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THE IMMUNOLOGY OF CELLULAR STRESS PROTEINS

Topic Editors
Willem Van Eden, Ruurd Van Der Zee and Cristina Bonorino

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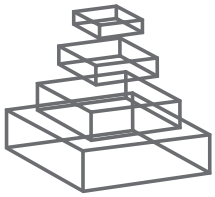

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THE IMMUNOLOGY OF CELLULAR STRESS PROTEINS

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Stress proteins or heat-shock proteins (HSP) are evolutionary conserved proteins present in every prokaryotic and eukaryotic cell. Their main function is to protect cells and proteins from damage under stressful circumstances. The latter circumstances do include the cell and protein damaging effects of inflammation.

The discovery of mycobacterial HSP60 being a critical antigen in the model of adjuvant arthritis, has led to studies that showed the immuno-dominance of microbial HSP60 and the potential of the microbial HSP induced repertoire of antibodies and T cells to cross-recognize the self-HSP homologues of stressed cells. Since then, the research in the immunology of stress proteins started to comprise a widening spectrum of topics with potential medical relevance. Interestingly, since stress proteins have their activities in both innate and adaptive immunity, they are key elements in the cross-roads between both arms of the immune system.

Stress proteins or HSP can be considered as functional 'biomarkers' of inflammation. They are up-regulated locally during inflammation and interestingly, they seem to function as targets for anti-inflammatory regulatory T cells. In experimental models of autoimmunity, mainly arthritis, administration of HSP peptides have been shown to suppress disease. First clinical trials have shown the anti-inflammatory nature of T cell responses to Hsp. In type I diabetes and in rheumatoid arthritis, parenteral and oral administration of Hsp peptides were shown to induce a bias in pro-inflammatory T cells, switching them in the direction of regulatory cytokine production (IL4, IL5 and IL10). In addition a raised level of a marker of natural T regulatory cells, the transcription factor FoxP3, was noted in the RA trial. Other inflammatory diseases or diseases with inflammatory components which feature the immune imprint of the up-regulated Hsp are atherosclerosis, inflammatory bowel diseases, multiple sclerosis and atopic diseases such atopic dermatitis and allergic asthma.

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The immunology of cellular stress proteins

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Stress proteins or heat shock proteins (HSP) are evolutionary conserved proteins present in every prokaryotic and eukaryotic cell. Their archetypical function is to protect cells and proteins from damage under stressful circumstances. The latter circumstances do include the cell and protein damaging effects of inflammation.

The discovery of mycobacterial HSP60 being a critical antigen in the model of adjuvant arthritis, has led to studies that showed the immuno-dominance of microbial HSP60 and the potential of the microbial HSP induced repertoire of antibodies and T cells to cross-recognize the self-HSP homologs of stressed cells. Since then, the research in the immunology of stress proteins started to comprise a widening spectrum of topics with potential medical relevance. Interestingly, since stress proteins have their activities in both innate and adaptive immunity, they are key elements in the cross-roads between both arms of the immune system.

Stress proteins or HSP can be considered as functional "biomarkers" of inflammation. They are up-regulated locally during inflammation and interestingly, they seem to function as targets for anti-inflammatory regulatory T cells (**Figure 1**). In experimental

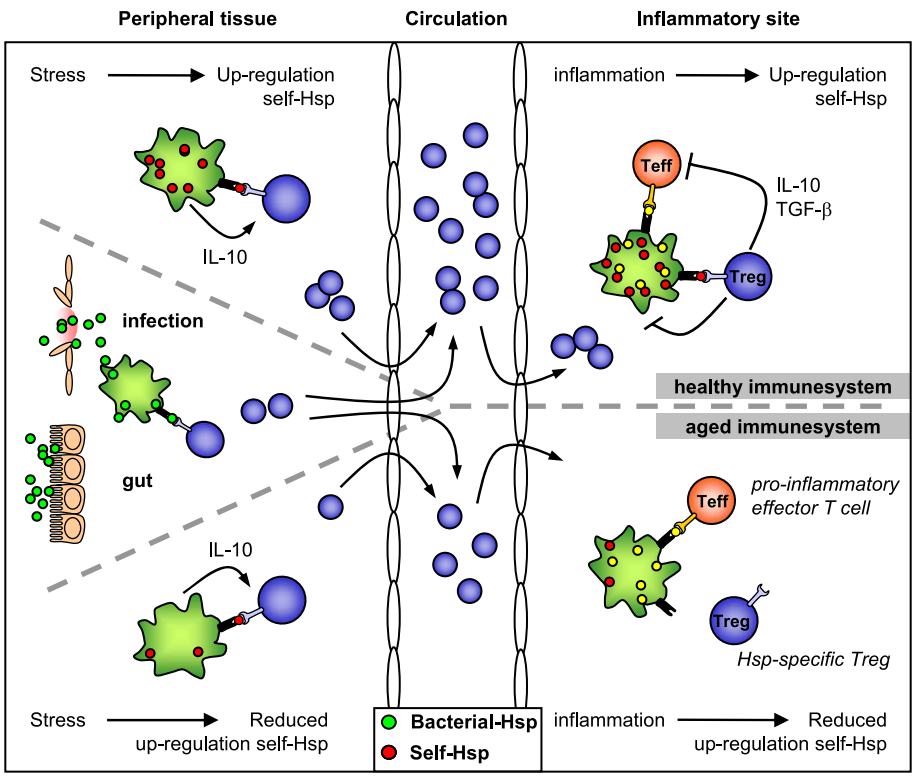

FIGURE 1 | HSP-specific immunoregulation in the healthy and aged immune system. Self-HSP-specific T cells reside in the circulation after escape from central tolerance in the thymus. Since HSP are highly conserved, these self-HSP-specific T cells can cross-recognize bacterial HSP. This T cell population can be expanded after exposure to bacterial-HSP at mucosal surfaces like the gut or during infection. At mucosal surfaces, these T cells will be directed toward a regulatory phenotype through mechanisms of mucosal tolerance. In addition, Treg induction and maintenance will be promoted by stress induced HSP expression in peripheral tissues, because up-regulation of self-HSP and presentation of HSP peptides by MHC class II can occur in the absence of co-stimulation. Treg induction will be enhanced by IL-10 produced in response to stress. Furthermore, self-HSP peptides can function as altered peptide ligands for bacterial HSP-specific T cells. During inflammation, HSP will be induced and presented on professional APCs at the inflammatory site, leading to full activation of HSP-specific Treg and local dampening ongoing inflammation. In the aged immune system stress induced HSP expression is decreased. Therefore, reduced HSP inducibility will probably influence both the induction of HSP-specific Treg in the periphery and their activation during inflammation. Ultimately this could result in reduced Treg numbers and function.

models of autoimmunity, mainly arthritis, administration of HSP peptides has been shown to suppress disease. First clinical trials have shown the anti-inflammatory nature of T cell responses to HSP. In type I diabetes and in rheumatoid arthritis, parenteral and oral administration of HSP peptides were shown to induce a bias in pro-inflammatory T cells, switching them in the direction of regulatory cytokine production (IL4, IL5, and IL-10). In addition a raised level of a marker of T regulatory cells, the transcription factor FoxP3, was noted in the RA trial. Other inflammatory diseases or diseases with inflammatory components which feature the immune imprint of the up-regulated HSP are atherosclerosis, inflammatory bowel diseases, multiple sclerosis, and atopic diseases such as atopic dermatitis and allergic asthma. The review by Borges et al. (2012) discusses the effects of HSP70 on the induction of tolerance at the level of antigen presenting cells and T cells. By this, HSP70 could lead to the development of innovative anti-inflammatory agents to use against autoimmunity and transplant rejection. Shields et al. (2012) have contributed with a review where inhibition and termination of immune responses using BiP (HSP70) are highlighted. They have introduced the term resolution promoting proteins for the aspects of HSPs (Resolution Associated Molecular Patterns or RAMPs). The function of small heat shock proteins (sHSP) in neurological diseases is discussed in the review by Brownell et al. (2012). It highlights the potential of using HSP as novel neuroprotective therapeutics. The ins and outs of HSP as immunoregulatory agents are discussed in more general terms in the review by Coelho and Faria (2012). Aalberse et al. (2012) have broadened the potential of anti-inflammatory effects of HSP in the area of atopic diseases. With the example of HSP60 it is argued that anti-microbial HSP immune reactivity may contribute to atopic disease resistance, suggesting that HSP immunity can constitute the molecular basis of the hygiene hypothesis.

Tumor associated stress proteins seem to qualify as prognostic biomarkers in many tumors. This may be caused by their activity as cellular stress-resistance enhancers. In addition, it may relate to the cell biology of metastasis or to their functions as targets of regulatory T cells. Despite the lack of membrane anchor sequences in HSP, there is ample evidence for cell surface expressed members of stress proteins. In the case of tumors they may then function as targets for NK cells. The review articles of Multhoff et al. (2012) and Calderwood et al. (2012) articulate two additional aspects of stress proteins in cancer development. Whereas both papers discuss the role of HSP as danger molecules, promoting anti-tumor inflammation, Multhoff et al. (2012) also discuss the tumor promoting anti-apoptotic effects of HSP. Calderwood et al. (2012) discuss the anti-tumor immune stimulatory effects of HSP on helper cells and antigen presenting cells. The issue of cell surface expression of HSP seems also relevant for endoplasmic reticulum (ER) stress proteins. As argued in the review by Morito and Nagata (2012), these proteins can also be cell surface expressed and have pathophysiological roles in autoimmunity and inflammation.

A controversy in the area has arisen concerning claims of stress proteins as danger molecules that have the innate quality of inducing inflammatory responses in dendritic cells or other antigen presenting cells. Apart from the possible contribution of contaminating LPS present in earlier recombinant HSP preparations, there is good evidence that cells may perceive stress proteins as danger molecules, indeed. The mechanisms involved in these stress protein activities are ready to be sorted out, amongst others motivated by findings that show additional potential of stress proteins as carriers for protein or oligosaccharide epitopes or as immune stimulatory adjuvants in vaccines. This issue of HSP seen as damage associated molecular patterns (DAMPs) is dealt with in the perspective by Land (2012). The argument in favor of HSP functioning as DAMPs is presented regardless of the final positive or negative regulatory function of HSP. Another aspect of the dual functional role of HSP in immune regulation is highlighted in the mini review by Lee and Repasky (2012). Based on findings that mild hyperthermia can lead to both pro- and anti-inflammatory effects in macrophages, it is proposed that activation state of macrophages is the determining factor in this.

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The anti-inflammatory mechanisms of Hsp70

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Immune responses to heat shock proteins (Hsp) develop in virtually all inflammatory diseases; however, the significance of such responses is only now becoming clear. In experimental disease models, Hsp administration can prevent or arrest inflammatory damage, and in initial clinical trials in patients with chronic inflammatory diseases, Hsp peptides have been shown to promote the production of anti-inflammatory cytokines, indicating immunoregulatory potential of Hsp. Therefore, the presence of immune responses to Hsp in inflammatory diseases can be seen as an attempt of the immune system to correct the inflammatory condition. Hsp70 can modulate inflammatory responses in models of arthritis, colitis and graft rejection, and the mechanisms underlying this effect are now being elucidated. Incubation with microbial Hsp70 was seen to induce tolerogenic dendritic cells (DCs) and to promote a suppressive phenotype in myeloid-derived suppressor cells and monocytes. These DC could induce regulatory T cells (Tregs), independently of the antigens they presented. Some Hsp70 family members are associated with autophagy, leading to a preferential uploading of Hsp70 peptides in MHC class II molecules of stressed cells. Henceforth, conserved Hsp70 peptides may be presented in these situations and constitute targets of Tregs, contributing to downregulation of inflammation. Finally, an interfering effect in multiple intracellular inflammatory signaling pathways is also known for Hsp70. Altogether it seems attractive to use Hsp70, or its derivative peptides, for modulation of inflammation. This is a physiological immunotherapy approach, without the immediate necessity of defining disease-specific auto-antigens. In this article, we present the evidence on anti-inflammatory effects of Hsp70 and discuss the need for experiments that will be crucial for the further exploration of the immunosuppressive potential of this protein.

Keywords: Hsp70, stress proteins, immunomodulation, adaptive immunity, innate immunity

Hsps ARE IMMUNODOMINANT PROTEINS

Heat shock proteins (Hsp) are highly conserved proteins, from microbes through mammals. They are preferentially induced in response to cell stresses including heat shock, oxidative stress, ultraviolet radiation, ischemia-reperfusion injury, viral infections, nutrient deprivation, and chemicals (Lindquist, 1986), protecting cells from injury and promoting refolding of denatured proteins. Hsp are grouped in families according to their molecular weight, and constitutive members of each family can be found in different cell compartments under non-stress conditions, performing chaperone functions (Lindquist and Craig, 1988).

Hsp70 is the most highly conserved protein known to date (Lindquist and Craig, 1988; Ellis, 1990; Feder and Hofmann, 1999). It was therefore surprising when Hsp, including Hsp70, were found to be immunodominant antigens. Early studies demonstrated that 10–20% of the T cells recognized Hsp60 of *Mycobacterium tuberculosis* after experimental mycobacterial immunization (Kaufmann et al., 1987). Hsp70 of *M. leprae* was shown to be a prominent antigen in humans infected with *M. leprae* (Kaufmann et al., 1987; Janson et al., 1991). Such mycobacterial-Hsp-specific T cell responses have also been observed in healthy individuals, not previously exposed to mycobacterial infections (Munk et al., 1989) and in cord blood (Fischer et al., 1992; Aalberse et al., 2011). Immunization with Hsp70 of *M. tuberculosis* (TB-Hsp70) led to a strong IgG response in 7 days without evidence of IgM production (Bonorino et al., 1998), suggesting that antigen-specific T cells able to provide help were already available in naïve mice. Interestingly, a detailed analysis of the peptides recognized by T cells, both in healthy and infected individuals, revealed that some of them were highly conserved (Quayle et al., 1992; Anderton et al., 1995).

Hsp70 AS AN IMMUNOMODULATORY AGENT

It was then hypothesized that, because of their homology with self, bacterial-Hsp would provoke autoimmunity through molecular mimicry with self-proteins. This idea was refuted by the finding that pre-immunization with bacterial-Hsp protected Lewis rats from adjuvant-induced arthritis (van Eden et al., 1988). Subsequently, immunoregulatory features of Hsp were demonstrated in various inflammatory diseases. The literature on immunomodulatory properties of Hsp is vast. In this review, we will focus on Hsp70. Although it may be tempting to generalize observations on different Hsp, it is important to consider that the different families of Hsp show no homology of sequence or structure, and are encoded by different genes, transcribed under the control

of different transcription factors, that are not always activated in coordinate manner. Rather, Hsp are grouped under the same banner because they are commonly induced in similar situations of stress, cooperating to promote cell recovery and protection from injury.

Hsp70 was demonstrated to have a disease suppressive role in experimental models of autoimmunity. One study demonstrated that T cells reactive to peptide 234–252 of TB-Hsp70 suppressed inflammatory responses against *Listeria monocytogenes* via production of IL-10 (Kimura et al., 1998). The same group later showed that pretreatment with peptide 234–252 of TB-Hsp70 suppressed the development of adjuvant-induced arthritis in Lewis rats, generating T cells that were specific for this peptide, and produced high levels of IL-10, but not IFN-g (Tanaka et al., 1999). Also the treatment with anti-IL-10 antibody abrogated protection. This peptide showed 58% amino acid identity between rat and mycobacterial Hsp70. Another study revealed that a different peptide of Hsp70, conserved between rat and mycobacteria, protected Lewis rats from development of arthritis when given intra-nasally (Wendling et al., 2000), preventing disease development by the induction of IL-10 producing T cells. Endogenous Hsp70 presence in the mouse, guaranteed by the presence of heat shock factor 1 (HSF1), its transcription factor, was found to protect from induced colitis (Tanaka et al., 2007). More recently, treatment with whole endotoxin-free TB-Hsp70 inhibited acute rejection of skin and tumor allografts (Borges et al., 2010). Consequently, disease suppressive effects have been observed in the case of both microbial and self (mammalian) Hsp70, some studies using whole protein, some studies using just the peptide, and IL-10 was always important.

How could the conservation of Hsp be reconciled with this apparent predisposition for recognition by the immune system? One idea was that the protective effects of microbial Hsp were related, at least in part, to their capacity to induce T cell responses which were cross-reactive with self-Hsp. Cohen proposed that, to avoid excessive immune responses to both self- and foreign-antigens, the immune system would be selective in its responsiveness and focus on particular immunodominant proteins: the so-called immunological homunculus (Cohen and Young, 1991; Cohen, 2007). Hsp were thus postulated to be such proteins. However, the regulatory capacity of Hsp could not be completely explained by immunodominance and homology between bacterial- and self-Hsp. This was demonstrated in studies using the adjuvant-induced arthritis model, in which Hsps, but not other highly immunogenic and conserved proteins of bacterial origin, were found to suppress disease development (Prakken et al., 2001). So, which additional features of Hsp would endow them with the capacity to suppress inflammatory responses? Along the years, different groups have collected evidence on Hsp70 involvement in innate and adaptive immune responses.

INNATE IMMUNE CELL MODULATION BY Hsp70 – EXTRACELLULAR Hsp70

The idea that Hsp70 could modulate innate cell function comes from studies that analyzed the interaction of Hsp70, either delivered extracellularly or present in the outer cell membrane/exosomes, with receptors on cells such as monocytes, dendritic cells (DCs) and myeloid-derived suppressor cells (MDSCs). This notion was surprising initially, because Hsp70 was then believed to be an intracellular chaperone. However, studies by Hightower and Guidon Jr. (1989) revealed that Hsp70 could be released from cells, in a mechanism that was independent of blockage of secretory pathways. A series of studies followed, revealing that soluble Hsp70 could be measured in the serum of both healthy and diseased individuals (Pockley et al., 1998); and that this extracellular Hsp70 could be either actively secreted by a non-classical pathway, or released from dying cells, review in De Maio (2011).

