aggregates of chemically or thermally denatured protein (76, 77), further highlighting a role of large HSP in molecular chaperoning and protein homeostasis. Hsp110 (78–81) and Grp170 (82–84) were recently proposed to serve as a nucleotide exchange factor (NEF) for Hsc70/Hsp70 and Grp78, respectively, during ATP hydrolysis of chaperoning cycle. Given the high efficiency of Hsp110 or Grp170 in protein holding, and their differential binding preferences for peptide residues compared to Hsc70/Hsp70 (85), we believe that Hsp110 or Grp170 do not function solely as a co-chaperone or a NEF in cellular functions.

## LARGE HSP-MEDIATED CYTOPROTECTION AND TISSUE HOMEOSTASIS

The intrinsic chaperoning property of large HSPs primarily contributes to their cytoprotective functions under stress conditions. Other mechanisms underlying their pro-survival activity may involve HSP-mediated suppression of the intracellular apoptotic pathways (86). We have demonstrated that both Hsp110 and Grp170 are significantly more efficient than Hsc70/Hsp70 in stabilizing and preventing irreversible aggregation of heat-damaged protein (50, 68, 72–74), which may be attributed to the large size of these two chaperones due to the substantial expansion of their C-terminuses (4, 5). The overexpression of Hsp110 in Rat-1 and HeLa cells conferred partial thermotolerance and promoted cell survival to a severe heat shock (68). Eroglu et al. recently reported that Hsp110-deficient mice exhibit more serious brain damage and edema in controlled cortical impact model for traumatic brain injury (TBI) compared to wild-type counterparts (22). Enhancing the expression of the Hsp110 by celastrol treatment effectively reduced injury at the impact site (22), suggesting a potential benefit of using drugs to induce Hsp110 for reducing the pathological effects of TBI. Similarly, Grp170 was highly upregulated in astrocytes undergoing hypoxic stress (47), suggesting a cellular protective role of Grp170. Grp170 overexpression in cultured neurons increased their resistance to hypoxic/ischemia stress, whereas astrocytes with reduced Grp170 expression became vulnerable (87). Upregulation of Grp170 also effectively ameliorated hepatic ER stress and hypercholesterolemia-related liver damage (88).

The accumulation of misfolded or aggregated protein that is non-functional and cytotoxic has been implicated in the pathogenesis of neurodegenerative diseases (89). While it has been well established that conventional HSPs (e.g., Hsp70 or Hsp90) suppress the neurotoxicity associated with protein misfolding both *in vitro* and *in vivo*, emerging evidence indicates an essential neuroprotective effect of large HSPs. Mutant human Cu/Zn superoxide dismutase 1 (SOD1) is associated with motor neuron toxicity and cell death in an inherited form of amyotrophic lateral sclerosis (ALS; Lou Gehrig disease). Song et al. showed that Hsp110 could prevent the aggregation of misfolded SOD1 species, resulting in abrogation of the neuron toxicity by rescuing the transport defect in transgenic mice and in axoplasm isolated from squid giant axons (90). A growing body of evidence suggests that soluble alpha-amyloid precursor protein (sAPPalpha), a cleavage product of APP, has neurotrophic and neuroprotective properties in Alzheimer disease (91). A recent study reported that Grp170 induced by sAPPalpha is part of this neuroprotective response (92). Erogu et al. provided first *in vivo* genetic evidence supporting the role of Hsp110 in pathogenesis of Alzheimer's disease (93). It was shown that Hsp110-deficient mice exhibited accumulation of hyperphosphorylated-tau and neurodegeneration. Crossing Hsp110-deficient mice with animal strain overexpressing mutant

APP resulted in selective appearance of insoluble amyloid β42. Analysis of brain tissues from patients with Alzheimer's disease showed the expression of Hsp110 in close proximity to Aβ plaques. Together, these results underscore an essential role for Hsp110 in maintaining the proper folding environment that is required for phosphorylation/dephosphorylation of tau and APP processing *in vivo*.

High expression of HSPs that has been well documented in a wide range of human cancer cells supports their crucial role in proliferation and survival of tumor cells. Moreover, the levels of HSPs have been proposed to be useful prognostic biomarkers for tumorigenesis in some cancers (94, 95). Hsp110 is one of the most highly upregulated proteins in a variety of human cancers (96) and can suppress cancer cell apoptosis by preventing the release of cytochrome *c* from the mitochondria (97–99). The small interfering RNA-mediated downregulation of Hsp110-sensitized human cancer cells to apoptotic induction (99). Yu et al. recently provided new insight into the molecular mechanism of Hsp110 overexpression in tumorigenesis (41). Hsp110 expression was correlated to upregulation of β-catenin and transcription of Wnt target genes in many cancers, including colorectal cancer and breast cancer. Knockdown of Hsp110 disrupted the integration of PP2A into the β-catenin degradation complex, resulting in degradation of β-catenin and inhibition of proliferation of colon cancer cell lines that harbor adenomatous polyposis coli (APC) mutation (41). Given that the majority of colon cancer patients have APC mutations and active Wnt/β-catenin signaling, these findings establish Hsp110 as a prognostic biomarker and as a potential therapeutic target for the treatment of colon cancer (100, 101).