Two new functions were then reported for extracellular Hsp70. One study demonstrated that (mammalian) Hsp70–peptide complexes purified from MethA sarcomas could lead to priming of cytotoxic T cell (CTL) responses against these tumors (Udono and Srivastava, 1993). That meant that Hsp70 could probably bind to a membrane receptor in antigen-presenting cells (APCs), and get access to the endogenous route of antigen processing and presentation in MHC class I – i.e., cross-priming. A different group later reported that human Hsp70 could bind to and activate human monocytes, promoting the secretion of inflammatory cytokines, such as TNF-α, IL-1β, and IL-6 (Asea et al., 2000a). Different groups went on to corroborate the findings of the cross-priming abilities of Hsp70 (Delneste et al., 2002; Kammerer et al., 2002; Ueda et al., 2004). However, the findings on the induction of pro-inflammatory cytokines were disputed (Gao and Tsan, 2004) when the removal of contaminating endotoxin of the recombinant preparations of human Hsp70 abrogated the induction of TNF-α by this protein. Hsp70 is a molecule with high affinity for hydrophobic moieties (Tsan and Gao, 2009) and the efficient removal of LPS and lipid-like contaminants from preparations of Hsp70 proved to be a challenge for those working with this protein. It is thus very likely that the ability of Hsp70 to bind cell surface receptors (see below) and be internalized, activating antigen presentation, which has been verified by independent groups, is independent of the induction of inflammatory cytokines by this protein, which, to this date, is still disputed.

The removal of contaminating endotoxin and lipopeptides by treatment with Triton X-114, a detergent, revealed that soluble Hsp70 had, in fact, anti-inflammatory properties. It was demonstrated that TB-Hsp70 could modulate cytokine production in blood and synovial cells of arthritis patients. *In vitro* treatment with endotoxin-free TB-Hsp70 for 48 h induced IL-10 production in peripheral blood mononuclear cells (PBMCs) from rheumatoid arthritis (RA) and reactive arthritis (ReA) patients as well as in normal controls PBMCs (Detanico et al., 2004). Concomitantly, PBMCs from these patients downregulated IFN-γ production (900-fold for RA patients and 750-fold for ReA patients when compared with untreated cells) and up-regulated IL-10 production (900-fold for RA patients and 500-fold for ReA patients). In addition, synovial cells incubated with TB-Hsp70 for 48 h showed a reversal of the inflammatory profile, with an induction of IL-10 [a 4.9-fold increase when compared with cells treated with bovine serum albumin (BSA) and LPS], correlating with a decrease in TNF-α and IFN-γ production. Synovial monocytes from the arthritis patients were the major source of IL-10 induced by TB-Hsp70. In accordance with these findings,

Luo et al. (2008) demonstrated that human Hsp70 downregulated in a concentration-dependent manner the TNF-α-induced production of pro-inflammatory mediators IL-6, IL-8, and MCP-1 in RA fibroblast-like synoviocytes when compared with OVA-treated cells. Thus, Hsp70, both bacterial and human, were shown to be associated with a protective phenotype in arthritis, corroborating the initial findings in adjuvant arthritis.

TB-Hsp70 could also modulate cytokine production in DCs. These cells provide a link between innate and adaptive responses, by presenting antigen to T cells, activating them, and shaping their differentiation into effector phenotypes (Heath and Carbone, 2009; Watowich and Liu, 2010). Production of IL-12 by DCs leads to a Th1 program of differentiation for the antigen-specific $CD4^+$ T cells, while IL-4 production induces a Th2 phenotype. Tolerogenic DCs, however, are characterized by low production of pro-inflammatory cytokines and high production of anti-inflammatory cytokines. It has been shown that cells expressing low levels of both MHC class II and T cell co-stimulatory molecules – such as CD80 and CD86, and that do or do not produce IL-10 and TGF-β, can be tolerogenic (Steinman et al., 2003; Rutella et al., 2006; Morelli and Thomson, 2007).

LPS-free TB-Hsp70 blocked the *in vitro* differentiation of DCs from bone marrow precursors. When murine bone marrow DCs (BMDCs) were treated with TB-Hsp70 for 24 or 48 h, an inhibition of maturation characterized by a failure to acquire MHC class II and CD86 expression was observed. TB-Hsp70-treated BMDCs had an eightfold increase in IL-10 production when compared with dexamethasone treated cells and produced 1,200-fold less TNF-α than LPS stimulated cells after 48 h of culture (Motta et al., 2007), suggesting not all transcription was inhibited in the treated BMDCs. More recently, a different group demonstrated that soluble inducible human Hsp70 (now known as HSPA1A) can also induce a regulatory phenotype in monocyte-derived DCs (MoDCs; Stocki et al., 2012). They tested three preparations of Hsp70, two commercial ones, with high or medium endotoxin levels, and one other with very low endotoxin levels. Only the Hsp70 preparations with high and medium endotoxin levels induced maturation of MoDCs in culture. The very low endotoxin level Hsp70, however, inhibited the maturation of MoDCs and reduced the capacity of those cells of stimulating allogeneic T cell proliferation. Together, these results indicated that both TB-Hsp70 and human Hsp70 produced a tolerogenic phenotype in DCs, provided that LPS contamination was eliminated.

These findings in DC have an important implication for a regulatory role of soluble forms of Hsp70. Tolerogenic DCs are known to contribute to the creation of a "suppressive environment" facilitating the peripheral generation of peripheral Tregs. Tregs play a crucial role in suppressing the excessive effector immune response that is harmful to the host (Sakaguchi et al., 2008). These cells can be divided into two subphenotypes. The first one is the Foxp3-expressing Tregs that develop in the thymus (nTregs; Feuerer et al., 2009). The second are the cells that can be induced in peripheral sites when given appropriate signals by the APCs (iTregs; Shevach, 2006). Tregs produce IL-10 or TGF-β, sometimes both, and actively suppress non-Treg proliferation (Vignali et al., 2008). Low levels of antigen presentation coupled to low co-stimulation have been linked to the differentiation of induced Tregs (iTregs; Jenkins et al., 1990; Steinman et al., 2000; Long et al., 2011). Thus, it was possible that, by modulating the APCs, Hsp70 could lead to the induction of Tregs in the periphery.

Confirming this prediction, soluble TB-Hsp70 was demonstrated to inhibit acute allograft rejection (Borges et al., 2010). When C57Bl/6 tumor cells or skin sections were pre-incubated in a solution with endotoxin-free TB-Hsp70 and then grafted onto a BALB/c host, the tumor cells formed a solid tumor, and skin rejection was delayed for 7–10 days, compared to controls. This effect was abrogated by depletion of Tregs, which were shown to infiltrate the accepted grafts. Interestingly, when soluble TB-Hsp70 was injected subcutaneously, this led to an increase in $CD4^+CD25^+Foxp3^+$ cells in the draining lymph node, which correlated to a diminished proliferation of lymph node cells in response to a T cell mitogen. The conclusion was that one single pretreatment with TB-Hsp70 could inhibit a powerful *in vivo* inflammatory process, and this correlated with the presence of Tregs.

The possibility that Hsp70 and Tregs are intimately linked is discussed in detail in the second part of this article (adaptive immunity). In the meantime, we wish discuss one more evidence that Hsp70 can act as an immunosuppressant – and this is related to another discovery, namely that Hsp70 could localize in membranes.

It was shown that Hsp70 (Vega et al., 2008), similarly to Hsc70 (Arispe and De Maio, 2000) could integrate into an artificial lipid bilayer, opening cationic conductance channels, and this ability was associated with the presence of phosphatidylserine (PS; Arispe et al., 2004). Other sphingolipids, such as globotriaosylceramide, have also been reported to enhance Hsp70 insertion into membranes (Gehrmann et al., 2008). This supported previous reports that Hsp70 could be found in the membrane of tumors (Ferrarini et al., 1992; Multhoff et al., 1995). Hsp70 was not simply associated with a receptor in the membrane, but rather inserted, because it could not be eluted by acid washes, or Triton X-1000 (Vega et al., 2008) and because only one antibody, recognizing a part of the C-terminus, but not antibodies that would recognize the N-terminus, would detect it (Botzler et al., 1998). The presence of Hsp70 in membranes of cells or exosomes of tumors presented one more way of extracellular interactions of Hsp70.

Myeloid-derived suppressor cells are a different, heterogeneous population of cells that are expanded during cancer, inflammation, and infection, with a remarkable ability to suppress T cell responses (Gabrilovich and Nagaraj, 2009). Chalmin et al. (2010) demonstrated, in mice and humans, that membrane-associated Hsp70 found in tumor-derived exosomes (TDEs) restrained tumor immune surveillance by promoting MDSCs suppressive functions. It was demonstrated that TDEs, contained in the tumor cell supernatant of three tumor cell lines, could mediate T cell-dependent immunosuppressive functions of MDSCs. The authors identified that the factor present on the TDEs that induced MDSCs activation was the inducible Hsp70 (HSPA1A) expressed on TDE cell surface. Hsp70 was only present on exosomal fractions, not in other microparticles. These findings indicated that immunomodulatory effects of tumor cells include their potential of inducing functional MDSCs by releasing exosomes expressing Hsp70.

Hsp70 PUTATIVE RECEPTORS AND RESPECTIVE SIGNALING PATHWAYS

Many studies asked the question of how would cells perceive the presence of extracellular Hsp. CD14 (Asea et al., 2000b), and Toll-like receptors (TLRs) 2 and 4 (Asea et al., 2002) were first proposed to be receptors for soluble extracellular human Hsp70 – and this was, as discussed above, disputed due to the contamination issue. CD40 (Wang et al., 2001) was then proposed as a receptor for mammalian Hsp70, however a different study (Binder, 2009) refuted this idea, demonstrating that Hsp70 would still bind to cells in CD40 knockout mice. CD91 (Basu et al., 2001) and LOX-1 (Delneste et al., 2002), two scavenger receptors, were shown to bind Hsp70–antigen complexes, increasing cross-presentation and eliciting a protective immune response against antigen-expressing tumor cells *in vivo*. Floto et al. (2006) suggested that TB-Hsp70 promoted DC aggregation, immune synapse formation between DCs and T cells, and an effector immune response the signaling through the CCR5 chemokine receptor. All these different results generated great confusion. A consistent finding among studies was the ability of extracellular Hsp70 to be internalized and interact with antigen presentation routes, inducing T cell responses to the peptides that associated with this protein. TLRs and CD40 are signaling receptors, rather than endocytic receptors. Scavenger receptors and lectin-like receptors are endocytic receptors, and the signaling events downstream binding and internalization that follows binding are not fully characterized.

A thorough study transfected Chinese hamster ovary (CHO) cells with cDNAs expressing each of these putative receptors, as well as other scavenger receptors and lectins, and studied their interaction with mammalian extracellular Hsp70 (Theriault et al., 2005). The authors verified no binding or internalization of Hsp70 with cells expressing TLR2, TLR4, CD40, or CD91. In a follow-up study, they used the same approach focusing on scavenger receptors (Theriault et al., 2006). They demonstrated that LOX-1, SREC-1, and FEEL-1 bind and internalize Hsp70. However, different forms of Hsp70 (peptide bound or ATP bound) interacted with each of these receptors with different affinities. In summary, while binding to signaling receptors was refuted by more than one study, different groups provided evidence for scavenger receptors as the likely receptors for extracellular Hsp70.

SIGNALING ROUTES ACTIVATED BY Hsp70

If extracellular Hsp70 indeed interacts with membrane-bound receptors, will it activate signaling pathways associated with these receptors? Few studies approached this issue.

Mitogen-activated protein (MAP) kinase cascade is one of the most ancient and evolutionarily conserved signaling pathways, which is also important for many processes in immune responses (Dong et al., 2002). TDE-associated Hsp70 was found to mediate the suppressive activity of the MDSCs via activation of STAT3 and ERK (Chalmin et al., 2010). An ERK-dependent route for IL-10 production by different immune system cells upon TLR stimulation has been described (Saraiva and O'Garra, 2010). It has been suggested that some TLR2 agonists are good inducers of IL-10 production (Dillon et al., 2006; Manicassamy et al., 2009; Saraiva and O'Garra, 2010; Yamazaki et al., 2011). It is an interesting feature of TLR2 that, depending on the nature of the ligand and the population of target cells, it can mediate either inflammatory or anti-inflammatory responses to the same infectious organism (Dillon et al., 2006; Frodermann et al., 2011), and the anti-inflammatory response is mediated by IL-10.

IL-10 is the main anti-inflammatory and immunosuppressive cytokine (Moore et al., 2001). However, depending on the situation, it can exert a pro-inflammatory role like in lupus erythematosus (Bussolati et al., 2000; Sharif et al., 2004). It has been suggested that type I interferons regulate the balance between anti- and pro-inflammatory role of IL-10 (Sharif et al., 2004). In monocytes of patients with systemic lupus erythematosus (SLE), it was demonstrated that IL-10 can stimulate production of platelet-activating factor (PAF) and this production was correlated with disease severity (Bussolati et al., 2000).

IL-10 production of by DCs stimulated via TLRs is diminished in presence of selective ERK inhibitors (Yi et al., 2002; Dillon et al., 2004; Kaiser et al., 2009) or in ERK-deficient cells (Agrawal et al., 2006). Besides, differences in IL-10 production by macrophages, myeloid DCs, and plasmacytoid DCs are correlated with different levels of ERK activation in these cells (Saraiva and O'Garra, 2010). Borges et al. (in preparation) observed that BMDCs treated with TB-Hsp70 showed a higher expression of phospho-ERK when compared with unstimulated cells, and inhibition of ERK expression with the specific ERK inhibitor PD98059 blocked IL-10 production upon incubation with Hsp70.

STAT3 is associated with IL-10 production and tolerance (Barton, 2006; Dhingra et al., 2011). Also, IL-10R recruits and activates JNK1-STAT3 pathway (Murray, 2006). In contrast, STAT3 can be activated by pro-inflammatory cytokines like IL-6, through IL-6R (Murray, 2007) and Oncostatin M (Halfter et al., 1999). Despite this duality in STAT3 activation, this transcription factor may be activated after IL-10 release induced by TB-Hsp70.

Based on this, we propose a model in which extracellular Hsp70 could regulate innate immune cell function, binding to cell surface receptors (a scavenger or lectin-like receptor), signaling through TLR2 via ERK to induce IL-10 production, resulting in an anti-inflammatory response. This model is depicted in **Figure 1**.

Is it possible to reconcile this model with what has been observed for the cross-priming and pro-inflammatory roles described for this protein? We believe that the next studies should test the possibility that extracellular Hsp70, upon binding to lectin-like or scavenger receptors, uses associated receptors to signal. It is possible that depending on the form of Hsp70 (associated with peptide; with membranes; with nucleotides; peptide-free) it will associate with a different receptor. Another issue that has to be considered is that, while in bacteria, Hsp70 comes from one gene, in mammals, there may be at least eight genes that code for Hsp70 (Kampinga et al., 2009). Bulk preparations of mammalian Hsp70 from cells contain not only the inducible, HSPA1A, but products from other genes as well. And this may also influence the outcome of the experiment. Finally, binding and internalization, followed by antigen presentation, may lead to inflammatory as well as to regulatory responses, depending on which receptor is engaged, as demonstrated in a recent study (Li et al., 2012). The authors verified that targeting an antigen to LOX-1 or DC-ASGPR on the surface of DCs led to internalization and cross-presentation of

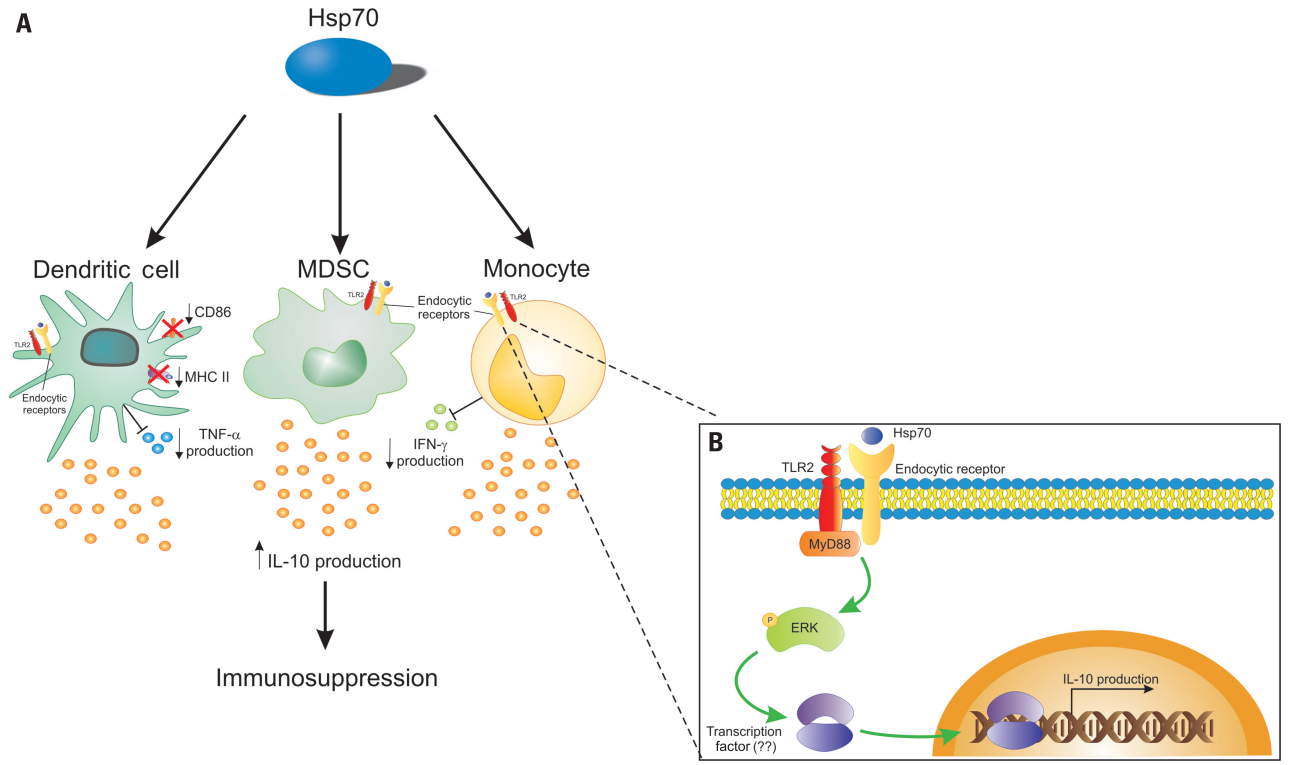

FIGURE 1 | Hsp70 can interact directly with innate immune cells. **(A)** A possible mechanism of the Hsp70 action is its interaction with dendritic cells (DCs), myeloid-derived suppressor cells (MDSCs), and monocytes. Hsp70 would bind to endocytic receptors, and be endocytosed, gaining access to routes of antigen presentation, modulating the cell phenotype toward a tolerogenic one, leading to the production of the anti-inflammatory cytokine IL-10 and consequently to immunosuppression. In DCs, Hsp70 downregulates CD86 and MHC class II expression, and inhibits TNF-α production. Also, Hsp70 can inhibit IFN-γ by monocytes. **(B)** Upon binding to an endocytic, Hsp70 signals through TLR2, resulting in MyD88 activation. The subsequent phosphorylation of ERK can trigger the activation of an undetermined transcription factor that will bind the *il-10* gene promoter leading to IL-10 production.

the antigen. However, while targeting to LOX-1 resulted in INF-gamma producing T cells, targeting to DC-ASGPR resulted in IL-10 producing CD4 T cells. Thus, it is possible that, depending on the form of extracellular Hsp70 and the target cell/tissue microenvironment, different outcomes may ensue. If this possibility is verified experimentally, that would in part explain some of the conflicting results previously discussed here. We are now left with the challenge to test these possibilities in order to elucidate the whole potential of Hsp70 as an immunomodulatory agent.