## LARGE HSPs IN INNATE IMMUNITY

Several reports have documented the effect of endotoxin free large HSPs (i.e., Hsp110 and Grp170) in inducing co-stimulatory signals and cytokine responses in innate immune cells, such as dendritic cells (DCs) (102, 103). Tumor cells engineered to produce a secretable form of Grp170-stimulated DCs to produce the pro-inflammatory cytokine TNF-α, which supports the extracellular Grp170 as a potential immunostimulatory "danger" molecule (104). Additionally, Hsp110 also induced mouse mammary carcinoma cells to elevate expression of IL-6, IL-12, and CD40 (102). A similar observation was made in mouse B16F10 melanoma cells treated with Grp170 (104).

The release of Hsp110 in subsets of human IECs into the intestinal lumen occurred as a consequence of epithelial renewal (61). The elevated levels of Hsp110 serve as an immunological "trigger," resulting in expression of CD1d and subsequent activation of natural killer T (NKT) lymphocytes (61). We recently demonstrated that extracellular Grp170 can function as a chaperone for microbial DNA, CpG oligodeoxynucleotides (CpG-ODN), which facilities immune-mediated recognition and clearance of pathogens (66). Grp170 was highly efficient in binding CpG-ODN and potentiated the endocytosis of CpG-ODN by macrophages, which led to the enhanced activation of toll-like receptor 9 (TLR9) (66). It should also be noted that Grp170 is able to directly interact with TLR9 and this interaction increases during stimulation of TLR9 signaling. Furthermore, Grp170–CpG-ODN complex initiated innate immunity enhanced the protection of mice from challenge with *Listeria monocytogenes* compared to CpG-ODN alone (66). This finding uncovered a new feature of extracellular large HSPs in modulating the immune sensing of pathogen-associated molecular patterns (PAMPs). Together, the active cooperation between the extracellular chaperone and TLR9 may be essential for amplification of innate immunity and maintenance of the host homeostasis.

Exposure of mononuclear phagocytes or macrophages to hypoxia-induced upregulation of Grp170 expression, which was restricted to foam cells within atherosclerotic lesions (105). The biological function of Grp170 in macrophages may be linked to the survival or function of this inflammatory cell population that is known to contribute to the pathogenesis of atherosclerosis (106). Additionally, the expression of Grp170 was highly increased in alveolar macrophages and lung epithelial cells in mice upon lipopolysaccharide (LPS) challenge (107). Overexpression of the Grp170 was able to confer resistance to LPS-induced acute lung injury (107). However, the possible role of intracellular Grp170 in LPS-induced inflammatory response has not been examined. Recently, Giffin et al. reported that Grp170 was associated with viral interleukin-6 (vIL-6), a cytokine homolog encoded by human herpes virus 8 (52). This interaction increased the ability of vIL-6 to bind gp130, thereby potentiating vIL-6-induced JAK/STAT signaling, survival as well as migration of endothelial cells (52). This suggests that Grp170 can modulate vIL-6 function and promote pro-inflammatory signaling, angiogenesis, and cell proliferation.

## LARGE HSPs IN ANTIGEN CROSS-PRESENTATION

In light of exceptional protein-holding capacity and superior immunostimulatory activity of the large HSPs (Hsp110, Grp170), we have created recombinant heat shock vaccines by complexing clinically relevant tumor protein antigens to these large chaperones (74, 108–112). These reconstituted complexes exhibited potent antitumor activities by eliciting antigen-specific cytotoxic lymphocytes (CTL) responses prophylactically and therapeutically. Due to the nature of these soluble antigen-targeted protein vaccines, it is believed that antigens-chaperoned by large HSPs are introduced into an antigen cross-presenting pathway in professional antigen-presenting cells (APCs), e.g., DCs (9, 113, 114). Intracellularly processed antigenic peptides are then loaded onto major histocompatibility complex (MHC) class I molecules, followed by presentation to $CD8^+$ T lymphocytes.