ADAPTIVE IMMUNITY REGULATION BY Hsp70

Besides the innate effects discussed above, several adaptive immunity associated mechanisms have been proposed for induction of Hsp-specific Tregs under physiological conditions.

The role of Hsp70 in adaptive immunity to mediate suppression through Tregs could be related to presentation of Hsp70 peptides, or to the modulation of the innate environment as described in the previous section, leading to the induction of Tregs.

The presentation of Hsp70 peptides in MHC molecules could result either from overexpression of endogenous Hsp70 in situations of physiological stress, or from endocytosis of extracellular Hsp; In response to physiological stress, intracellular levels of Hsp70 will rise in the stressed cells which can lead to presentation of Hsp peptides on MHC class I via the default MHC loading route for cytosolic proteins. This pathway includes degradation of the protein by the proteasome, transporter associated with antigen presentation (TAP) mediated translocation to the endoplasmic reticulum and subsequent loading of the peptides on MHC class I molecules (Neefjes et al., 2011). As will be discussed in more detail below, it is now becoming clear that via autophagy, intracellular Hsp can also be loaded on MHC class II molecules. Peptides derived from extracellular Hsp (pathogen-associated or secreted endogenous Hsp) can be presented via endocytic pathways by MHC class II molecules on APCs or on non-APCs upon stimulation with factors like IFNγ.

The mechanisms leading to production of Hsp-specific Treg can be manifold. Continuous encounter of bacterial-Hsp, in mucosal surfaces such as the gut can be a way to induce bacterial-Hsp-specific Treg, contributing to Hsp-specific mucosal tolerance (van Eden et al., 2005, 2007). Another possibility is the up-regulation of self-Hsp on non-professional APCs in response to various forms of stress in tissues. In the gut lamina propria of many species, MHC class II is also found to be present on non-professional APCs (Stokes et al., 1996). In addition, the inflammatory mediator IFN-γ is known to induce MHC class II in various cell types. Thus, MHC class II presentation of Hsp fragments in the absence

of proper co-stimulation may add to the production of tolerogenic or regulatory T cell responses. In addition, presentation of self-Hsp70 conserved peptides in presence of TGF-β (Sela et al., 2011) could lead to Treg induction and/or expansion (Rosenblum et al., 2011). Also, because some self-Hsp70 peptides are not completely identical to their bacterial homolog peptides, such presented self-peptides could function as altered peptide ligands for bacterial-Hsp-specific cells leading to induction of a partially agonistic and therefore downmodulated T cell response (Wauben et al., 1993). Finally, induction of Treg might be reinforced by the increased levels of the immunoregulatory cytokine IL-10, induced upon stress in multiple tissues (Stordeur and Goldman, 1998).

AUTOPHAGY, LOADING Hsp PEPTIDES ON MHC CLASS II

To activate $CD4^+$ T cells, peptides should be presented by MHC class II molecules. Cytosolic proteins, like Hsp70, are by default loaded on MHC class I molecules while extracellular proteins will be presented on MHC class II. Thus, another fundamental question can be raised; how do Hsp peptides end up to become presented by MHC class II? The distinct localization between MHC class I and MHC class II loading pathways has been proven incorrect because cytosolic proteins have been eluted from MHC class II and vice versa (Schmid et al., 2007). Autophagy has been initially found as a process to sustain metabolic fitness during food deprivation through bulk protein degradation (Kuma et al., 2004). The role of autophagy in the immune system is only now becoming clear (Schmid and Munz, 2007; Munz, 2009). Two pathways can result in loading of intracellular peptides on MHC class II. First, intracellular proteins can be incorporated in autophagosomes that subsequently fuse with lysosomes for degradation of their cargo (macroautophagy). In addition, cytosolic proteins can be transported via LAMP2a directly into the lysosome (chaperone mediated autophagy; Munz, 2006; Schmid et al., 2007; Strawbridge and Blum, 2007). Recently, the role of autophagy in loading Hsp70 peptides has been described; in human HLA-$DR4^+$ B cells a striking increase of especially Hsp70 peptides was eluted from HLA-DR4 upon induction of autophagy by amino acid deprivation (Dengjel et al., 2005). Autophagy induction coincided with elevated Hsp70 mRNA levels. In other words, especially under conditions of cell stress, fragments of Hsp70 will be presented on APCs to T cells, possibly initiating a regulatory T cell response.

PHENOTYPE OF Hsp-SPECIFIC Treg

The phenotype of Hsp-specific Treg has not been studied in detail. However, since Hsp-specific T cells have been observed in cord blood, some of them will probably be thymus derived $CD4^+CD25^+Foxp3^+$ natural Treg (Sakaguchi et al., 1995; Tang and Bluestone, 2008). Also, Hsp-specific Treg can be induced in the periphery, which potentially leads to induction of several induced Treg subsets. For example, $Foxp3^-$ Tr1 cells, which are induced by repetitive stimulation with antigen in the presence of IL-10 (Groux et al., 1997; Roncarolo and Battaglia, 2007). Alternatively, mucosal exposure of Hsp can produce iTregs, expressing a $CD4^+CD25^+Foxp3^+$ phenotype (Chen et al., 1994; Weiner, 2001). Or, conversion of naïve $CD4^+$CD25-$Foxp3^-$ cells into induced $CD4^+CD25^+Foxp3^+$ can occur in the presence of IL-2 and TGF-β at low levels of pro-inflammatory cytokines (Horwitz et al., 2008).

The phenotype of the Hsp-specific Treg may depend on the exposure route. Intraperitoneal (i.p.) immunization with endotoxin-free TB-Hsp70 or OVA as a control resulted in $CD4^+CD25^+$ T cells from Hsp70 immunized mice expressing slightly enhanced levels of regulatory cytokine IL-10, but not increasingly expression of Foxp3 (Wieten et al., 2009a). In contrast, in a study in a mouse atherosclerosis model, oral Hsp administration increased Foxp3 expression (van Puijvelde et al., 2007). Enhanced Foxp3 expression, both systemically in the spleen and locally in the inflamed joint, was also found upon up-regulation of endogenous Hsp70 in Peyer's patches of carvacrol (a co-inducer of Hsp70) fed mice (Wieten et al., 2010). The finding that Foxp3 levels were increased in cells obtained from joint synovial fluid suggested that induced Treg could have actually migrated to the site of inflammation.

In a recent study, after local injection of whole TB-Hsp70, a higher percentage of $CD4^+CD25^+Foxp3^+$ cells in draining lymph nodes compared with local injection with OVA was observed. Moreover, TB-Hsp70 inhibition of lymph node cell proliferation was superior to the inhibition induced by dexamethasone after PHA stimulation. The authors also observed that inhibition of acute rejection induced by TB-Hsp70 was dependent on $CD4^+CD25^+$ T cells in a skin allograft model (Borges et al., 2010).

To study the phenotype of Hsp-specific Treg in more detail, the expression of the transcription factor Helios in Tregs elicited by Hsp70 treatment, to verify if they are nTregs or iTregs (Thornton et al., 2010), since peripherally induced Tregs do not usually express this molecule. It will also be interesting to see if T cells found at the site of inflammation are Hsp70 specific, and if they indeed express special homing receptors. Future studies should tell us the relative proportions of nTregs and iTregs in Hsp70-specific Tregs, as well as what are the mechanisms by which they can mediate suppression in each of these models.

SUPPRESSIVE MECHANISM OF Hsp-SPECIFIC Treg

Hsp-specific Treg will probably use similar suppressive mechanisms as other antigen-specific Treg, like the production of anti-inflammatory cytokines, cell contact dependent suppression or killing of effector T cells and conversion of APC into a tolerogenic state (Vignali et al., 2008). Most Treg subsets use IL-10 for suppression (Bluestone, 2005). It has been recently demonstrated that Treg IL-10 is important for local responses, and not for the systemic suppression of inflammation (Rubtsov et al., 2008). In previous studies, we showed that cross-reactive Hsp-specific T cell responses coincided with the production of IL-10 (Anderton et al., 1995; Wendling et al., 2000; Prakken et al., 2001). Subcutaneous injection of soluble TB-Hsp70 increased IL-10 production and the number of Tregs in draining lymph nodes when compared with OVA injection (Borges et al., 2010). Moreover, while addressing the role of IL-10 in modulation of Proteoglycan-induced arthritis (PGIA) upon i.p. immunization with TB-Hsp70 and after nasal administration of Hsp70 peptides, it was observed that both treatment strategies enhanced Hsp70-specific T cell proliferation and IL-10 production. TB-Hsp70 immunization failed to rescue IL-10

deficient mice from PGIA development. In both wild type and IL-10 deficient mice, Hsp70-specific T cell responses were found, but only in wild type mice these responses suppressed arthritis (Wieten et al., 2009a). In addition, increased PG-specific T cell proliferation, IFN-γ and IL-10 production were found in wild type, but not in IL-10 deficient mice. This illustrates that Hsp70 immunization also modified the PG response to a more anti-inflammatory response. It is therefore possible that Hsp70-induced Tregs generated a tolerogenic micro-milieu by their cytokine production that enabled the outgrowth of new Tregs with antigen specificities beyond Hsp and that IL-10 was required for this effect.

These findings emphasize that Hsp-specific Tregs use mechanisms of infectious tolerance for modulation of inflammation. This has been shown before in transplantation (Qin et al., 1993; Borges et al., 2010), type-1 diabetes (Tarbell et al., 2007), and experimental autoimmune encephalomyelitis (EAE; Mekala et al., 2005) models. Besides IL-10, the role of other cytokines associated with Tregs, like IL-35 has not been addressed but might be relevant.

HOW IMPORTANT IS STRESS-INDUCED Hsp EXPRESSION?

Hsp expression is up-regulated in virtually every inflammatory condition. Also in autoimmune disease this has been reported; enhanced expression of Hsp60 has been shown in synovial and mononuclear cells of juvenile idiopathic arthritis (JIA) patients (Boog et al., 1992; de Graeff-Meeder et al., 1995). In addition, increased expression of inducible Hsp70 and HSF1 has been shown in the inflamed joint of RA patients (Schett et al., 1998). This has also been seen for BiP, an ER restricted Hsp70 family member (Blass et al., 2001) and interestingly enhanced expression in RA synovium was also seen for the constitutive Hsc70 (Schick et al., 2004).

As mentioned before, stress-induced Hsp expression has been proposed to be important for induction, maintenance, and activation of Hsp-specific Treg. If indeed so, reduced expression of Hsp – like with aging, as also depicted in **Figure 2**, where a reduced HSF activity leads to a relatively poor capacity to up-regulate Hsp (Rao et al., 1999; Njemini et al., 2003) – can be expected to influence Hsp mediated immune homeostasis and therefore might contribute to development of chronic inflammatory diseases. In fact, Hsp70 polymorphisms have been associated with inflammatory or autoimmune diseases such as Crohn's disease (Debler et al., 2003), Alzheimer's disease (Clarimon et al., 2003), pancreatitis (Balog et al., 2005) and with development of graft versus host disease upon allogeneic hematopoietic stem cell transplantation (Bogunia-Kubik and Lange, 2005).

Decreased Hsp expression has been observed in several immune disorders. A low Hsp70 response has also been described in a subtype of Biobreeding (BB) rats with a high susceptibility for development of autoimmune (Bellmann et al., 1997). Similar results have been found in human PBMC from patients with newly diagnosed type-1 diabetes. In that study, stress responses were found to become re-established again in patients with longstanding diabetes, more than 8 months after disease manifestation. So, defective Hsp70 induction coincided with beta cell directed inflammatory activity, and seemed modulated by pro-inflammatory cytokines rather than metabolic factors (Burkart et al., 2008).

To amplify stress-induced Hsp70 expression, a study tested multiple food-derived compounds for their effect on Hsp70 expression (Wieten et al., 2009b). One of the compounds, carvacrol, was identified as a potent enhancer of stress-induced Hsp70 both *in vitro* and *in vivo*. Also *in vivo*, intragastric (i.g.) gavage of carvacrol enhanced Hsp70 expression in Peyer's patches (Wieten et al., 2010). Carvacrol was used to boost Hsp levels in APCs and this enhanced Hsp-specific T cell hybridoma activation. We also addressed the immunomodulatory potential of carvacrol *in vivo* and found that i.g. carvacrol treatment specifically boosted Hsp70-specific T cell responses. The finding that adoptive transfer of T cells, isolated from carvacrol treated donor mice, suppressed PGIA, were indicative of the induction of Treg.

The above mentioned findings suggested that the immune system can recognize and react on altered expression of these proteins.

PERSPECTIVES

Hsp expression or Hsp-specific T cell responses have been positively associated with a better disease prognosis in several inflammatory conditions (de Graeff-Meeder et al., 1991; de Kleer et al., 2003). In addition, the immunosuppressive action of Hsp has been demonstrated in multiple rodent disease models. So, it is attractive to speculate that simply enhancing Hsp mediated immunoregulation in either way could be used as therapy.

Apparently, this is oversimplified. Depending on multiple factors such as disease etiology and inflammatory status, patient age and genetic background, difficulties will be encountered. In general, defects in for example positive or negative selection in the thymus, IL-2 production by effector T cells or IL-10 or TGF-β production by Tregs can lead to loss of peripheral tolerance as a result of decreased T cell numbers or functioning (Brusko et al., 2008). Some of these defects might also influence Hsp-specific Treg. For example, the findings that Hsp70-induced suppression of arthritis failed in the absence of IL-10 (Wieten et al., 2009a), illustrated that defects in IL-10 production will also influence Hsp-specific Treg. Furthermore, as disease progresses, severe ongoing inflammation has been described to obstruct the effectiveness of antigen-specific Tregs (Valencia et al., 2006; Peluso et al., 2007). It is currently not known if Hsp-specific Treg can also be hampered by ongoing inflammation. Recent experiments performed by us (Lotte Wieten, Martijn J. C. van Herwijnen, Femke Broere, Ruurd van der Zee, and Willem van Eden) have indicated that this is not the case, however. Transfer of Hsp70 peptide-induced Tregs were found to suppress ongoing experimental arthritis (van Herwijnen et al., in preparation). Recently, it has been reported that iTreg but not natural Treg can convert into Th17 cells after exposure to IL-6 and TGF-β (Horwitz et al., 2008). Besides Th1 cells, Th17 cells are major pathogenic effector cells in many autoimmune diseases. Whether Hsp-specific Treg can convert into Th17 cells has not been studied, but if so, timing and route of boosting the Hsp response could be important to avoid exacerbation of disease instead of induction of regulation.

Earlier studies have emphasized the pro-inflammatory nature of stress proteins such as the Hsp70 family members. In this

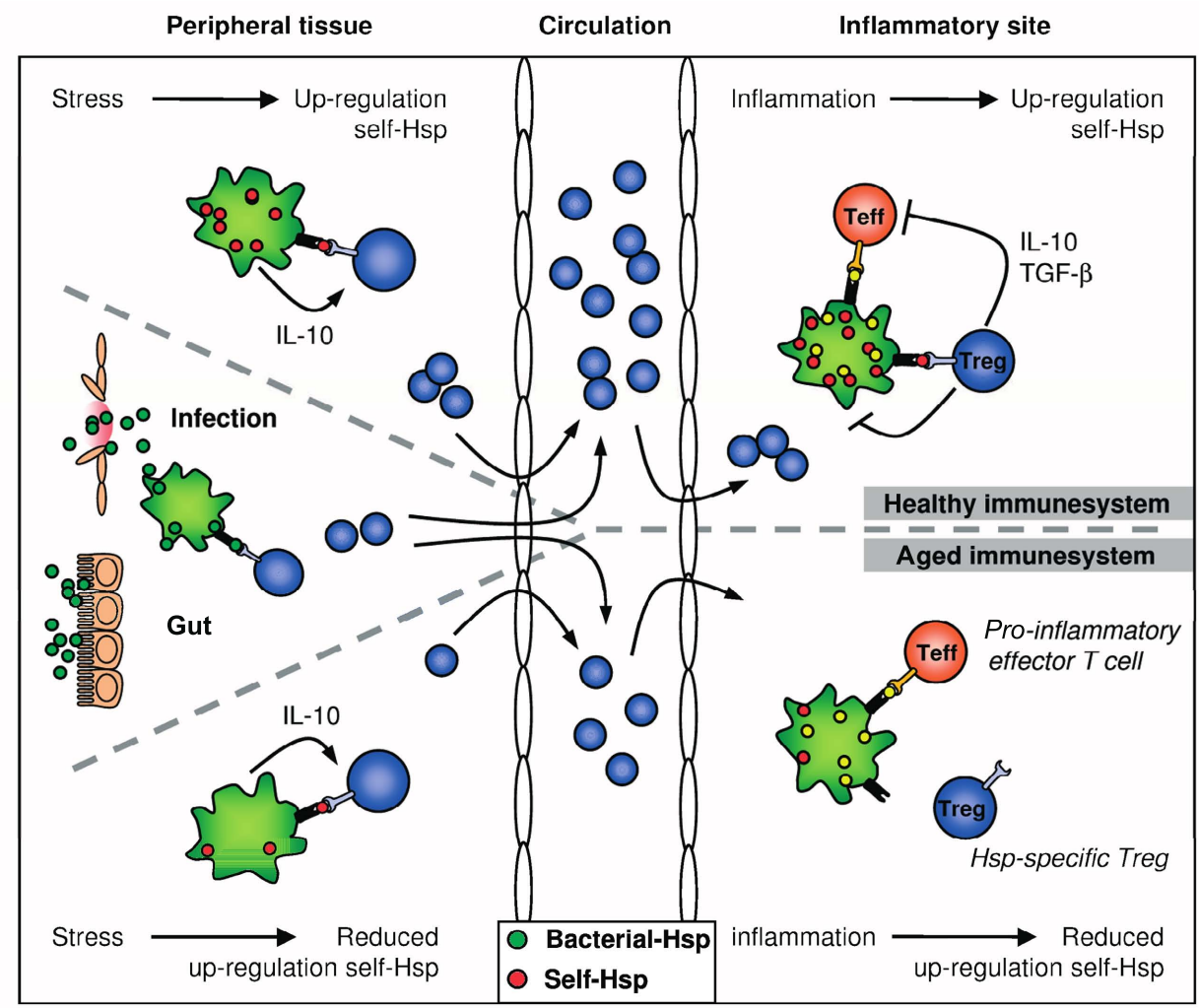

FIGURE 2 | Hsp-specific immunoregulation in the healthy and aged immune system. Self-Hsp-specific T cells reside in the circulation after escape from central tolerance in the thymus. Since Hsp are highly conserved, these self-Hsp-specific T cells can cross-recognize bacterial-Hsp. This T cell population can be expanded after exposure to bacterial-Hsp at mucosal surfaces like the gut or during infection. At mucosal surfaces, these T cells will be directed toward a regulatory phenotype through mechanisms of mucosal tolerance. In addition, Treg induction and maintenance will be promoted by stress-induced Hsp expression in peripheral tissues, because up-regulation of self-Hsp and presentation of Hsp peptides by MHC class II can occur in the absence of co-stimulation. Treg induction will be enhanced by IL-10 produced in response to stress. Furthermore, self-Hsp peptides can function as altered peptide ligands for bacterial-Hsp-specific T cells. During inflammation, Hsp will be induced and presented on professional APCs at the inflammatory site, leading to full activation of Hsp-specific Treg and local dampening ongoing inflammation. In the aged immune system, stress-induced Hsp expression is decreased. Therefore, reduced Hsp inducibility will probably influence both the induction of Hsp-specific Treg in the periphery and their activation during inflammation. Ultimately, this could result in reduced Treg numbers and function.

sense, they were often mentioned as prime examples of so-called DAMPs or damage-associated molecular patterns. It is possible that contaminating microbial components present in partially purified recombinant proteins used in the experiments have contributed to this (Bausinger et al., 2002; Gao and Tsan, 2004; Motta et al., 2007). Besides this, there are other arguments to make against a pro-inflammatory role of Hsp (Broere et al., 2011). As discussed here above, experimental evidence in favor of an immunomodulatory role for Hsp70 is accumulating and therefore Hsp70's immunosuppressive potential seems to constitute a real phenomenon. A more detailed characterization of the molecular pathways activated by Hsp70 in different cell subpopulations is needed. Such studies will allow us to understand and maximize the use of Hsp70 as an anti-inflammatory agent.

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A new-age for biologic therapies: long-term drug-free therapy with BiP?

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Heat shock proteins (HSPs) and other members of the much broader stress protein family have been shown to play important roles in coordinating multiple phases of immunological reactions; from facilitating immunological recognition, to promoting and regulating immunological responses and finally augmenting the resolution of inflammation and return to immunological homeostasis. In this review, we consider the challenges facing the stress protein field as we enter 2012; in particular we consider the role that HSPs and stress proteins may play in the initiation and termination of immunological responses. Special attention is afforded to the resolution-associated molecular pattern, binding immunoglobulin protein (BiP, also known as glucose regulated protein-78). We review the evidence that resolution-promoting proteins such as BiP may herald a new generation of biologics for inflammatory disease and reflect on the challenges of achieving clinical remission in rheumatoid arthritis with novel therapeutics and correlating clinical remission with immunological parameters of resolution of inflammation.

Keywords: binding immunoglobulin protein, resolution-associated molecular patterns, inflammation, resolution of inflammation, immunotherapy, rheumatoid arthritis, immune networks

INTRODUCTION

Heat shock proteins (HSP) and stress proteins are a collection of highly evolutionarily conserved proteins, grouped by molecular weight, with intracellular functions involving protein chaperoning and folding and protection of the cell during physiologically stressful conditions, including heat-shock, hypoxia, hypoglycemia, and intracellular electrolyte abnormalities. The term "stress protein" provides an umbrella for all those proteins upregulated in response to physiological stress, while HSP refers to the sub-family of proteins that have specific promoters allowing them to respond to heat shock. Stress proteins may be intracellular, cell surface expressed, and/or extracellular proteins and, therefore, have access to multiple subcellular and extracellular compartments. Consequently, there has been much discussion around potential roles that stress proteins may play in innate and adaptive immune responses.

Immunological responses may be grouped into four overlapping and interdependent phases: recognition, response, regulation, and resolution. Much data suggest that stress proteins play important roles in all of the phases of the immunological response. In this review, we briefly discuss how stress proteins can influence the resolution of inflammation, in particular, with respect to binding immunoglobulin protein (BiP, also known as glucose regulated protein-78). We also explore the concept of third generation biologic therapies for immunological diseases such as rheumatoid arthritis (RA). The third generation of biologics for immune-mediated diseases will be therapeutic agents that significantly modulate the dysregulated immune system, restoring immune homeostasis for prolonged periods without further biologic administration. We believe that BiP and other members of the recently defined resolution associated molecular pattern (RAMP) family (Shields et al., 2011) may herald a new generation of such novel biologics.

ELEPHANTS IN THE LABORATORY; A 2011 VIEW OF THE STRESS PROTEIN FIELD

Extensive research has now shown that stress proteins are involved in multiple stages of the immune response (**Figure 1**): stress proteins participate in the recognition of immunological danger, facilitate peptide carriage between cells and subsequent cross presentation of antigen, can modulate the immunological profile of myeloid lineage cells in both a pro- and anti-inflammatory direction and promote the regulation and resolution of inflammation. However, research in the stress protein field has been dogged by two controversies for many years. Firstly, there remains suspicion that stress protein family members possess no intrinsic immunological activity and all observed activity is the consequence of bacterial contaminants, particularly endotoxin, within the protein preparation (Wallin et al., 2002; Gao and Tsan, 2003a,b; Ye and Gan, 2007). Secondly, compounding this problem, is the ever-increasing variety of cell surface receptors identified for HSP. Using HSP70 as an example, the broad array of putative receptors include, TLR2, TLR4, CD14, CD40, CD91, CCR5, LOX-1, SREC-1 (Calderwood et al., 2007). However, there remain inconsistencies when trying to replicate HSP binding to null cells transfected with some of these specific molecules, such as CD14, TLR2, and TLR4, thus throwing doubt on whether they are true receptors (Theriault et al., 2005).

The major challenge facing the stress protein field is consolidating and refining the results of the extensive body of research conducted to date into a more comprehensive theory of how stress protein networks facilitate the initiation, progression, and resolution of an immunological event. To achieve this, careful

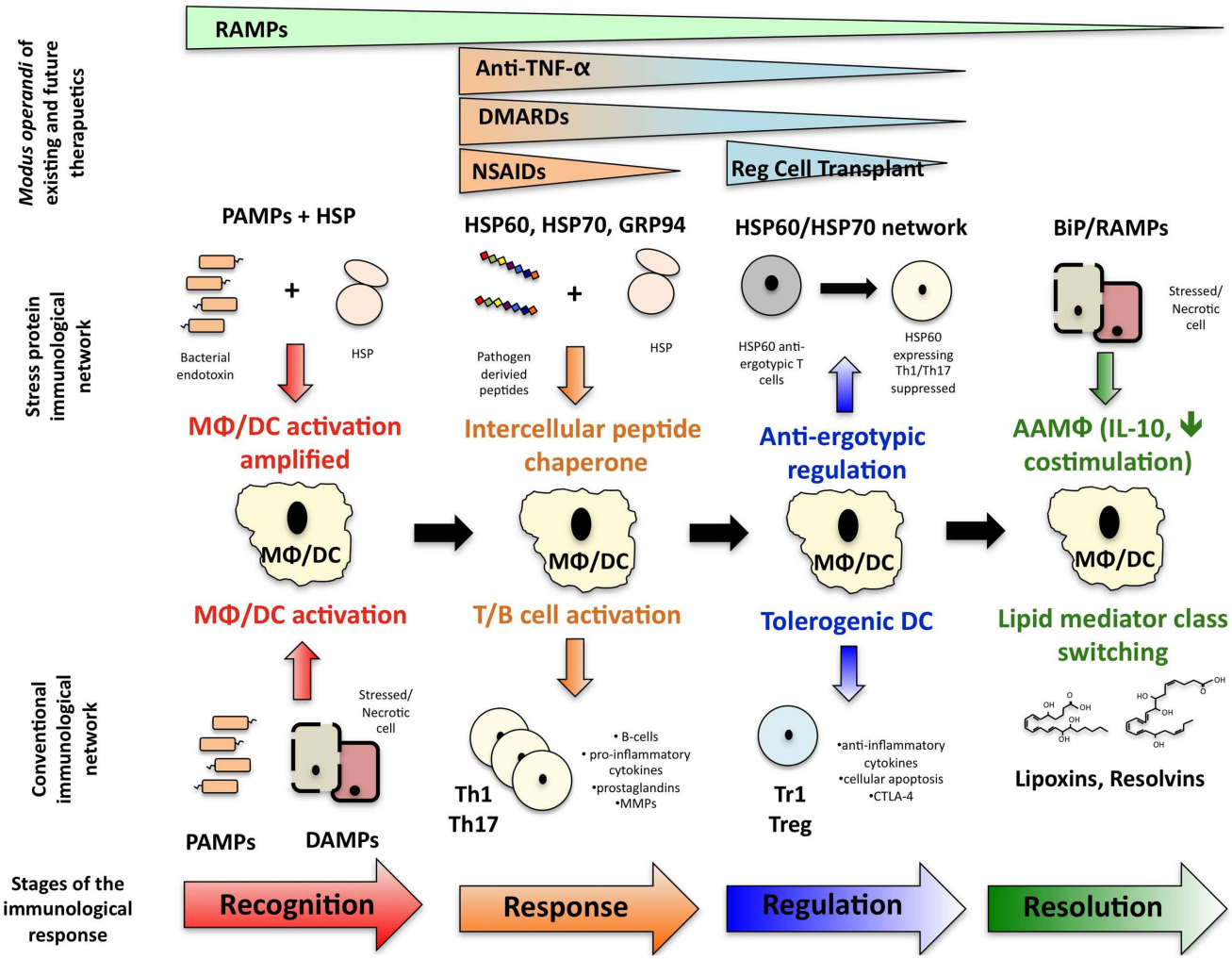

FIGURE 1 | How stress protein networks influences immunological reactions. Immunological events can be broken down into a series of interdependent phases: immunological recognition, an immunological response following by regulation of that response and resolution of inflammation leading to a return to homeostasis. The stress protein network influences each phase of the immunological event and ultimately, resolution-associated molecular patterns may help facilitate the return to immunological homeostasis. The different phases at which existing and future therapeutics act are also illustrated.

distinctions should be made between the immunological activity of mycobacterial HSP and mammalian HSP and, more pertinently, between the immunological effects derived from whole extracellular stress protein molecules and those effects caused by peptides derived from stress protein molecules coordinating the generation or expansion of self-reactive HSP T cells.

Moreover, stress protein researchers must address the elephants in their experimental data. It is increasingly clear that stress proteins bind bacterial products; however, the potential immunological implications of stress protein–bacterial product interactions have been over-shadowed by heated debate as to the immunological activity of ultra-pure stress protein preparations and their role as damage-associated molecular patterns (DAMP; Gao and Tsan, 2003a,b; Osterloh et al., 2004; Henderson et al., 2010). More recently, further discussion has arisen surrounding subtle differences between danger-associated and DAMP (van Eden et al., 2011), with van Eden et al. convincingly arguing that HSP should be excluded from the DAMP family given the paucity of evidence that they alone can activate an immunological response. Yet, the very fact that HSP are upregulated in response to cellular stress and can associate with pathogen associated molecular patterns (PAMP) is of great immunological significance in it's own right, particularly with respect to the danger hypothesis, for the following reasons:

(1) Cellular stress is synonymous with potential threats to tissue viability and is therefore synonymous with immunological danger, in accordance with the danger hypothesis (Matzinger, 2002). Cellular stress can arise from infectious and non-infectious sources (Macario and Conway de Macario, 2005; Rath and Haller, 2011) and results in the upregulation and redistribution of stress proteins, including members of the

HSP family, to the cell surface and beyond, into the extracellular fluid (Multhoff and Hightower, 1996; Delpino and Castelli, 2002; Mambula and Calderwood, 2006; Corrao et al., 2010; Merendino et al., 2010; Sreekumar et al., 2010).

(2) Cell surface HSP and other stress proteins (e.g., gp96) signal immunological danger by inducing and/or enhancing inflammatory cytokine production, dendritic cell (DC) maturation, and NK cell activity (Chen et al., 2002; Liu et al., 2003; Osterloh et al., 2004). While this does not qualify the molecules as DAMPs, as laid out in the criteria defined by Kono and Rock (2008), it emphasizes their importance in signaling immunological danger. Furthermore, while on the cell surface, it has been hypothesized that stress proteins associate with PAMPs and thus serve as a primitive antigen presentation system (Li et al., 2002).

(3) There is considerable evidence to suggest that extracellular HSP60, HSP70, HSP90, and gp96 bind to LPS and potentiate its immunostimulatory effects (Triantafilou et al., 2001; Warger et al., 2006; Osterloh et al., 2007). High-mobility group box protein-1 has shown similar properties (Pisetsky et al., 2008). This suggests that stress proteins may facilitate the recognition of a pathogenic infection, particularly if that infection has caused direct cellular insult. This may be of significance in maintaining immunological homeostasis at epithelial surfaces.

(4) Beyond the cell, stress protein family members appear to bind multiple extracellular protein and receptor targets (Calderwood et al., 2007). The immunological significance of these protein–protein interactions remain unclear. However, HSP do appear to be "sticky" proteins. Given their interaction with PAMPs, this promiscuity may facilitate the containment of shed bacterial products, preventing their dissemination, while providing a localized cache of PAMPs permitting a more anatomically targeted immunological response.

In summary, the specific properties of an extracellular stress protein are likely to depend both on the protein itself, the surrounding extracellular environment and the nature of the tissue from which the stress protein was released. Furthermore, the possibility that the extracellular environment modulates the activity of stress proteins during the course of an immunological response requires further attention; one could envisage a paradigm where extracellular stress proteins evolve from poachers, inciting inflammation, and tissue destruction, into gamekeepers, promoting regulation, and resolution during the course of an immunological event. Indeed a post-translational modification of high-mobility G protein-1 (HMGB1) caused by oxidation attenuates pro-inflammatory functions and makes the protein anti-inflammatory (Urbonaviciute et al., 2009). However, since stress proteins exhibit such radically different extracellular functions it should not be surprising that they show different affinities for a variety of receptors and may even bind different receptors depending on cell type. Such activity would enhance their flexibility and diversity of function (Calderwood et al., 2007). Hence, a greater understanding of how the stress protein network interacts with wider immunological networks would be extremely beneficial, however, a systems biology approach will likely be necessary to extend our knowledge further.

THE THIRD GENERATION OF BIOLOGICS: PROMOTING RESOLUTION

At present all therapies for immune-mediated diseases – autoimmune or allotransplant – require either continuous administration of immunosuppressive drugs or intermittent dosing at frequent intervals. For example, despite the effectiveness of anti-TNF-α therapy at suppressing inflammation in RA, cessation of therapy is associated with clinical relapse and radiological progression (Quinn et al., 2005) and although there is some *in vitro* evidence that anti-TNF-α therapy may modulate adaptive immune responses, particularly with respect to regulatory T cells (Ehrenstein et al., 2004), there is little evidence that long-term therapy alters the underlying immunological mechanisms that contribute to the chronic autoimmune state. Indeed, even after achieving low disease activity following long-term infliximab treatment, 45% of patients displayed further radiological disease progression within just a year of cessation of therapy (Tanaka et al., 2010).

Nevertheless, the transplantation field has offered hope that physicians can induce a state of tolerance to alloantigen in the absence of long-term immunosuppression. The best-characterized example of this phenomenon is liver transplantation: 20% of liver transplant recipients are capable of achieving a state of clinical operational tolerance (defined as a well functioning graft, without histological signs of rejection following the complete cessation of immunosuppression for over 1 year; Orlando et al., 2009). Tolerance is rarer with other solid-organ transplants, but has been reported in renal transplantation (Orlando et al., 2010); immunologists are beginning to describe biomarkers and molecular signatures that characterize and can be used to monitor clinical operational tolerance which include reduced co-stimulatory molecule expression, immune quiescence, apoptosis, and memory T cell responses are important in the maintenance of operational tolerance (Brouard et al., 2007; Hernandez-Fuentes and Lechler, 2010).

With respect to RA, the clinical goal is not a tolerogenic state *per se*, but the induction of drug-free remission. The ACR–EULAR agreed definition of disease remission in RA is a simplified disease activity index (SDAI) of $\leq$13.3 at any one time, or a total joint count of $\leq$1, and a swollen joint count $\leq$1, and a CRP $\leq$1 mg/dl and a patient global assessment score of $\leq$1 (Bykerk, 2011). Remission of RA, may indeed involve the re-establishment of immunological tolerance to cognate autoantigens. However, extensive cell–cell interactions between immune and stromal cells within the synovial architecture add a layer a complexity beyond the extracelluar cytokine and stress protein networks that drive the pathogenesis and maintain chronic inflammation within the rheumatoid joint (McInnes and Schett, 2011).

Currently, rheumatologists do not possess any immunological biomarkers to predict which patients will achieve or maintain a state of disease-free remission or indeed the mechanisms by which clinical remission is achieved (Isaacs, 2010). Thus, the attainment of drug-free remission in RA is the premier challenge facing rheumatologists and immunologist in the twenty-first century.

Early, aggressive intervention in the disease process is now the gold-standard for achieving maximum clinical response and potential remission in the RA patient (Quinn et al., 2005). B-cell depletion therapy has also offered a glimpse that more permanent

changes to the immunological phenotype are possible. For example, rituximab, a B-cell depleting anti-CD20 monoclonal antibody, induces enduring clinical responses that do not correlate with peripheral blood levels of $CD20^+$ B cells after treatment (Breedveld et al., 2007). Multiple potential therapies and possible mechanisms for the re-establishment of immune tolerance and subsequent resolution of chronic inflammation have been discussed at length elsewhere (Albani et al., 2011). Needless to say that, like the conditions necessary for operational tolerance of renal grafts, tolerance in the rheumatoid patient involves modification of DC function such that T regulatory cells, of various phenotypes and mode of action are generated.