Despite the fact that these large HSPs efficiently facilitate a $CD8^+$ T cell response to associated antigens, the mechanism underlying large HSP-enhanced antigen cross-presentation during interaction with DCs is not completely understood. The prolonged existence or stability of antigens is an important prerequisite for cross-presentation after internalization because antigen degradation by endo/lysosomal proteases can rapidly destroy putative antigenic epitopes (115). Hsp110 and Grp170

are significantly more efficient than other chaperones in stabilizing heat-denatured protein substrates (50, 72), which may help to protect antigenic epitopes from being degraded. In addition, these large HSPs may be able to influence intracellular trafficking of antigens to promote cross-presentation. Kutomi et al. showed that Grp170 shuttled the chaperoned peptide into the $Rab5^+EEA1^+$ static early endosomes and transferred the peptide onto the recycling MHC class I molecules (116).

Using a clinically relevant melanoma protein antigen (i.e., gp100), we recently investigated the trafficking pathway of Grp170–gp100 vaccine complex internalized (117). We showed that the Grp170 directed the antigen gp100 efficiently to the ER following uptake into DCs. Grp170-facilitated antigen processing and presentation was dependent on the endoplasmic reticulum-associated protein degradation (ERAD) pathway involving Sec61, which targets gp100 for proteasome-mediated degradation in the cytosol and subsequent integration into the conventional MHC class I restricted antigen-processing pathway. Grp170 can assist protein antigen to escape from lysosomal degradation and shuttle the antigen into the ER from the early endosomal compartment, possibly *via* a previously reported "ER–endosome fusion" process (118–120). Internalized Grp170 may be directly involved in the ERAD, because Grp170 in the complex enhanced the interaction of gp100 with several molecules known to participate in this pathway (e.g., Sec61α, VCP/97, CHIP, Grp78). It is conceivable that partially unfolded gp100 protein during the vaccine preparation serves as an ERAD target once accessing the ER. Since endogenous Grp170 also binds to Sec61α, it is possible that internalized Grp170 collaborates with other ER chaperones to guide antigen retrotranslocation. Interestingly, cytoplasmic chaperone Hsp110 was recently shown to regulate folding as well as quality control of cystic fibrosis transmembrane conductance regulator (CFTR) in the ER (62). It is of interest to determine whether endogenous Hsp110 is also involved in Grp170-enhanced antigen cross-presentation by DCs.

It has been established that antigen cross-presentation promoted by HSPs, including Hsp110 and Grp170, requires uptake of the HSP–antigen complexes by pattern recognition scavenger receptors (SR), such as CD91, LOX1, scavenger receptor A (SRA), and SREC (121–127). Although SRA serves as a binding structure on DCs for exogenous Hsp110 or Grp170, the loss of SRA does not seem to significantly alter the overall capacity of DCs to process and present Hsp110-associated antigen (i.e., gp100) (108, 127, 128). On the contrary, the lack of SRA increased the ability of DCs pulsed with large HSP–gp100 complex to stimulate gp100-specific naive T cells (108). Given that LOX-1 and SREC have been shown to potentiate Hsp70-mediated antigen cross-presentation (121, 125), we postulate that SRs are functionally distinct upon interactions with HSPs that carry antigens (129).

## USE OF LARGE HSPs FOR DEVELOPMENT OF CANCER VACCINES

Numerous studies have documented the immunogenicity of tumor-derived HSPs, which has been attributed to the individually distinct array of antigenic peptides associated with these chaperone proteins (130–138). Our early study showed that vaccination of mice with Hsp110 or Grp170 purified from methylcholanthrene-induced fibrosarcoma resulted in a complete regression of the tumor (139). Moreover, tumor-derived Hsp110 or Grp170 appeared to elicit a more potent antitumor response on a molar basis than Hsp70 (139), which may be explained by their superior antigen chaperoning capability. Several reports suggest that the affinity with which the chaperone binds antigen determines its ability to induce a CTL response (140–142). Using a more aggressive and less immunogenic mouse B16 melanoma model, we demonstrated that tumor-derived Grp170 preparations delayed tumor progression and reduced pulmonary metastases (143).

HSP–peptide complexes derived from patient tumors represent an autologous or personalized vaccine. However, the yield of such a vaccine in the clinic is low for certain types of cancer due to requirement for patient specimen (144). To overcome this limitation, we developed a recombinant chaperone vaccine by reconstituting Hsp110/Grp170-tumor protein antigen complexes under heat shock conditions (72, 110, 112, 145). A "natural chaperone complex" between Hsp110 and the intracellular domain (ICD) of human epidermal growth factor receptor 2 protein (HER-2)/neu elicited both $CD8^+$ and $CD4^+$ T-cell responses against ICD. The Hsp110–ICD complex also significantly enhanced ICD-specific antibody responses relative to that seen with ICD alone (112). Subsequent studies showed that the Hsp110–ICD complex was able to inhibit the development of spontaneous mammary tumors in FVB-neu (FVBN202) transgenic mice (145). Consistent with this finding, immunization with melanoma antigen gp100 complexed with Hsp110 exhibited therapeutic efficacy against established B16 melanoma (72). Strikingly, Hsp110 was more effective than complete Freud's adjuvant in inducing an antitumor immune response (72), which is likely to be due to the unique capability of the large HSPs in promoting antigen cross-presentation. The extensive preclinical studies by us and others have validated this recombinant chaperone vaccine approach that uses large HSPs to target tumor antigens to professional APCs (74, 108–111, 146–148), which provide strong scientific rationale for exploiting these large HSPs to develop synthetic and non-toxic vaccines for cancer immunotherapy.