Since stress proteins have pleiotropic functions, and have been called "moonlighting" proteins (Huberts and van der Klei, 2010), it may be that the immune down regulating properties of some of them are due to an effect on DCs. Hence when used for the treatment of human disease they may be able to produce prolonged drug-free disease remissions or even tolerance. What is the evidence for this dramatic claim? Examination of the multiple extracellular functions of BiP may provide an answer.

BiP – A BRIEF HISTORY

Binding immunoglobulin protein is a member of the HSP70 family and an ubiquitously expressed, endogenous protein. BiP is constitutively expressed in the endoplasmic reticulum (ER), and essential for the correct folding of many nascent peptides (Gething, 1999). BiP is also the master regulator of the unfolded protein response (UPR), a transcriptional program designed to relieve ER stress by promoting the correct folding of ER luminal proteins (Hendershot, 2004). BiP, therefore, is regulated at two levels, constitutive and stress induced. Stress induced upregulation occurs in environments of high cellular activity in inadequately vascularized tissue, for example, during inflammation or neoplasia, pathologies characterized by relative hypoglycemia and hypoxia (Lee, 2007). Principally, during perturbation of the ER, BiP protects the cell from the accumulation of misfolded and denatured proteins and, thus, prevents apoptosis. Testament to the fundamental importance of BiP is the fact that BiP knock-out mice and mice where BiP is constitutively targeted to the incorrect subcellular compartment are not viable (Luo et al., 2006; Mimura et al., 2007). Many previous reviews have covered the intracellular chaperone function and protective role of BiP, during health and disease, so these aspects of BiP biology will not be reviewed here.

In contrast, our work for the last decade has focused exclusively on the extracellular properties of cell-free human BiP and its immunoregulatory role in inflammation. Like many other stress proteins, BiP is now known to be cell surface expressed and detectable at relatively high concentrations in serum, synovial fluid and oviductal fluid (Delpino and Castelli, 2002; Corrigall et al., 2004; Marin-Briggiler et al., 2010). As such, BiP has several physiological properties. Although BiP is a member of the HSP70 family we have previously hypothesized that, unlike HSP70 itself, BiP acts as a RAMP (Shields et al., 2011). RAMPs are protein molecules released alongside DAMPs from stressed or necrotic cells, which provide negative inputs into immunological networks, antagonizing pro-inflammatory mediators, and helping restore the immune system to homeostasis. Unlike the "DAMPing" or regulatory effect that HSPs exert on the immune system which appear to act via HSP derived peptides expanding sets of HSP-specific regulatory T cells (van Eden et al., 2005; Quintana and Cohen, 2011), the RAMP family members act predominantly on the myeloid lineage, setting the scene for the resolution of inflammation. Furthermore, what makes these molecules unique, is that unlike the increasing number of resolution inducing molecules being described (e.g., lipoxins and resolvins), the protein members of the RAMP family are constitutively expressed and thus able to affect the course of inflammation from the outset (Shields et al., 2011).

BiP: DRIVING RESOLUTION OF INFLAMMATION

What sets BiP apart from other potential biologic therapeutics appears to be its mode of action. Our research provides evidence that BiP offers long-lasting prophylactic, and therapeutic protection from disease in the murine model of collagen induced arthritis (CIA; Corrigall et al., 2001; Brownlie et al., 2006). Adoptive transfer studies confirm that the end-point of the mode of action involves immunological changes to cell function and suggests that regulatory T cells are induced rapidly by BiP either in naïve animals, following intravenous injection of BiP, or *in vitro*, when their splenocytes and lymph nodes are cultured in the presence of BiP (Corrigall et al., 2001; Brownlie et al., 2006). Importantly, the message from these studies is that BiP-treated cells, when adoptively transferred into arthritic mice are therapeutic and give long-term relief in the absence of repeated administration.

Another indicator that BiP is a potentially successful therapeutic in RA arises from our experimental pre-clinical investigation into a xenogeneic model where small pieces of inflamed synovium from RA patients were transplanted subcutaneously into severe combined immunodeficient (SCID) mice (Yoshida et al., 2011). Following successful vascular anastomosis of the graft, a single intravenous dose of BiP significantly reduced histological features of inflammation in the synovial explants. In addition, the histological expression of pro-inflammatory cytokines (TNF-α and IL-6) and the co-stimulatory molecules HLA-DR and CD86 was significantly down regulated in the grafts from the BiP-treated animals (Yoshida et al., 2011). The down-regulation of co-stimulatory molecules would significantly reduce the efficiency of antigen presentation. This SCID/RA synovial membrane chimera has been used to validate other biologic therapies, anti-TNF-α neutralizing antibody inhibitor and anti-soluble IL-6 receptor, that are now in the clinic.

Binding immunoglobulin protein treatment induces key changes in T cell and monocyte development. Firstly, T cell development is skewed to a Th2 profile with the production of IL-4, IL-5, and IL-10 (Bodman-Smith et al., 2003). Human BiP-specific T cell clones derived from healthy PBMC are predominantly $CD8^+$ and produce little or no interferon γ while splenocyte and lymph node cell suspensions from BiP-treated CIA mice also show a Th2 profile of cytokine release, IL-4, IL-5, and IL-10, on re-stimulation with BiP *in vitro* (Brownlie et al., 2006). Serum samples from these mice suggest that BiP suppresses the production of the pathogenic anti-collagen type II antibodies, which drive CIA, while stimulating the production of non-pathogenic IgG1 isotype antibodies

indicative of a Th2 environment (Brownlie et al., 2006). Secondly, BiP is capable of binding to a receptor expressed by >95% human peripheral blood monocytes, up to 50% B cells and 10% T cells (Corrigall et al., 2003). Currently, the identity of this receptor(s) remains elusive. The immediate result of BiP stimulation, in human peripheral blood mononuclear cells, is the attenuation of TNF-α production after 7 h and increased IL-10 production to a plateau lasting over 96 h (Corrigall et al., 2004). On being stimulated by BiP, monocytes show phenotypic changes similar to deactivated macrophages. Macrophage deactivation is achieved via the stimulation of macrophages with IL-10, TGF-β, steroids, or interactions between CD200 and CD200R and CD47 and CD172a; the consequences of deactivation include increased production of IL-10, TGF-β, PGE-2, and reduced expression of MHC-II molecules (Gordon and Taylor, 2005). The various differentiation pathways and activation states of the monocyte–macrophage lineage remain under intense investigation, however, BiP induced changes are temporally different from those induced exclusively by IL-10. The major effect is the complete inhibition of TNF-α production with increased production of IL-10, soluble TNF receptors, and IL-1 receptor antagonist (Corrigall et al., 2004). Ultimately, the consequence of BiP stimulation is non-phlogistic activation of the monocyte–macrophage. Monocytes cultured in the presence of IL-4 and GM-CSF and BiP fail to differentiate into mature DC (Corrigall et al., 2009). Remarkably these BiP-treated cells were highly positive for indoleamine 2,3-dioxygenase (IDO), a characteristic of tolerogenic DC. When these DCs were co-cultured with autologous T cells they induced regulatory CTLA-4^+ T cells. This process was attributable to IDO up regulation because CTLA-4 up regulation was reversible in the presence of 1 methyl tryptophan, the IDO inhibitor. On separation from the DC the CTLA-4^+ T cells showed regulatory T cell function capable of suppressing T cell stimulation by anti-CD3. Although BiP was essential for the induction of the DCs, no additional BiP was required in the secondary cultures in which regulatory T cell function was assessed (Corrigall et al., 2009). The mechanism underlying the induction of regulatory T cells by BiP is under investigation by our laboratory. There are three possible scenarios: firstly, that there is direct action on the T cell via a receptor mediated process, but this is unlikely given the relative lack of extracellular BiP protein in the culture system during DC–T cell col-culture; secondly, that BiP drives the induction of DC of a tolerogenic phenotype, including increasing IDO expression, which we have shown directly leads to upregulated CTLA-4^+ regulatory T cells, either in the presence or absence of peptide presentation. Finally BiP-specific peptide presentation may expand existing regulatory T cells in a peptide dependent process. Cross-reactivity with peptides from other HSP70 family members is a possibility in this case as 68% amino acid homology exists between HSP70 and BiP. If cross-reactivity between HSP70 and BiP occurs and is responsible for any of the T-cell dependent regulatory effects of either protein, it lends further weight to the hypothesis that an intricate network of extracellular stress proteins exists which facilitates the maintenance of immunological homeostasis (Panayi et al., 2004).

These studies are all in immunological models. In the TNF-α transgenic mouse a spontaneous arthritis develops that resembles RA in many of its features (Li and Schwarz, 2003) although the joint inflammation and destruction is independent on immune mechanisms. However, since BiP deactivates monocytes both at the transcriptional and the translational level a prediction would be that BiP would also have a therapeutic effect in this model. Indeed a single intraperitoneal dose of BiP was able to significantly suppress joint inflammation and systemic bone damage for several weeks (Corrigall et al., manuscript submitted).

The spontaneous arthritis observed in the transgenic mice is almost certainly induced by TNF-α activation of transcription factors including NF-κB, which is responsible for the induction of many of the major inflammatory cytokines. Intracellularly, BiP and NF-κB are counter regulated (Pahl and Baeuerle, 1995). A recent review by Kitamura (2011) has reported that although early UPR activity may drive NF-κB activation later UPR involvement tends to attenuate NF-κB function. As an extracellular protein we have shown that BiP acts to inhibit MAPK phosphorylation (Corrigall et al., manuscript submitted) and downregulates protein levels of these signaling molecules. As a stress protein, BiP is upregulated intracellularly in response to ER stress prior to its release into the extracellular environment. Thus, both the intracellular and extracellular actions of BiP may operate to diminish the pro-inflammatory effects of NF-κB thus helping the resolution of acute inflammation.

THE OTHER RESOLUTION-ASSOCIATED MOLECULAR PATTERNS

Binding immunoglobulin protein is just one member of a family of proteins we have recently defined as the RAMPs. We have extensively reviewed the properties of the RAMP family elsewhere (Shields et al., 2011). Needless to say, we believe this family of proteins, whose founder members include HSP10, HSP27, and α-B-crystallin may have great potential as resolution-promoting therapeutics. Resolution-promoting regulatory signals are subtly different from immunoregulatory immunological signals; resolution-promoting signals are those that specifically promote the non-phlogistic activation of macrophages, the phagocytosis of apoptotic neutrophils, the prevention of influx of inflammatory cells and the restoration of parenchymal cells to the non-inflammatory state (Serhan et al., 2007). The crux of resolution is, therefore, centered around myeloid lineage cells and the parenchyma. This is why we believe the RAMP family, which principally exert their immunological effects via the myeloid lineage (thus affecting antigen presentation, co-stimulatory molecule expression, myeloid cell differentiation, and anti-inflammatory cytokine secretion (De et al., 2000; Corrigall et al., 2004, 2009; Banerjee et al., 2011; Shields et al., 2011)), have the potential to exert resolution-promoting effects, rather than simply immunoregulatory effects.

Clinical trials of HSP10 have already yielded promising results (Vanags et al., 2006; Williams et al., 2008) and pre-clinical models have shown α-B-crystallin to be an extremely effective therapeutic in a variety of different inflammatory disease models including experimental autoimmune encephalomyelitis, ischemic optic neuropathy and stroke (Ousman et al., 2007; Arac et al., 2011; Pangratz-Fuehrer et al., 2011). Further investigations are necessary to fully understand how the RAMP family exert their immunological effects and under what circumstance they might be used in clinical practice.

CONCLUSION

To develop the third generation of biologics, a great deal of investment will need to be made in correlating the clinical parameters of remission from chronic inflammatory disease and the immunological parameters of resolution from inflammation. Experience from the rheumatological field has shown us that aggressive immunosuppressive and biological therapy can induce remission in early RA. However, the longer the disease progresses, the more unlikely this strategy is to succeed because the mechanisms controlling the regulation and resolution of inflammation fail in chronic inflammation. Therapy in chronic inflammation can reduce ongoing inflammation but does not resolve the underlying immunological defect.

Evidence from our laboratory suggests that a single administration of BiP in models of inflammatory arthritis is sufficient to regulate and resolve chronic inflammation. The planned Stage I/II clinical trial of BiP in RA patients will teach us more regarding the properties of BiP as a therapy in human disease. However, it may be that resolution-promoting biologic therapies will have to be administered in conjunction with other biologics, which can control ongoing inflammation and set-the-scene for immunological resolution and restoration of homeostasis to occur.

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The protective and therapeutic function of small heat shock proteins in neurological diseases

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Historically, small heat shock proteins (sHSPs) have been extensively studied in the context of being intracellular molecular chaperones. However, recent studies looking at the role of sHSPs in neurological diseases have demonstrated a near universal upregulation of certain sHSPs in damaged and diseased brains. Initially, it was thought that sHSPs are pathological in these disease states because they are found in the areas of damage. However, transgenic overexpression and exogenous administration of sHSPs in various experimental disease paradigms have shown just the contrary – that sHSPs are protective, not pathological. This review examines sHSPs in neurological diseases and highlights the potential for using these neuroprotective sHSPs as novel therapeutics. It first addresses the endogenous expression of sHSPs in a variety of neurological disorders. Although many studies have examined the expression of sHSPs in neurological diseases, there are no review articles summarizing these data. Furthermore, it focuses on recent studies that have investigated the therapeutic potential of sHSPs for neurological diseases. Finally, it will explain what we think is the function of endogenous sHSPs in neurological diseases.

Keywords: small heat shock proteins, neuroinflammation, neurological diseases, HSPB1, HSPB5, sHSPs

OVERVIEW OF SMALL HEAT SHOCK PROTEINS

Small heat shock proteins (sHSPs) have molecular weights between 12 and 43 kDa, distinguishing them in size from large heat shock proteins (Ganea, 2001; Arrigo et al., 2007). There are 10 human sHSPs: HSPB1–HSPB10 (Kappé et al., 2003). They share common structural characteristics, including a highly conserved 90 amino acid long HSP20 domain, often referred to as the alpha-crystallin domain, and the capacity to form large dynamic oligomers (Poulain et al., 2010). sHSPs are intracellular molecular chaperones (Horwitz, 1992; Van Montfort et al., 2001). As chaperone proteins, sHSPs bind misfolded proteins and prevent them from aggregating. However, they are unable to actively re-fold the protein themselves due to their lack of ATPase activity. Instead, sHSPs sequester the misfolded proteins within the cell to prevent aggregation until a large heat shock protein can assist in refolding (Jakob et al., 1993).

Although sHSPs share both common structural and functional characteristics, they differ in tissue distribution and expression patterns (**Table 1**). HSPB1, HSPB5, HSPB6, HSPB7, and HSPB8 are ubiquitously expressed, and are constitutively present in the brain at low levels (Quraishe et al., 2008). HSPB4 is expressed in the lens of the eye, composing nearly 50% of the protein mass in the human lens (Andley, 2007). HSPB2 and HSPB3 are expressed in muscle and heart (Quraishe et al., 2008), although a recent study indicates that they also have some expression in the brain (Kirbach and Golenhofen, 2011). HSPB9 and HSPB10 are expressed in the testes (Quraishe et al., 2008). Notably, only three members of the sHSP family (HSPB1, HSPB5, and HSPB8) are induced in response to challenges such as heat (Morimoto and Santoro, 1998; Zhang et al., 2002), glucocorticoids (Nédellec et al., 2002), prostaglandins (Ito et al., 1997), and interferon-gamma (Oba et al., 2008), rendering them true sHSPs that are upregulated in response to cellular stress.

Biochemical, biophysical, and crystallography studies have elucidated the structure of HSPB5 and key residues important for quaternary structure and chaperone function (Bagnéris et al., 2009; Jehle et al., 2009, 2010, 2011). Naturally occurring mutations in conserved regions in several human sHSPs have functional consequences including myopathies (Vicart et al., 1998; Simon et al., 2007), cataracts (Litt et al., 1998), and Charcot Marie Tooth disease (Evgrafov et al., 2004; Ackerley et al., 2006). The crystal structures of sHSPs give us insights into understanding how some of these mutations have pathological consequences. However, mounting evidence over the past two decades suggests that sHSPs may not only play a role in maintaining a healthy body, but that they also have protective functions in disease or injury to the central nervous system (CNS; Sun and MacRae, 2005; Arrigo et al., 2007; Steinman, 2008). This insight has illuminated the possibility of using sHSPs as a novel class of neuroprotective agents.

ROLE OF ENDOGENOUS sHSPs IN NEUROLOGICAL DISEASES

Altered regulation of sHSPs has been seen in many neurodegenerative and neuroinflammatory diseases in both human and rodent brain tissue. A summary of published studies is shown in **Table 2**.

TAUOPATHIES: ALZHEIMER'S DISEASE AND PICK'S COMPLEX

Tauopathies are neurological diseases that involve abnormal aggregation of the tau protein in the brain (Ballatore et al., 2011). The healthy tau protein stabilizes microtubules, which are necessary

Table 1 | Endogenous expression of sHSPs in the brain.

sHSP	Alternate name	Constitutive expression in brain
BI	Hsp 27 (human)	Quraishe et al. (2008)
	Hsp 25 (mouse)	Armstrong et al. (2001), Kirbach and Golenhofen (2011)
B2	MKBP	Kirbach and Golenhofen (2011), limited expression
B3	–	Kirbach and Golenhofen (2011), limited expression
B4	Alpha-A crystallin (cryaa)	
B5	Alpha-B crystallin (cryab)	Quraishe et al. (2008), Kirbach and Golenhofen (2011), Dubin et al. (1989)
B6	Hsp 20	Quraishe et al. (2008), Kirbach and Golenhofen (2011)
B7	–	Quraishe et al. (2008), mRNA, not protein
B8	Hsp 22	Quraishe et al. (2008), Kirbach and Golenhofen (2011)
B9	–	
B10	–	

for the proper transportation of proteins and neurotransmitters along neuronal axons. However, when tau becomes defective and hyperphosphorylated, it can aggregate and forms neurofibrillary tangles (NFTs) that interfere with normal neuronal function and ultimately lead to cell death.