The similar principle has been used to prepare a recombinant vaccine to induce an immune response to infection. McLaughlin et al. showed that recombinant Hsp110 can efficiently bind to complete viral antigens and enhance monocyte-stimulated proliferation of recall $CD4^+$ T cells *in vitro*. However, the complexes failed to improve primary immune response *in vivo* (149). Therefore, more studies are necessary to understand immunoregulatory activity of the large HSPs in vaccine design directed against infectious diseases.

Using colon-26 (CT26) cancer cells stably transfected with Hsp110, we showed that Hsp110 overexpression markedly enhanced the immunogenicity of the tumor *in vivo*. Immunization of mice with irradiated CT26-Hsp110 cells caused growth inhibition of unmodified CT26 tumor, associated with increased frequency of tumor-specific T cells (150). Similarly, engineering of TRAMP-C2 mouse prostate cancer cells to secrete Grp170 profoundly enhanced tumor immunogenicity, indicated

by increased levels of tumor-infiltrating $CD8^+$ T cells, enhanced cytolytic activity, and improved control of distant tumors (104), suggesting that the induction or manipulation of large HSPs for secretion may help break immune tolerance to cancer cells. We also found that the secreted Grp170 chaperoned full length tumor protein antigens, which can be potentially captured by DCs in the tumor microenvironment (151). Considering abundant tumor antigens present in cancer cells, we investigated the feasibility of intratumoral delivery of Grp170 using an adenovirus to promote antitumor immunity. We showed that the adenovirus encoding a secretable form of Grp170 elicited a tumor-reactive CTL response (152). Furthermore, this Grp170-expressing adenovirus combined with an adenovirus encoding melanoma differentiation-associated gene-7/interleukin-24 (*mda*-7/IL-24), a cancer-specific, apoptosis-inducing gene (153), led to a synergistic systemic antitumor effect as shown by improved control of both treated and untreated prostate cancers (152).

The significance of pathogen-sensing TLR signaling in enhancing antigen presentation by specialized APCs (e.g., DCs) and in bridging innate and adaptive immune responses has been well established (154). Incorporating pathogen-associated molecules or TLR agonists into therapeutic vaccines can potentially augment immune activation. Recently, we engineered a chimeric chaperone by fusing Grp170 with a defined NF-κB-activating domain of the TLR5 agonist flagellin. This chimeric molecule, termed Flagrp170, combined action of the large HSP in facilitating antigen cross-presentation and a microbial immunostimulatory signal for functional activation of DCs (155). Intratumoral administration of an adenovirus expressing Flagrp170 restored systemic antitumor immunity against B16 melanoma and distant lung metastases compared to either unmodified Grp170 or Flagellin. The therapeutic potency of Flagrp170 was also confirmed in mouse models of prostate cancer and colon cancer. The mechanistic studies showed that Flagrp170-provoked activation of tumor-reactive T cells required $CD11c^+$ DCs and $CD8^+$ DCs. Although research is needed to better understand the molecular and cellular bases of immunoregulation by this chimeric chaperone in the immunosuppressive tumor environment, our results support the use of this new generation chaperone molecule for future design of optimized vaccines to achieve improved treatment outcomes. In a separate study, Chen et al. reported that Grp170-HPV16 $E7_{49–57}$ peptide complex plus the TLR3 agonist polyinosinic-polycytidylic acid or poly(I:C), a synthetic analog of double-stranded RNA and a molecular pattern associated with viral infection, induced synergistically an immune response to cervical cancer in mice (156). Therefore, strategic inclusion of a microbial component in large HSP-based vaccine regimen will further strengthen its immunostimulatory capacity in driving an effective CTL response for tumor eradication.