The most common tauopathy is Alzheimer's disease, a neurodegenerative disease characterized by NFTs and amyloid-rich plaques in the brain that ultimately results in cognitive decline and dementia (Ballard et al., 2011). The endogenous regulation of sHSPs in Alzheimer's disease has been well examined. HSPB1 levels are elevated in the cortex of Alzheimer's patients, with higher levels corresponding to increased severity and duration of dementia (Renkawek et al., 1994a). In fact, HSPB1 levels correlate significantly with levels of phosphorylated tau (Shimura et al., 2004; Björkdahl et al., 2008), suggesting that it may play a role in protecting the cell from the pathological effects of hyperphosphorylated tau.

HSPB5 is also upregulated in Alzheimer's disease (Iwaki et al., 1992; Shinohara et al., 1993; Renkawek et al., 1994b) and highly expressed in ballooned neurons (Lowe et al., 1992). Mao et al. (2001) found that HSPB5 is highly expressed in neurons in the vicinity of extracellular NFTs, but less so in classical senile plaques, diffuse plaques, and intracellular NFTs.

Three other sHSPs have been shown to be elevated in Alzheimer's disease: HSPB2, HSPB6, and HSPB8. Using immunohistochemistry, Wilhelmus et al. (2006b) found small, but not significant, elevations in HSPB2 and HSPB6 in Alzheimer brains. HSPB6 localizes to both diffuse and classic senile plaques, whereas HSPB2 was only present in the classic senile plaques. An additional study by Wilhelmus et al. (2006a) showed that HSPB8 is found in classic senile plaques from AD brains. These data collectively indicate that certain members of sHSPs are elevated in Alzheimer's disease, but it is unknown whether they are playing a protective or pathological role in the disease process and there are no studies to date that investigate the therapeutic potential of sHSPs in Alzheimer's disease.

Less common tauopathies include Pick's complex, which is now more commonly referred to as frontotemporal dementia (FTD; Weder et al., 2007). This collection of tauopathies includes frontotemporal lobar degeneration, corticobasal degeneration (CBD), progressive supranuclear palsy (PSP), and familial tauopathy FTD with parkinsonism linked to chromosome 17 (FTDP-17; Kertesz, 2003; Tolnay and Probst, 2003).

In many of these conditions, tau pathology is not limited to neurons and instead extends to glial cells such as astrocytes and oligodendrocytes (Komori, 1999). Both HSPB1 and HSPB5 were found to be elevated in brains of patients with olivary hypertrophy, a condition characterized by the enlargement of neurons and neuronal loss in response to a lesion in the dentatoolivary pathway. This can occur as a result of trauma, tumors, cerebrovascular disease, and PSP, a component of Pick's Complex (Hanihara et al., 1998). HSPB5 has been shown to be upregulated early in disease progression in neurons and later in astrocytes, but HSPB1 was only elevated in astrocytes later in the disease course (Fukushima et al., 2006). However, another study did not find upregulation of HSPB1 or HSPB5 in neurons, but rather that localization of these sHSPs is specific to glial cells in sporadic CBD and PSP and familial FTDP-17 (Dabir et al., 2004). The specificity to astrocytes was confirmed by a study that found elevated levels of HSPB1 in astrocytes in PSP and CBD (Schwarz et al., 2010). It is currently unknown why there are elevated levels of sHSPs in these diseases; there have been no published studies determining the effects that a loss of sHSP (i.e., using genetically deficient mice) or a gain of sHSP (i.e., exogenous administration or overexpression) would have on these diseases.

OTHER PROTEIN AGGREGATION DISEASES: PARKINSON'S AND ALS

Parkinson's disease is a neurodegenerative disease that is caused by the loss of dopamine-producing neurons in the substantia nigra. The decrease in dopamine is accompanied by an accumulation of alpha-synuclein protein, forming inclusions called Lewy bodies (Burn, 2006). Clinical features of Parkinson's disease are described by the acronym TRAP: tremor at rest, rigidity, akinesia or bradykinesia (loss of movement and slowness of movement, respectively), and postural instability (Jankovic, 2008).

HSPB1 and HSPB5 are upregulated in the cortex of Parkinson's disease patients (Renkawek et al., 1994a, 1999). These sHSPs are associated with an increased number of tangles in the hippocampus of these patients. Mouse data corroborated the human data, and showed similar elevations in both HSPB1 and HSPB5 using the transgenic Parkinson's model, alpha-SynA53T (Wang et al., 2008).

Amyotrophic lateral sclerosis (ALS), also known as Lou Gehrig's disease, is a neurodegenerative disease characterized by the progressive loss of motor neurons. ALS is associated with the formation of intraneuronal proteinaceous inclusions that are non-amyloid, many of which include hyperphosphorylated and ubiquitinated TAR DNA-binding protein 43 (TDP-43; Perry et al., 2010). Approximately 95% of ALS cases appear to be sporadic and the remaining 5% are familial (Traub et al., 2011). Twenty percent of the familial cases can be attributed to a mutation

Table 2 | Small heat shock protein expression in human and rodent models of neurological diseases.

Disease	sHSP	Model system	Regulation	
			Up	Down
Amyotrophic lateral sclerosis (ALS)	B1	Mouse	Vleminckx et al. (2002), Wang et al. (2008)	Maatkamp et al. (2004)
	B5	Mouse	Vleminckx et al. (2002), Wang et al. (2008)	–
		Human	Iwaki et al. (1992)	–
Alexander's disease	B1	Human	Head et al. (1993), Iwaki et al. (1993)	–
	B5	Human	Head et al. (1993), Iwaki et al. (1989), Iwaki et al. (1992), Iwaki et al. (1993)	–
Alzheimer's disease	B1	Human	Renkawek et al. (1994b), Björkdahl et al. (2008), Shinohara et al. (1993), Wilhelmus et al. (2006b)	–
	B2	Human	Wilhelmus et al. (2006b)	–
	B5	Human	Björkdahl et al. (2008), Shinohara et al. (1993), Lowe et al. (1992), Renkawek et al. (1994a), Iwaki et al. (1992), Wilhelmus et al. (2006b)	–
	B6	Human	Wilhelmus et al. (2006b)	–
	B8	Human	Wilhelmus et al. (2006a)	–
Epilepsy	B1	Human	Bidmon et al. (2004)	–
Huntington's disease	B5	Mouse	–	Zabel et al. (2002)
Multiple sclerosis	B1	Human	Aquino et al. (1997), Han et al. (2009)	–
	B5	Human	Sinclair et al. (2005), Bajramovic et al. (1997), Iwaki et al. (1992)	–
Other tauopathies	B1	Human	Fukushima et al. (2006), Dabir et al. (2004), Schwarz et al. (2010)	–
	B5	Human	Fukushima et al. (2006), Dabir et al. (2004), Lowe et al. (1992), Iwaki et al. (1992), Kato et al. (1992)	–
Parkinson's disease	B1	Human	Renkawek et al. (1994b), Renkawek et al. (1999)	–
		Mouse	Wang et al. (2008)	–
	B5	Human	Renkawek et al. (1999), Iwaki et al. (1992)	–
		Mouse	Wang et al. (2008)	–
Prion disease	B1	Mouse	Tortosa et al. (2008)	–
		Sheep	Vidal et al. (2009)	–
	B5	Human	Renkawek et al. (1992), Kato et al. (1992), Iwaki et al. (1992)	–
Spinocerebellar ataxias	B1	Human cell lines	Chang et al. (2005)	Tsai et al. (2005), Wen et al. (2003), Chang et al. (2005)
	B1	Mouse	Chang et al. (2005)	–
	B1	Human	Chang et al. (2005)	–
Stroke	B1	Rat	Imura et al. (1999)	–
	B5	Rat	Piao et al. (2005)	–
		Human	Minami et al. (2003), Lowe et al. (1992)	–

in the superoxide dismutase 1 (SOD1) enzyme (Rosen et al., 1993).

Iwaki et al. (1992) found that eight human ALS brains had higher immunoreactivity against HSPB5 compared with healthy brains. This result is corroborated in a study using the mutant SOD mouse model of familial ALS, in which $SOD1^{G93A}$ mice exhibited higher levels of HSPB5 in the cytoplasm of reactive glial cells (Vleminckx et al., 2002). This study found that levels of HSPB1 were upregulated in mouse neurons and glial cells as well. However, the story is complicated by the fact that Maatkamp et al. (2004) found that protein levels of HSPB1 were downregulated just before the degeneration of motoneurons in the mutant SOD mouse model. They also found that mRNA levels of HSPB1 remained unchanged despite lower protein levels, highlighting the need for further studies to clarify what the role of sHSPs might be in ALS.

POLY-GLUTAMINE DISEASES: HUNTINGTON'S AND SPINOCEREBELLAR ATAXIAS

Poly-Q diseases are caused by genetic mutations that lead to a trinucleotide repeat of CAG, the triplet that encodes the amino acid glutamine. This increases the number of glutamines in the protein from as few as 20 to as many as 306 residues depending on the disease. These diseases are known as poly-Q diseases based on the one letter amino acid abbreviation of glutamine, and they include Huntington's disease (HD), dentatorubral-pallidoluysian atrophy (DRPLA), spinobulbar muscular atrophy (SBMA), and the spinocerebellar ataxias (SCAs; Paulson et al., 2000).

Huntington's disease is a neurodegenerative disease caused by a poly-glutamine expansion of the huntingtin (Htt) gene (Eidelberg and Surmeier, 2011). It typically appears during middle age and is characterized by chorea, or involuntary, explosive, fidgeting movements.

The SCA diseases are a group of neurodegenerative disorders that are also caused by an expanded poly-glutamine repeat. They manifest as a loss of gait and coordination difficulties. There are distinct subtypes, each caused by a specific mutation in a gene encoding an ataxin protein; around 30 different genes have been identified to date (Di Donato et al., 2001).

Contrary to findings in other protein aggregation diseases of the CNS, elevation of sHSPs have not been observed in the poly-Q diseases. One study using the R6/2 mouse model of HD found reduced levels of HSPB5 at the end of the disease course (Zabel et al., 2002). However, studies on HSPB1 in SCA found that HSPB1 is downregulated early in the disease (Wen et al., 2003; Chang et al., 2005; Tsai et al., 2005), but upregulated during later stages of the disease (Chang et al., 2005). These controversial data might uncover a dynamic property in the expression of sHSPs, indicating the need to examine sHSP expression at multiple time points in all diseases discussed above.

INFECTIOUS PROTEIN AGGREGATION DISEASE: PRIONS

Misfolded prions are infectious proteins that are responsible for the transmissible spongiform encephalopathies (Prusiner, 1998; DeArmond and Prusiner, 2003). These include bovine spongiform encephalopathy (BSE, commonly referred to as "mad cow disease") in cattle, scrapie in sheep, and Creutzfeldt–Jakob disease (CJD) in humans.

Small heat shock proteins are elevated in prion disease. HSPB1 is increased in scrapie in sheep (Vidal et al., 2009) and in a mouse model of BSE (Tortosa et al., 2008). Research on HSPB5 has been limited to humans; HSPB5 has been shown to be dramatically elevated in both glia and neurons from CJD brains (Kato et al., 1992; Renkawek et al., 1992). Given the similarities in protein aggregation between tauopathies and prions, it is likely that sHSPs are playing similar roles in both disease states.

LEUKODYSTROPHIES: ALEXANDER'S DISEASE

Alexander's disease is a rare genetic disease, a leukodystrophy with abnormal development of the myelin sheath, resulting from a mutation in the glial fibrillary acidic protein (GFAP; Messing et al., 2010). Alexander's disease is typified by the appearance of Rosenthal fibers – fibrous, eosinophilic deposits in the brain that are involved in the pathogenesis of the disease. It is a progressive, neurodegenerative disease that is usually fatal.

Both HSPB1 and HSPB5 are upregulated in Alexander's disease. Head et al. (1993) showed that both mRNA and protein levels of HSPB1 and HSPB5 were elevated in Rosenthal fibers in astrocytes taken from human patients, which was corroborated by Iwaki et al. (1989, 1993). Additionally, mouse models of Alexander's disease that lacked HSPB5 demonstrated greater mortality, indicating that the presence of alpha-B crystallin in the astrocytes is protective (Hagemann et al., 2009).

AUTOIMMUNE DISEASES: MULTIPLE SCLEROSIS

Multiple sclerosis (MS) is an autoimmune demyelinating disease of the CNS that manifests as lesions predominantly in the white matter (Rejdak et al., 2010). It is characterized by a T cell mediated attack on the myelin sheath surrounding the axons of neurons. Although protein aggregation has not been observed in MS, sHSPs are elevated and appear to play a protective role during the course of the disease.

van Noort et al. (1995) first identified that HSPB5 is involved in MS pathogenesis when they pinpointed this molecule as the most immunodominant myelin T cell antigen in this disease. These findings suggested that HSPB5 might be an autoantigen in MS and that immune cells attacked endogenous HSPB5 as part of the pathology of the disease. This theory was supported by data that showed high levels of HSPB5 in astrocytes and oligodendrocytes in MS lesions (Iwaki et al., 1992; Bajramovic et al., 1997). Although subsequent studies found that HSPB5 was the most abundant transcript in MS lesions (Chabas et al., 2001), attempts to induce experimental autoimmune encephalomyelitis (EAE), the predominant mouse model of MS, with HSPB5 as an antigen, rather than using a myelin antigen, were never successful (Verbeek et al., 2007). Further research indicated that although HSPB5 is upregulated during the course of the disease, its purpose is protective rather than pathological. A 2007 study conducted by Ousman et al. (2007) found that mice deficient in HSPB5 suffered from more severe EAE than wild type mice and that treatment with exogenous HSPB5 ameliorated EAE. This study demonstrated that the absence of HSPB5 results in a more pro-inflammatory state of immune cells and a higher level of immune cell infiltration into the brain. Furthermore, treatment of HSPB5 deficient EAE mice or WT EAE mice with exogenous HSPB5 decreases immune infiltration into the brain and shifts the phenotype of these immune cells to an anti-inflammatory state. Additional studies have validated the initial reports that HSPB5 is elevated in the MS brain (Sinclair et al., 2005) and that HSPB5 is elevated in the blood of MS patients (Rothbard et al., 2012).

HSPB1 has also been shown to be upregulated in MS. Using immunohistochemistry, HSPB1 was found to be elevated in astrocytes and oligodendrocytes in the plaques (Aquino et al., 1997). A recent study found elevated levels of HSPB1 circulating in the blood of MS patients, peaking during relapses (Ce et al., 2011).

ACUTE NEUROLOGICAL INSULT AND INFLAMMATION: STROKE AND EPILEPSY

Small heat shock proteins are not just upregulated in long-term, chronic diseases of the CNS; recent studies have shown an

upregulation in acute inflammatory conditions such as stroke and epilepsy.

Stroke is the result of the lack of blood supply to the brain, leading to brain injury. The most common type is ischemic stroke, which is defined as a blocked blood vessel to the brain. Inflammatory mediators exacerbate acute stroke. These mediators infiltrate the injured area upon reperfusion. Several studies have been conducted that examine the expression of HSPB5 in human and mouse models of stroke. HSPB5 is found in ballooned neurons at the edge of cerebral infarcts (Lowe et al., 1992) and elevated in 68% of human stroke brains, specifically in the neurons (Minami et al., 2003). Studies using rodent models of cerebral ischemia indicate that HSPB5 is transiently upregulated in neurons a few hours after reperfusion and followed by gradual sustained increase in astrocytes (Piao et al., 2005). HSPB5 has also been shown to be elevated in human and mouse plasma post-stroke (Arac et al., 2011). Notably, HSPB1 is also upregulated in both rat and mouse models of ischemia (Imura et al., 1999) and overexpression of HSPB1 is neuroprotective in cerebral ischemia models (van der Weerd et al., 2010).

Epilepsy is a neurological disorder characterized by seizures. These are due to abnormal, excessive, or synchronous neuronal activity in the brain (Vezzani et al., 2011). Only one study has found that HSPB1 is elevated in epileptic human neocortex (Bidmon et al., 2004). Using immunohistochemistry, they found that HSPB1 was located in both astrocytes and the walls of blood vessels.

PROOF OF CONCEPT THERAPEUTIC EXPERIMENTS

Many studies have shown that endogenous sHSPs are elevated in neurological diseases, with some studies showing the lack of sHSPs leads to worse disease, implying a protective role for these molecules. This has led to the examination of these molecules as potential novel therapeutics with proof of concept experiments in mice using three routes of administration: transgenic overexpression, viral administration, and exogenous treatment (**Table 3**).

PARKINSON'S DISEASE AND ALS

Using a viral vector, HSPB1 has been shown to have a positive effect in an *in vitro* model of Parkinson's disease by protecting against alpha-synuclein induced cell death (Zourlidou et al., 2004). However, no studies have been conducted on the therapeutic effects of HSPB1 on *in vivo* models of PD.

Overexpression studies using transgenic mouse models of ALS have shown mixed results. One study demonstrates that overexpression of transgenic HSPB1 in the $SOD1^{G93A}$ mouse model of ALS by crossing $SOD1^{G93A}$ mice with HSPB1 overexpressing mice did not delay disease onset or decrease disease severity (Krishnan et al., 2008), despite the fact that HSPB1 overexpression was protective in acute motor neuron injury (Sharp et al., 2006). However, another study by Sharp et al. (2008) indicates that HSPB1 does have a positive effect early in the disease course in the same mouse model of ALS. They show that $SOD1^{G93A}$/HSP27 double transgenic mice had delayed decline in motor strength and increased survival of spinal motor neurons compared to $SOD1^{G93A}$ single transgenics during the early phase of disease. *In vitro* experiments using SOD1 mutant cells demonstrate that HSPB1 has an anti-apoptotic function (Patel et al., 2005). This suggests that HSPB1 does have an effect, but the levels in the overexpressing mouse may not be high enough to combat the chronic disease state. It may also suggest that the cellular upregulation of sHSPs represents only one part of the protective response and that the presence of sHSPs in the plasma may be a crucial factor in ameliorating disease.

ALEXANDER'S DISEASE

A study conducted by Hagemann et al. (2009) using a mouse model of Alexander's disease demonstrated that mice lacking HSPB5 exhibited increased mortality, but restoring HSPB5 specifically in astrocytes using a GFAP promoter reversed this effect. Additionally, transgenic overexpression of HSPB5 protects mice from death in a second model of Alexander's disease that typically causes the mice to die at one month of age (Hagemann et al., 2009).

MULTIPLE SCLEROSIS

Mice lacking HSPB5 were found to suffer from more severe disease in the experimental autoimmune encephalomyelitis (EAE) model of MS, and intravenous administration of exogenous HSPB5 ameliorated disease (Ousman et al., 2007). EAE mice treated with HSPB5 showed a dampened immune response, including less proliferation of immune cells and a lower production of pro-inflammatory cytokines (Ousman et al., 2007). Furthermore, we believe that this anti-inflammatory effect is based on HSPB5's ability to act as a chaperone extracellularly, binding pro-inflammatory molecules, including members of the acute phase, coagulation, and complement pathways (Rothbard et al., 2012). Additional experiments using other members of the sHSP family have indicated that all members are therapeutic in EAE and they act through anti-inflammatory effects (unpublished data). This further expands the research supporting the therapeutic possibilities of sHSPs in MS (Steinman, 2008).

STROKE

Transgenic mice that overexpress HSPB1 exhibited 30% smaller lesion sizes after undergoing a permanent MCAO model of stroke (van der Weerd et al., 2010). This corroborated previous work conducted in a cardiac ischemia model that showed that transgenic overexpression of HSPB1 was protective (Hollander et al., 2004). Virally administered HSPB1 was also shown to be protective in cerebral ischemia, although the mechanism was not clear (Badin et al., 2006). The researchers of that study speculated that the protective effect of HSPB1 might be due to its chaperone function. Finally, recent work has shown that HSPB5 is therapeutic when treating mouse models of stroke 12 h post-insult (Arac et al., 2011).

MECHANISMS OF sHSP NEUROPROTECTION

A substantial number of studies have indicated that endogenous sHSPs, particularly HSPB1 and HSPB5, are upregulated in CNS injury and disease. Studies investigating both sHSP deficiency and overexpression support the conclusion that these molecules are serving neuroprotective roles rather than pathological ones.

A caveat to the studies done using the HSPB5 deficient mice: these mice also lack the sHSP HSPB2. HSPB2 is not thought to be

Table 3 | Overexpression and exogenous administration of sHSPs.

Disease	sHSP	Model system	Treatment type	Therapeutic efficacy
ALS	B1	$SOD1^{G93A}$ mouse model of ALS	Tg overexpression	No effect (Krishnan et al., 2008) Effective in early, but not late disease (Sharp et al., 2008)
		SOD1 mutant neuronal cell lines	Viral vector	Anti-apoptotic (Patel et al., 2005)
Alexander's disease	B5	$GFAP^{Tg}$ and $GFAP^{Tg}$; $GFAP^{+/R236H}$ mouse models of Alexander's disease	Tg overexpression	Rescue of lethal phenotype in $GFAP^{Tg}$/cryab null mice by induction of Cryab under GFAP promoter (Hagemann et al., 2009)
Huntington's disease	B1	R6/2 mouse model of Huntington's disease	Tg overexpression	No effect (Zourlidou et al., 2007)
		COS-7 (monkey kidney) or SK-N-SH (human neuroblastoma) cells transfected with huntingtin exon 1 (httEx1) fused to EGFP	Cellular co-transfection	Prevented poly-Q mediated cell death, but did not prevent protein aggregation (Wyttenbach et al., 2002)
Multiple sclerosis	B5	EAE mouse model of MS	Exogenous administration	Decreased clinical score when administered at peak of disease (Ousman et al., 2007; Rothbard et al., 2012) Reduced apoptosis in CNS (Ousman et al., 2007)
Parkinson's disease	B1	Cells expressing alpha-synuclein	Viral vector	Anti-apoptotic (Zourlidou et al., 2004)
Ischemia/reperfusion (stroke)	B1	Permanent middle cerebral artery occlusion (MCAO) mouse model of cerebral ischemia	Tg overexpression	Reduced infarct size (van der Weerd et al., 2010)
			Viral vector	Reduced infarct size (Badin et al., 2006)
			Exogenous administration	Reduced infarct size (Arac et al., 2011)
Acute nerve injury	B1	Neonatal nerve injury, mouse	Tg overexpression	Rescues motor neurons 5–6 months following injury (Sharp et al., 2006)

inducible, so the levels of HSPB2 are typically not elevated in neurological diseases (for an exception see Wilhelmus et al., 2006b). Although we believe that the phenotype we see in HSPB5 deficient mice is due to the lack of HSPB5 alone, it is possible that the lack of HSPB2 is contributing to the effects observed in these mice. However, HSPB5/HSPB2 deficient mice have worse disease, which supports the idea that sHSPs are serving protective roles whether that is due to HSPB2 or HSPB5. An additional point to consider is whether the phenotype observed in HSPB5/HSPB2 deficient mice is due to the lack of the sHSPs or the altered levels of another molecule that is dependent on normal sHSP function. If isogenic mouse strains are not used as controls, then it is possible that polymorphisms in other genes could be the cause of differences seen between the WT and HSPB5/HSPB2 deficient mice.

How sHSPs are neuroprotective is still a subject of debate. It could be due to its molecular chaperone properties that prevent protein aggregation, in particular for protein aggregation diseases such as Alzheimer's or Parkinson's. However, sHSPs are also protective in non-aggregation diseases such as acute ischemia. Several studies have indicated an anti-apoptotic role for sHSPs, which might be the reason they are protective. HSPB5 and HSPB1 have been shown to be anti-apoptotic when overexpressed by either a transgene or virus (Akbar et al., 2003; Kalwy et al., 2003; Li et al., 2005). HSPB5 confers protection against apoptosis through the regulation of caspase-3, a proapoptotic factor (Shin et al., 2009) and has also been shown to sequester the p53 tumor suppressor, thus preventing apoptosis (Liu et al., 2007).

However, in light of recent studies that have indicated that sHSPs have an anti-inflammatory role (Ousman et al., 2007; Arac et al., 2011), the anti-apoptotic effects of sHSPs are unlikely to be the whole story. Although historically the brain was thought to be immune privileged, research over the past few decades has shown that the immune system is an important factor in many neurological diseases previously thought to be independent of the immune system, including Alzheimer's and stroke. Endogenously upregulated sHSPs may be playing dual roles as both anti-apoptotic and anti-inflammatory molecules in these diseases. In fact, Arac et al. (2011) investigated the relative effects of the deficiency of HSPB5 in the immune system and the brain post-stroke by doing a bone marrow chimera experiment. By irradiating WT and HSPB5$^{-/-}$ mice, depleting their immune systems, and reconstituting their immune systems with immune cells from either WT or HSPB5 deficient mice, they created four different types of mice: WT mice with WT immune cells, WT mice with HSPB5$^{-/-}$ immune cells, HSPB5$^{-/-}$ mice with WT immune cells, and HSPB5$^{-/-}$ mice with HSPB5$^{-/-}$ immune cells. After inducing stroke and comparing infarct size, they found that deficiencies of HSPB5 in either the brain or immune system alone increases infarct size and together result in a synergistic effect (Arac et al., 2011). They did not demonstrate any specificity to particular brain cells and this is an area of future investigation to fully understand the contribution of HSPB5 in the brain compared to the immune system.

The mechanism of action by which sHSPs are anti-inflammatory is currently under investigation. A study conducted by Rothbard et al. (2012) suggests that HSPB5 acts extracellularly as a molecular chaperone, binding acute inflammatory molecules. Notably, they showed that HSPB5 binding was temperature-dependent and binding increases with an increase in temperature, making HSPB5 more effective at sites of inflammation. Exactly how endogenous HSPB5 is released from cells is currently a matter of speculation. HSPB5 does not possess a signal sequence, so it is not secreted by the normal secretory pathway. However, a recent paper showed that HSPB5 could be released via exosomes (Gangalum et al., 2011). Additionally, if damage is occurring in the brain, it is very likely that cells are undergoing apoptosis or necrosis and releasing HSPB5 upon death. However, whether HSPB5 is released in a regulated manner through a specific process or leaked out of damaged or dying cells, the specific mechanism by which it becomes extracellular does not affect our interpretation that it can act as an extracellular chaperone.

Although we do see higher endogenous levels of sHSPs in neurological diseases, in particular at the site of damage, studies investigating the therapeutic effect of sHSPs using exogenous administration of sHSPs have been focused only on mouse models of MS and stroke. Using varying concentrations of human recombinant HSPB5 administered both intravenously and intraperitoneally, a therapeutic effect of HSPB5 has been shown in mouse models of MS (Ousman et al., 2007; unpublished data). This therapeutic effect has been demonstrated in EAE mice with different genetic backgrounds and we have seen a similar therapeutic effect from the administration of other members of the sHSP family (unpublished data). HSPB5 has also been shown to be therapeutic when administered intraperitoneally in a mouse model of stroke (Arac et al., 2011). We believe that it is likely that exogenous administration of HSPB5 will be therapeutic in other neurological diseases, but the experiments have yet to be done.

The vast literature indicating that endogenous sHSPs, particularly HSPB1 and HSPB5, are protective in neurological diseases opens the door for the possibility that these molecules could be developed as novel therapeutics. To date, therapeutic strategies utilizing sHSPs have been conducted solely in mice; however, the data generated from these experiments have provided the foundation to pursue this exciting avenue of therapy.

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HSP60: issues and insights on its therapeutic use as an immunoregulatory agent

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Heat shock proteins 60 (HSP60) is one of the most well studied member of the HSP family. Although found to be a target self antigen in pathological autoimmunity and HSP60-reactive T and B cells are part of immune responses in several infectious diseases, there is consistent experimental evidence that HSP60 displays dominant immunoregulatory properties. There are a series of reports on animal models showing that the administration of HSP60 can modulate inflammatory diseases. However, HSP60 has both immune-regulatory and inflammatory properties placing it as an essentially homeostatic antigen, but with potentially harmful effects as well. There have been a series of reports on the successful use of HSP60 and its peptides as immune-modulatory agent for several models of autoimmune diseases and in some clinical trials as well. We believe that the potential risks of HSP60 as a therapeutic agent can be controlled by addressing important factors determining its effects. These factors would be route of administration, appropriate peptides, time point of administration in the course of the disease, and possible association with other modulatory agents.

Keywords: heat shock proteins, HSP60, homeostasis, inflammation, immunoregulation

HSP60 AS AN IMMUNODOMINANT HOMEOSTATIC ANTIGEN

Heat shock proteins (HSP) are highly conserved proteins during evolution. Besides being upregulated in stress conditions, they are constitutively and abundantly present in all living beings and are profoundly involved in various intracellular and systemic homeostatic functions in different species (Hemmingsen et al., 1988; Karlin and Brocchieri, 2000). This feature can be illustrated by their capacity to prevent protein degradation in salamanders or in tropical trees that live exposed to very high temperatures (Kusukawa and Yura, 1988), or by their capacity to interact with the immune system in mammalians, contributing to the fine tuning of inflammation (Pockley et al., 2009), and participating in cell survival signaling pathways (Chun et al., 2010). These robust homeostatic properties and their marked evolutionary conservation lead us to think that HSP can be considered as dominant homeostatic molecules in evolution. Taken the diversity of HSP families (classified according to their molecular weight), it is possible that different HSP families play differential roles in the complex integrated homeostatic network.

Heat shock proteins 60 is one of the most well studied HSP, especially in the interaction with the immune system. It was first described as intracellular chaperone, assisting the folding of polypeptides into proteins and the transporting of proteins (Bukau et al., 2006), but later it was found to be a dominant antigen recognized during infections, together with HSP70 (Kaufmann, 1990; Zügel and Kaufmann, 1999). These properties place these two proteins in a special category as antigens. Indeed, HSP60 has been intensely studied in various immunologic contexts such as in autoimmune diseases (Danielle et al., 1992; Tanaka et al., 1999; Dieude et al., 2004; Quintana et al., 2004, 2008; Van Eden et al., 2005) – often considered as an important target molecule in the pathogenesis – in tumors (Michaluart et al., 2008; Victora et al., 2009) and in transplantation (Qian et al., 1995; Birk et al., 1999; Granja et al., 2004). But, HSP60 does not seem to be a relevant antigen only in pathological conditions. Autoreactivity to HSP60 is also found in health and a high frequency of B and T cell clones reactive to self HSP60 is found in physiological conditions in mammalians (Munk et al., 1989; Fischer et al., 1992; Abulafia-Lapid et al., 1999; Pockley et al., 1999).

Heat shock proteins have been considered by Irun Cohen (Cohen and Young, 1991) as immunodominant antigens for self immune responses and as key players in physiological autoimmunity, likely to be involved in homeostasis (Cohen and Lohse, 1991; Cohen, 1992). In line with this idea, self-HSP-reactive T and B cell clones can be seen as an important part of a network of regulatory cells and molecules in the immune system engaged in homeostatic activities (Cohen, 2007). These activities would include tissue maintenance and repair, but also limiting clonal expansion and controlling inflammation.

Since the discovery of AIRE and its role in the expression of various tissue specific antigens by thymic medullary epithelial cells (MEC; Anderson et al., 2002), it became clear that the different levels of expression of these antigens placed them at a hierarchical order of relevance for central tolerance (Kyewski and Klein, 2006). All highly expressed antigens would compose a particular set of "dominant" antigens relevant for the immune homeostasis (Coutinho and Haas, 2001). In this sense, HSPs can be considered as strong candidates to be part of this immunodominant

homeostatic self. They are ubiquitous antigens expressed both in housekeeping as well as in stressed circumstances, and they are highly expressed in the thymus (Mamalaki et al., 1996). Taken that transgenic expression of HSP60 in the thymus does not lead to the deletion of HSP60 specific T cell clones (Minter and Osborne, 2003), it is possible that central tolerance to HSPs relies on the generation of natural regulatory T cells (nTregs) akin to the way tolerance is imposed to many other self antigens (Coutinho et al., 2005; Aschenbrenner et al., 2007). Indeed, nTregs do recognize self antigens and HSPs, including HSP60, are likely to be among these self antigens. In addition, HSP60 has a strong effect in the survival and function of CD4+CD25+Foxp3+ regulatory T cells (Zanin-Zhorov et al., 2006) and they were shown to efficiently drive the differentiation of CD4+CD25− T cell clones derived from Juvenile idiopathic arthritis (JIA) patients into CD4+CD25^{high} regulatory T cells expressing GITR, CTLA-4, and Foxp3 (de Kleer et al., 2004).

In addition to the reported immunomodulatory effect of HSP60, it is clear that this molecule also displays inflammatory actions. HSP60 activates both innate and adaptive arms of the immune response being considered as a danger signal that amplifies inflammation and influence a wide range of immune reactions (Chen et al., 1999; Wallin et al., 2002; Habich and Burkat, 2007). Anti-HSP antibodies and HSP60-reactive T cells are part of immune responses in several infectious diseases (Kaufmann, 1990). Approximately 10–20% of the specific T cells in mice immunized with *Mycobacterium tuberculosis* are against the bacterial HSP65 (Kaufmann et al., 1987). Antibodies to HSP60 of *Chlamydia trachomatis* have been detected at high levels in the sera of infected patients (Sanchez-Campillo et al., 1999), and immunodominant responses to HSP60 are present in other fungal infections (Matthews et al., 1998). This strong immune response directed to HSP60 during infection can be explained by its critical role in cellular homeostasis and by its upregulation in host tissues as a result of stress during infection. In addition, antibodies as well as effector pathological T cells reactive to self HSP60 were found in different autoimmune and inflammatory diseases including type 1 diabetes (Elias et al., 1991; Birk et al., 1996; Abulafia-Lapid et al., 1999) rheumatoid arthritis (RA), multiple sclerosis (Quintana et al., 2008), lupus erythematosus (Dieude et al., 2004), atherosclerosis (Xu et al., 1993), Behcet's disease (Tanaka et al., 1999), inflammatory bowel disease (Stevens et al., 1992), and inflammatory skin disorders (Bayramgurler et al., 2004).

Although HSP60 was first found to be a mitochondrial protein (McMullin and Hallberg, 1988), it is now known that, in eukaryotes, it can also locate in the cytosol (Chandra et al., 2007), at the cell surface (Wand-Wurttenberger et al., 1991; Soltys and Gupta, 1997), in the extracellular space (Davies et al., 2006), and soluble in the peripheral blood (Cappello et al., 2008). In addition, HSP60 peptides can be also be presented in MHC class I and class II molecules (Koga et al., 1989; Anderton et al., 1995), activating specific CD8+ and CD4+ T cells (Van Eden et al., 2005). Thus, we believe that many of its immunologic functions may be triggered/modulated by soluble HSP60 and its peptides present in the microenvironment. In this respect, several synthetic HSP60 peptides have been shown to be immunologically active *in vitro* (Paul et al., 2000; Caldas et al., 2004; Van Eden et al., 2005) and *in vivo* (Elias and Cohen, 1995; Huurman et al., 2007), but the repertoire of HSP60 peptides actually generated *in vivo* in different physio and pathological contexts is still unknown.

Immunologic activation induced by any type of stimulus triggers a variety of both inflammatory and anti-inflammatory mechanisms, and the capacity to both induce and control inflammation is critical to homeostasis. HSP60 has both properties placing it as an essentially homeostatic antigen, but with potentially harmful effects as well. Thus, we think it is crucial to always look at both functional sides of the immune response in any given immunologic context. We have proposed the term REG/INFLAMMA to denote functional activities predominantly immunoregulatory or proinflammatory of any given immune molecule. In our descriptive model, a REG/INFLAMMA molecular panel of immunologically relevant transcription factors (such as GATA-3, RORγt, T-bet, Foxp3) and cytokines (such as IL-6, TGF-β, IL-10, IFN-γ, TNF-α) is used as a way to evaluate the overall immunologic activity of a given molecule or of a particular pathological context (Moraes-Vieira et al., 2012). We believe this may be a useful strategy to evaluate the functional activities of HSP60 peptides, for selecting adequate candidates for clinical application. As a matter of fact, for the specific selection of HSP60 peptides, the REG/INFLAMMA panel could be expanded to also include relevant innate molecules, since HSP60 and its derived peptides also interact with a variety of these molecules.

Some of the HSP60-autoreactive T cell clones have been already isolated from mice (Birk et al., 1996), rats (Feige and Cohen, 1991), and humans (Caldas et al., 2006), under a variety of inflammatory conditions, showed REG and INFLAMMA properties *in vitro* and *in vivo*. This dual immunologic activity places Hsp60 and its derived peptides in a privileged position as potential therapeutic immunomodulators, to either amplify or control inflammation. On the other hand, the same duality also poses a concern for clinical application because it is still unclear what determines a predominant REG or INFLAMMA outcome induced by HSP60 and derived peptides, as previously discussed (Coelho et al., 2008).