The levels of HSPs, including large HSPs, are generally elevated in various human tumors possibly due to the increased requirement for chaperone molecules to stabilize the mutated or oncoproteins in cancer cells (41, 157). A recent study established a direct correlation between Hsp110 expression and lymphoma aggressiveness (63). Treatment of human B-NHL cell lines with an anti-Hsp110 antibody had no direct effect on cell cycle or apoptosis, but significantly reduced the tumor burden in xenotransplanted immunodeficient mice (63). Hsp110 was reported as a prognostic biomarker for the poor survival of patients with breast cancer and melanoma (41). Similarly, both the protein and mRNA levels of Grp170 were significantly upregulated in several cancer cell lines compared to their normal counterparts (158). Induced overexpression of Grp170 inhibited the senescence and apoptosis of human breast cancer cells, not normal cells (158). Considering the potential involvement of these large HSPs in oncogenesis, Hsp110 or Grp170 itself may represent a novel tumor-associated antigen for immunotargeting. Indeed, vaccination with Hsp110 cDNA resulted in growth inhibition of colorectal CT26 and melanoma B16 tumors, which was associated with stimulation of both Hsp110-specific $CD4^+$ and $CD8^+$ T cells (159). Although our recombinant chaperone vaccine does not appear to induce a significant cellular or humoral response to the Hsp110 (112), such a response may be ideal for those cancers with high expression of the large HSPs.

## LARGE HSPs AND INFLAMMATORY DISEASES

Inflammatory disease is a term that collectively describes a group of apparently unrelated conditions that have common inflammatory pathways leading to inflammation, which may result in various organ damages (160, 161). Although each of these diseases has distinctive epidemiology and pathophysiology, the dysregulated inflammatory response is believed to be pivotal to the disease pathogenesis. In addition to conventional inflammatory disorders, such as inflammatory bowel disease (IBD), diabetes, rheumatoid arthritis (RA), and multiple sclerosis (MS), some other diseases, such as idiopathic pulmonary fibrosis (IPF), myocardial infarction (MI), were recently shown to involve inflammatory responses as well. Despite that HSPs have been implicated in inflammatory and immune-mediated diseases over the past few decades (162–166), the roles of the large HSPs in disease pathogenesis, especially in immune-related processes, remain less investigated. Understanding of molecular actions of Hsp110 and Grp170 in classical inflammatory diseases and those that involve an inflammatory process may provide new insights into disease mechanisms and lead to new strategies for prevention and therapy of these diseases.

An early study by Colgan et al. showed that Hsp110 released from IECs contributes to CD1d surface expression in a novel autocrine pathway, suggesting that Hsp110 regulation of CD1d represents the "physiologic inflammation" in mucosal tissue sites (61). Since CD1d presents self and microbial lipid antigens to NKT cells, it is possible that the Hsp110–CD1d axis might contribute to the pathogenesis of inflammatory diseases, e.g., IBD. Surprisingly, a recent study from this group reported that bone-marrow-derived CD1d signals induced NKT cell-mediated intestinal inflammation; however, engagement of epithelial CD1d elicits protective effects through the STAT3-dependent regulation of IL-10, Hsp110, and CD1d itself (167), which highlights a role of Hsp110 in IEC-dependent maintenance of gut homeostasis. In line with these observations, pharmacological stimulation of Hsp110 expression may be exploited to prevent

colorectal inflammation and favorably affect the progression of IBD. Indeed, human HSP A4 (HspA4, also called Apg2), a member of the Hsp110 family, is inducible by chronic inflammation (164, 168). A recent study showed that increased HspA4 inhibited apoptosis of inflammatory cells, thereby augmenting immune response in the gut through the upregulation of Bcl-2 and IL-17 expression, which led to treatment resistance in IBD (164). This result also suggests that HspA4 may be used as a potential biomarker for refractory IBD. Interestingly, Zebrafish HspA4 was also upregulated in the intestinal epithelium within the gut under inflammatory stress conditions (169). Using HspA4-deficient mice and human tissue samples, Sakurai et al. showed that the expression of HspA4 was inversely correlated to gastric ulcer healing induced by endoscopic submucosal resection (170). Further studies revealed that HspA4 downregulated the expression of stromal cell-derived factor 1 (SDF-1, also known as CXCL12), which signals through its cognate receptor CXC chemokine receptor 4 (CXCR4). The resultant inhibition of cell migration delayed gastric ulcer healing (170).

Obesity and associated insulin resistance predispose individuals to develop chronic metabolic diseases, such as type 2 diabetes. Emerging evidence supports an important role for Grp170 in insulin release (171–173) or insulin resistance in diabetes (174, 175). Kobayashi et al. showed that Grp170 was highly expressed on pancreatic beta cells and decrease in serum glucose concentration by fasting strongly suppressed Grp170 expression in the pancreas concomitant with decreased insulin level in the serum (172). Interestingly, titer of autoantibodies against Grp170 was elevated during high-fat diet feeding (171). A significant increase in autoantibodies to Grp170 was also observed in patients with type I diabetes (176). However, the pathophysiological effect of these autoantibodies to Grp170 in diabetes remains unclear.

Involvement of Grp170 in improving insulin sensitivity of the skeletal muscle and liver was established in type 2 diabetes (175). Overexpression of Grp170 was shown to delay the onset of disease and improve insulin sensitivity, subsequently ameliorated glucose tolerance in diabetic animals. Conversely, reduction of Grp170 in the liver facilitated the disease progress and decreased insulin sensitivity (174). Mechanistic studies suggested that the improved insulin sensitivity by Grp170 was executed though attenuation of oxidative stress and augmentation of insulin signaling in the skeletal muscle and liver (174). Recently, Deng et al. showed that Grp170 expression on pancreatic beta cells decreased gradually during the pathogenesis of acute necrotizing pancreatitis (177). Dekki et al. reported that transthyretin (TTR), which promotes insulin release and protects against pancreatic beta cell death, was associated directly with Grp78, Grp94, and Grp170 in pancreatic beta cells (178). Treatment of beta cells with physiological concentrations of TTR triggered a pronounced increase in intracellular calcium concentration (179). The TTR-induced change in calcium levels was abolished when cells were treated with an antibody against Grp78 (178). Although Grp170 was not directly studied in this model, it is likely that Grp170 along with other chaperone molecules in these multimeric complexes collaboratively regulate the internalization or activity of TTR, thereby affecting insulin release. Several studies have shown that intracellular Grp170 was involved in regulation of calcium signaling or calcium homeostasis (58, 180, 181). However, it remains to be determined if such a pathway alters the function of pancreatic beta cells and inflammatory processes associated with diabetes.

Idiopathic pulmonary fibrosis is a progressive chronic disease associated with inflammatory responses, fibrosis, and lung dysfunction. Treatment of mice with bleomycin-induced lung damage with concurrently enhanced expression of Grp170 in the lung (182). Despite a modest exacerbation of inflammatory responses in Grp170$^{+/-}$ mice, these animals showed significantly ameliorated pulmonary fibrosis, alteration of respiratory dysfunction compared to wild-type counterparts. Although Grp170 appeared to be a protectant against bleomycin-induced lung injury, it promoted lung fibrosis by increasing levels of TGF-β1 and myofibroblasts (182).

Myocardial infarction (i.e., heart attack) is the irreversible myocardial cell damage or death, which occurs during prolonged ischemia caused by blockage of a coronary artery. Grp170 overexpression significantly reduced the hypoxia/reoxygenation-induced cardiomyocyte death by inhibiting activation of capase-3 and release of mitochondrial cytochrome C. This protective effect of Grp170 also inhibited injury caused by myocardial ischemia–reperfusion *in vivo* (181). In addition, increased levels of Grp170 and Grp170-derived peptide fragments was shown in the plasma of patients after MI, which was associated with enhanced ER or hypoxic stress and suggested to be prognostic marker that predicts a poor outcome (183). Accumulating evidence indicates that MI-triggered inflammatory response is involved in injury, repair, and remodeling of the infarcted heart (184, 185). Recent study showed that stimulation of inflammasomes induced the caspase activation associated with maturation and secretion of biologically active IL-1β, which can cause additional loss of functional myocardium and heart failure in mouse model of MI (186). While intracellular Hsp90 has been reported to regulate activation of inflammasome (187), the potential effect of intracellular or extracellular Grp170 on inflammasome activity as well as in the pathophysiology of MI remain to be examined. Elucidating the inflammatory pathways and their contributions to the pathogenesis of MI may lead to novel therapies for preventing post-infarction heart failure.

Although large HSPs, like other chaperone molecules, function to primarily promote and restore cellular or tissue homeostasis, they may be involved in immune pathology through several mechanisms given their potential immunoregulatory effects (**Figure 1**). Of note, extracellular Grp170 secreted from intact cells or released from injured cells facilitate the delivery of Grp170–antigen complex to DCs *via* interaction with surface SRs, thereby enhancing the cross-presentation of the HSP-bound antigens for T cell activation (9, 113, 114, 117). In addition to functioning as "danger" molecules that alert the immune system of tissue damage (102, 103), the extracellular Grp170 has a capacity to amplify the inflammatory response triggered by microbial signal (66) and possibly endogenous damage-associated molecular patterns (DAMPs) as well. Indeed, host-derived DAMPs, such as RNA (188) and DNA (189, 190), can also be recognized by TLRs or other pattern recognition receptors that provokes sterile inflammation or inflammatory diseases (191–194). Compared

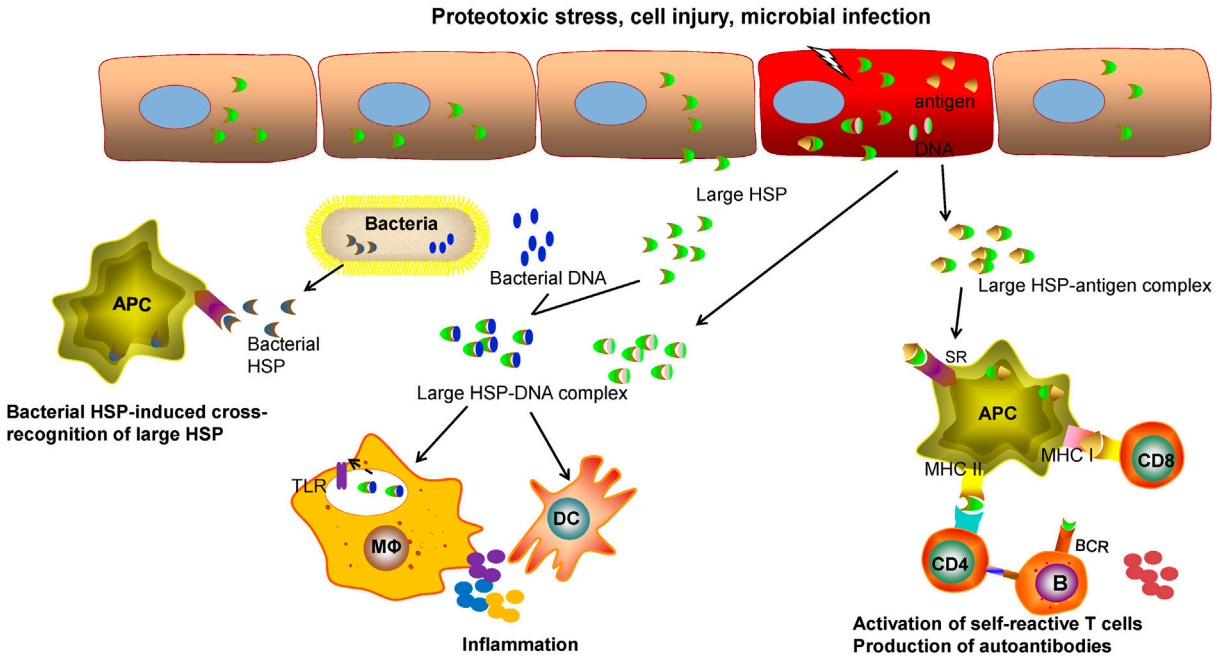


**FIGURE 1 | The potential role of large HSPs in inflammatory or immune-related diseases**. Intracellular HSPs typically function as molecular chaperones to promote proteostasis, counteract cellular stresses, and improve cell survival. The extracellular large HSPs, released from the host cells, caused by proteotoxic stresses, cell injury, or microbial infection, may be involved in regulation of immune pathways that contribute to immune pathologies. The large HSPs complexed with self-antigens can be captured by professional antigen-presenting cells (APCs) through scavenger receptor (SR)-mediated endocytosis. Large HSP-based chaperoning enhances the cross-presentation of associated antigens and activation of self-reactive $CD8^+$ T cells. The processing of these HSP–antigen complexes may result in activation of $CD4^+$ T cells and production of autoantibodies by B lymphocytes. It is also likely that a cellular or humoral response directed to the large HSP itself can be induced in certain pathological conditions. The released large HSPs from injured cells, if complexed with self-DNAs or with microbial DNAs in the case of infection, can provoke and amplify inflammation or an innate immune response triggered by toll-like receptors (TLRs) present on sentinel cells, such as macrophages (Mφ) or dendritic cells (DCs). Due to the conserved sequence, pathogen-derived microbial HSPs from (e.g., bacteria) may induce an immune response that cross-reacts with self HSPs during chronic infection.

with healthy individuals, serum from patients with autoimmune diseases, patients with trauma, and children with septic shock shows high concentrations of Hsp70 (59), even though Hsp110 or Grp170 was not examined in this study.

Cross-recognition of the self and the mycobacterial HSPs by T cells suggests that HSPs may act as a link between infection and autoimmunity (195). Although the controversy remains, the molecular mimicry caused by structural similarity between microbial HSPs and mammalian HSPs may lead to autoimmunity to self HSPs following bacterial infection (196–198). Consistent with this idea, Hsp110 itself can be immunogenic in certain contexts, such as DNA vaccination against Hsp110 that resulted in priming of T cells reactive with Hsp110 on tumor cells *in vivo* (159). Increased levels of anti-Hsp110 antibodies and enhanced expression of Hsp110 have been observed in mice with experimental autoimmune encephalomyelitis (EAE) as well as patients with MS (199), which support a potential role of Hsp110 in inflammatory autoimmune pathology.

Although therapeutic inhibition of Hsp90 has been evaluated in the context of cancer treatment (200), growing evidence indicates that Hsp90 inhibitors also provide benefits for treatment of inflammatory disorders. Inhibition of Hsp90 using a small molecular inhibitor prevented LPS-induced NF-κB activation and nitric oxide production, and attenuated the inflammatory response in EAE (201). Inhibition of Hsp90 reduced the activation of the transcription factors STATs and NF-κB by pro-inflammatory cytokines in atherosclerotic mice (202). These results suggest involvement of Hsp90 and potentially other HSPs (e.g., Hsp110, Grp170) in an inflammatory response possibly through modulating key regulators of immune signaling pathways such as JAK/STATs, TLR4, and NF-κB. We recently showed that UPR response enhanced the production of inflammatory cytokines (e.g., IFN-β) by DCs stimulated by poly(I:C), which involved both TLR3 and melanoma differentiation-associated gene-5. This enhanced inflammatory response was associated with increased activation of NF-κB and IRF3 signaling as well as the splicing of X-box-binding protein-1 (XBP-1), a transcription factor known to regulate ER chaperone genes such as Grp170 (203). It is not clear if the induction of intracellular Grp170 and/or secretion of Grp170 contribute to UPR-amplified inflammatory response in this context. Moreover, Grp170 is known to bind with immunoglobulin in B cells (51) and was suggested to functionally compensate for Grp94/Gp96 to facilitate the assembly of immunoglobulin (204), implicating a potential role of Grp170 in B cell functions. Strikingly, lupus-like autoimmune disorder and systemic inflammation are induced in Grp94/Gp96

**TABLE 1 | The activity of large heat shock proteins in multiple diseases.**

| | Pathology | Activity | Reference |
|---|---|---|---|
| Hsp110 | Traumatic brain injury | Reduce injury at impact site | (22) |
| | Amyotrophic lateral sclerosis | Prevent the neuron toxicity mediated by mutant SOD1 protein | (90) |
| | Alzheimer's disease | Maintain a proper folding environment for phosphorylation and dephosphorylation of tau and APP processing | (93) |
| | Cancer | Upregulate β-catenin and transcription of Wnt-targeted genes; suppress cancer cell apoptosis | (41, 97) |
| | Inflammatory bowel disease | Impair CD1d signal induced NKT cell-mediated intestinal inflammation; enhance treatment resistance by upregulating Bcl-2 and IL-17 | (164, 167) |
| | Gastric ulcer healing | Delay wound healing by suppressing the expression of stromal cell-derived factor 1 | (170) |
| | Multiple sclerosis | Increased levels of anti-Hsp110 antibodies | (199) |
| Grp170 | Alzheimer's disease | Maintain neuroprotective functions of sAPP alpha | (92) |
| | Acute lung injury | Protect alveolar cells after LPS exposure | (107) |
| | Obesity and diabetes | Promote insulin release and enhance the insulin Sensitivity; increased autoantibodies to Grp170 | (171, 173–176) |
| | Myocardial infarction | Inhibit activation of capase-3 and release of mitochondrial cytochrome C | (181) |
| | Idiopathic pulmonary fibrosis | Protect against bleomycin-induced lung injury; promote lung fibrosis by elevating levels of TGF-β1 | (182) |

transgenic mice, in which Grp94/Gp96 was engineered for cell surface expression (205). Thus, development of pharmacological inhibitors selectively targeting Hsp110 or Grp170 may provide new opportunities to treat certain inflammatory or immune-related diseases (**Table 1**).

## CONCLUSION

Like other chaperone molecules involve in intracellular protein quality control, Hsp110 and Grp170 play essential roles for maintaining and restoring protein homeostasis under physiological and stress conditions. The upregulation of these large HSPs as part of stress response (e.g., heat shock response, UPR) generally provide a cytoprotective effect *via* assistance with protein refolding. Therefore, their manipulation may be strategically used to slow progress of certain diseases associated with protein misfolding or aggregation (e.g., neurodegeneration) or treat cancers that highly express Hsp110 or Grp170 to maintain oncogenic signaling for sustained proliferation and survivals. Due to their superior capacity to hold and target protein antigens for DC-mediated cross-presentation, these large HSPs have been used to develop antigen-targeted cancer vaccines and to enhance the immunogenicity of cancer cells. The efficient generation of an antitumor immune response by large HSP-based immunotherapy in animal models of several cancers has led to an ongoing phase I clinical trial in patients with advanced melanoma. Despite that these large HSPs are typically considered to be homeostatic factors in maintaining cellular function and tissue integrity, it is not surprising that they are involved in certain immunopathologies (e.g., inflammatory or immune-related diseases) given the well-documented immunoregulatory activities of surface or extracellular HSPs including the large HSPs at the interface of innate and adaptive immunity. A better understanding of multifunctional roles of Hsp110 or Grp170, defined by their expression compartmentalization as well as the nature, magnitude, or duration of stress signals in different contexts, will offer important insight into their pathogenic relevancies and assist in the design of new potential therapies for treatment of inflammatory disorders (e.g., IBD, MS).

## AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct, and intellectual contribution to the work, and approved it for publication.

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