HSP60 REGULATORY ACTIVITY IN INFLAMMATORY DISEASES

Although found to be a target self antigen in pathological autoimmunity, there is consistent experimental evidence that HSP60 displays dominant immunoregulatory properties. There are a series of reports on animal models showing that the administration of HSP60 can generate T cell responses that modulate inflammatory diseases (arthritis, type 1 diabetes, atherosclerosis, EAE, asthma, lupus, dermatomyositis; Quintana and Cohen, 2011).

Administration of HSP60 and its peptides by oral, nasal, intraperitoneal, and subcutaneous routes has been extensively studied in experimental models of arthritis in rats (Van Eden et al., 2005). The protective effects of these protocols lead some groups to explore the reactivity of T cells of patients with JIA and RA to HSP60. Interestingly, HSP60-reactive T cells isolated from JIA, but not from AR, patients display a regulatory phenotype and secrete predominantly cytokines such as IL-10 e TGF-beta (de Kleer et al., 2004).

Intranasal and oral administration of HSP65 from *Mycobacterium tuberculosis* has been also tested with success in the

Ldlr−/− mice which develop atherosclerosis upon feeding a hypercholesterolemic diet (Harats et al., 2002; Maron et al., 2002). HSP65-treated mice showed a co-relation between plaque reduction and IL-10 expression at the aortic arch (Maron et al., 2002).

In the NOD mice, a type 1 diabetes model, systemic treatment with HSP65 from Mycobacteria as well as with HSP60 p277 peptide trigger immunoregulatory pathways that result in suppression of disease (Elias et al., 1991; Elias and Cohen, 1995; Birk et al., 1996). Phases II and III clinical trials using HSP60 p277 in patients with type 1 diabetes have been successful in the preservation of islet beta-cell function (Raz et al., 2001, 2007) and, more recently, also in a randomized phase III trial with over 400 newly diagnosed type 1 diabetes patients. Other clinical trials employing HSP60 peptides arrested the autoimmune destruction in patients with RA (van Roon et al., 1997; de Kleer et al., 2004), and autoimmune uveitis (Stanford et al., 2004).

The capacity of HSP60 to control inflammation in the context of transplantation seems to be a harder task. May be this is related to the more robust inflammatory response, simultaneously triggered by multiple alloantigens, in an already highly inflammatory milieu induced by surgery, in contrast to the more insidious development of inflammation in autoimmune diseases, together with the insidious occurrence of epitope spreading to newly exposed self antigens. Nonetheless, our group has been investing on the identification of potent HSP60 immunoregulatory peptides for use in allotransplantation. We have been able to prolong allograft survival across both minor (Luna et al., 2007) and major (unpublished data from V. Coelho laboratory) alloantigen disparities but so far, we have been unsuccessful in inducing transplantation tolerance. Nevertheless, other investigators have been able to induce transplantation tolerance in a mouse model of skin allograft (Birk et al., 1999), but publications in this field are scarcer. Despite the difficulties, we believe it is worth investing on the use of HSP60 peptides as immunomodulators in allotransplantation; may be not alone, but in combination with other drugs and immunoregulatory molecules. If HSP60 and its peptides are, indeed, endogenous immunoregulators, we should find ways to enhance/activate endogenous immunoregulatory networks, in a synergic manner with other therapies. This strategy seems particularly relevant to transplantation, due to the multicity of antigens involved in triggering inflammation; each pair of donor/recipient comprise a different set of alloantigens inducing aggression against the graft. If we are able to identify combinations of conserved dominantly immunoregulator HSP60 peptides, they could be used in allotransplantation, alone or in combination with other immunoregulatory therapies, irrespective of specific alloantigen disparities. A broad immunoregulatory effect is likely to occur once human HSP60 DNA vaccination has been able to control disease in NOD diabetes, inducing a Th2-like immune response not only to HSP60 but also to other relevant target self antigens, such as GAD (glutamic acid decarboxylase) and insulin (Quintana et al., 2002).

IMMUNOREGULATORY MECHANISMS INDUCED BY HSP60

Heat shock proteins 60 and derived peptides are able to exert an important role in the fine balance between promoting and controlling inflammation through a variety of mechanisms. It is very likely even that HSP60's REG and INFLAMMA functional activities occur simultaneously within an inflammatory microenvironment, probably also mediated by its different peptides, affecting different cell types, and mobilizing different molecular pathways. However, it is not known whether specific molecular signals, either immune cell or tissue-derived, influence HSP60's functional activity in the course of inflammation. Maybe there is also a time course difference – a proinflammatory predominance at the initial phase of inflammation and a regulatory one at a later time point – as we previously suggested in the context of human renal transplantation (Granja et al., 2004).

Heat shock proteins 60 can be viewed as a protein antigen that will be processed and presented to T cells inducing a typical lymphocyte reaction. However, HSP60 and its various peptides have the capacity to interact with a variety of immune molecules, intracellularly (Cappello et al., 2008; Chun et al., 2010) and at the cell surface such as with TLR2, TLR4 (Vabulas et al., 2001), and MHC molecules (Newcomb and Cresswell, 1993), bridging innate and adaptive immune responses. Therefore, plasticity and multiple connectivity are also striking immunologic properties of HSP60.

An important issue that may be raised regarding this plasticity is the relationship between the dual role (regulatory and inflammatory) of HSP60 with its binding molecules. The signaling of an endogenous protein expressed in low amounts chronically may lead to distinct patterns of activation of TRL-expressing cells such as dendritic cells, macrophages, and T cells. In this respect, we would like to highlight the complex diversity of effects triggered by different molecules through the same receptor on different cell types. For instance, it has been shown that both HSP60 and HSP70 signaling, through TLR2 and NF-κB activation, on murine cardiomyocytes induce, *in vitro*, contractile dysfunction and cell death (Mathur et al., 2011). The treatment of human CD4+CD25+ Tregs with human HSP60 or its p277 peptide before anti-CD3 activation also enhanced their ability to down-regulate proliferation and production of IFN-γ and TNF-α by CD4+CD25− or CD8+ target T cells. In addition, the enhancing effects of HSP60 costimulation on Tregs involved innate signaling via TLR2, led to activation of PKC, PI3K, and p38, and were further enhanced by the inhibition of ERK. HSP60-treated Tregs suppressed target T cells both by cell-to-cell contact and by secretion of TGF-β and IL-10 (Zanin-Zhorov et al., 2006). HSP60 is also a ligand that activates B cells via TLR4. Simultaneous ligation of TLR4 and BCR in HSP60 specific B cells induces antibody secretion (Cohen-Sfady et al., 2005; Herlands et al., 2008) and this may be an important way to induce the anti-HSP60 IgM autoantibodies that are prevalent in physiologic conditions (Merbl et al., 2007). TLR4 ligation in epithelial cells are also known to play a protective role in the intestinal mucosa. On the other hand, high concentrations of HSP60 usually trigger TLR4 signaling and inflammatory activation of monocytes (Pockley et al., 2009).

Adding more complexity to the plethoric immunobiology of HSP60, it was recently reported that HSP60 interacts with beta-catenin, increasing its transcriptional activity and protein expression, and favoring metastasis (Tsai et al., 2009). Taken that beta-catenin also enhances cell survival of several cell types including Tregs (Ding et al., 2008), it is plausible that this may represent an

additional pathway by which HSP60 and derived peptides promote immunoregulatory activity.

THERAPEUTIC USE OF HSP60 FOR INFLAMMATORY DISEASES: ADVANTAGES AND RISKS

Since administration of Hsp60 has a well documented regulatory effect in inflammatory disease models, it has been proposed by several groups that this antigen may be used as a therapeutic tool in diseases where inflammation is undesirable such as autoimmune and allergic diseases and transplantation.

Despite the concerns about the potential risks of inducing inflammatory undesirable effects, different formulations of HSP60 and derived peptides have been used for immunoregulation in a variety of animal models, and no evidence of pathological autoimmunity was detected. Accordingly, we have not found histopathological signs of inflammation in over 10 tissues following the use of the HSP65-DNA vaccine, in mice (Lima et al., 2009). Nevertheless, we believe that the use of HSP60 peptides displaying a predominant immunoregulatory function, instead of the whole molecule, may reduce potentially harmful effects. In addition, this strategy may provide an opportunity to combine different peptides which trigger distinct immunoregulatory mechanisms. Nonetheless, depending on the route of administration, the quantity of peptides required may be a limiting issue for clinical application.

Another risk that can be foreseen for the use of HSP60 is a possibility of immunosuppression to protective responses against infection. Experiments carried out by our group have shown that mice treated with HSP65 by the oral route for 4 days have decrease immune responses to HSP65 but show a normal response to *Salmonella* infection (Rezende et al., unpublished results). The course of infection was not altered by the treatment suggesting that protective responses to infectious agents may rely on other potent pathogen-associated antigens.

On the contrary, in infectious contexts, immune response to bacterial antigens might benefit from the fact that there is a high frequency of self-HSP-reactive T and B cell clones in a normal repertoire. These HSP-reactive lymphocytes would be recruited and take part in the ongoing immune response, exerting mainly a regulatory role and contribute to reestablishing homeostasis (Cohen and Young, 1991). Indeed, immunomodulation of inflammatory responses during infection is critical for the outcome of the infectious disease. There are several examples of infectious diseases in which severity of disease is mostly related to a high prevalence of proinflammatory cytokines (especially TNF-α) and poor immune-regulatory components (such as IL-10 or Tregs). This is true for Chagas disease (Vitelli-Avelar et al., 2008), Schistosomiasis (Wamachi et al., 2004), and Leishmaniasis (Oliveira et al., 2011).

It has been shown that effector CD4+ T cells that secrete large amounts of IL-2 are able to efficiently recruit CD4+CD25+Foxp3+ regulatory T cells (Curotto de Lafaille et al., 2004; Almeida et al., 2006). IL-2 is a critical cytokine for Treg function and this is one of the mechanisms triggered during inflammatory events capable of modulating the degree of tissue damage without compromising needed inflammatory responses. Therefore, the REG/INFLAMMA balance is an essential part of all inflammatory responses. HSPs fit very well to this picture since they are self proteins ubiquitously expressed and also they are upregulated in stress conditions.

The fact that HSP60 is an ubiquitous molecule, on the other hand, may represent another benefit. Its use would circumvent the need for the identification of the target antigens involved in the induction of each inflammatory disease. In this regard, it has been demonstrated that tolerance induced to an antigen can recruit regulatory T cells and molecules that would spread their effect in a bystander fashion (Miller et al., 1991). This bystander effect depends on the antigen presenting cells that induce the regulatory T cells and it recruits for the modulatory immune network most the antigens presented in the same context.

In view of these potential risks and benefits, we believe that some issues have to be addressed on the therapeutic use of HSP60 as an immunoregulatory agent: route of administration, form of the protein (whole molecule, peptides), time point in the course of the disease, possible association with other modulatory agents.

ROUTE OF ADMINISTRATION

The majority of studies on the use of HSPs and HSP60 as modulatory agents in several models of inflammatory diseases have used the parenteral (subcutaneous, intraperitoneal, intravenous, and intramuscular) route of administration (Van Eden et al., 2005), probably because of its easy translation into a later clinical setting. However, we believe it is time to reexamine the pros and contras of the routes in the light of new knowledge on HSP60 immunobiology.

It has been extensively described that the oral route is a very efficient way to induce peripheral tolerance in animal models (Faria and Weiner, 2006) and also in humans (Mestecky et al., 1996). Oral tolerance is a well known phenomenon that probably accounts for the robust balance that keeps the homeostasis of the gut mucosa to the daily challenge of microbiota and dietary antigens (Faria and Weiner, 2005). Indeed, we are all tolerant to the food proteins we ingest and also to our microbiota and this has been documented in mice and humans (Andrade et al., 2006; Round et al., 2010). This is especially interesting regarding HSP because bacterial components of our microbiota do express HSPs and they are likely to be involved in immunoregulatory networks in the gut. Although many of these proteins are intracellular, we now know that HSP60 can also be expressed at the cell surface and in the extra cellular space, therefore providing a variety of molecular forms and pathways by which HSP60 could affect the immunologic milieu in the gut. In addition, it is plausible that some of the luminal contents (including dead bacteria) in contact with the abundant lymphoid tissue of the gut mucosa would induce regulatory T cells and oral tolerance. Therefore, oral tolerance to our microbiota can be envisaged as a peripheral form of homeostasis reinforcement of tolerance involving HSPs.

Of all the feeding regimens already tested for oral tolerance, continuous feeding has been shown to be the most efficient way to induce tolerance. Continuous feeding of antigen, but not gavage, can render otherwise refractory animals, such as aged mice, tolerant. Moreover, oral tolerance induced by continuous feeding of antigen lasts longer and, in mice, do not require any type of reinforcement during their lifetime (Faria et al., 1998, 2003).

Since continuous feeding protocols are very cumbersome for human studies, the use of a probiotic vehicle has been designed to continuously deliver *Mycobacterium leprae* HSP65 in the gut mucosa upon a single administration daily. This strategy associates the immunomodulatory and tolerogenic potential of HSP65 and of the gut mucosa. We observed that oral administration of Hsp65-producing *Lactococcus lactis* prevented EAE in mice (Rezende et al., unpublished results). Histological analysis showed no inflammatory cell infiltrate and no tissue destruction in the spinal cord of HSP65-treated mice. Moreover, increased frequency of regulatory T cells correlated with the treatment. Thus, this strategy may constitute a promising alternative therapy for the treatment of autoimmune and inflammatory diseases.

Nasal administration of antigens is also an effective form of inducing long living tolerance (Mestecky et al., 1996; Faria and Weiner, 2006). Regulatory elements triggered by nasal versus oral administration of antigen may be distinct but both routes have been described as efficient ways to modulate inflammatory diseases by HSP60, including reducing atherosclerotic plaque formation in the Ldlr−/− mice (Maron et al., 2002) and prolonging allograft survival in mice (Luna et al., 2007). We would like to point that other forms of continuous delivery of HSP60 have been explored with success. Accordingly, intranasal administration of HSP60 peptide p277 encapsulated into polylactide-co-glycolide acid microspheres resulted in increased skin graft survival in two combinations of murine strains with minor H-2 disparities (Luna et al., 2007).

We believe that the use of strategies such as continuous delivery of antigen by mucosal routes is an appropriate way to favor anti-inflammatory activities of HSPs by mimicking natural routes of tolerance induction and should be further explored.

WHOLE PROTEIN OR PEPTIDES?

An important caveat regarding the *in vitro* functional studies using recombinant HSP60 is endotoxin contamination which, even at very low concentrations, may still influence cytokine production and immune cell functional activity. This seems particularly important because HSP60 and its derived peptides bind to TLRs, including TLR4 which is also a ligand to endotoxin, raising controversies regarding HSP60 specific immunologic effects. This issue has been extensively discussed in the literature and several investigators claim that there is sufficient evidence to support HSP60 specific signaling actions (Henderson et al., 2010). Indeed, working with low-endotoxin HSP60 recombinant preparations has minimized the effect of bacterial contaminants. However, we believe this issue will only be clearly solved when HSP60 produced in endotoxin-free systems is used for functional assays. Taking this into consideration, as well as HSP60 REG/INFLAMMA properties, several groups and ours have been exploring the immunologic functions of HSP60 synthetic peptides in a variety of pathological and physiological (Prakken et al., 1997; Luna et al., 2007) contexts, and have identified immunologically active peptides, effective in, at least partially, downregulating inflammation in animal models (Elias et al., 1991; Thompson et al., 1998; Luna et al., 2007) and in humans in the context of diabetes (Raz et al., 2001, 2007).

Using a panel of HSP60 synthetic peptides and a gene panel of predominantly REG/INFLAMMA molecules, we have found several peptides displaying the capacity to simultaneously modify the gene expression of a variety of immune molecules, *in vitro*, and have identified some predominantly REG and others INFLAMMA, using both mouse splenocytes and human peripheral blood mononuclear cells (PBMC; unpublished data from V. Coelho's laboratory). Interestingly, some predominantly regulatory HSP60 peptides were shared by the two species, suggesting evolutionary functional conservation.

TIME POINT OF THE DISEASE

For oral administration protocols, it is well established that the regulatory mechanisms of oral tolerance is very efficiently triggered before inflammation onset and their efficiency declines progressively after sensitization. Therefore, for oral administration of hsp65, early diagnosis and treatment would be the ideal protocol. This will be critical for HSP60 administration considering the dual functional properties of the antigen (REG–INFLAMMA). Another way to circumvent this caveat of oral treatment with HSP60 might be the association with a delivery system that could act as a modulatory adjuvant boosting the tolerogenic effect of HSP60.

As mentioned before, it is not known whether specific molecular signals from the tissues influence HSP60's functional activity in the course of inflammation. It is possible that a time course difference exists as we have observed in patients who had renal transplants (Granja et al., 2004). Autoreactivity to HSP60 displayed a proinflammatory effect at the initial phase of transplantation and a regulatory one at a later time point.

ASSOCIATION WITH OTHER MODULATORY AGENTS

Since immune tolerance mediated by HSP60 could be more difficult to induce in sensitized diseased individuals, the use of modulatory adjuvants might be helpful. If the oral route is chosen, probiotic bacteria would be excellent candidates since they are well known as agents with immunomodulatory properties. Several probiotic bacteria are already in use for this purpose (*Lactobacillus*, *Bifidobacteria*, *Lactococcus*) and the ones that were genetically engineered to express immune-relevant proteins are specially interesting (Pontes et al., 2011). A trial on IL-10-producing *L. lactis* is currently been carried out after promising results in animals models of inflammatory bowel disease (Braat et al., 2006). Hsp65-producing *L. lactis* would also fulfill these requirements since the bacteria *L. lactis* has modulatory properties on its own (unpublished data) besides their effect as delivery agent.

Another interesting strategy recently reported is the use of a pathologically relevant antigen along with HSP60 to induce immunomodulation. Nemirovsky and coworkers used amyloid beta peptide Aβ1–15 conjugated with HSP60 peptide p458 in a subcutaneous vaccination protocol for a murine experimental model of Alzheimer disease. The combined peptide vaccine resulted in a significant decrease in cerebral amyloid burden and inflammation in the brain (Nemirovsky et al., 2011). A similar approach could be designed for autoimmune diseases such as type 1 diabetes, arthritis, myasthenia gravis, and others for which a target self molecule has already been identified.

CONCLUDING REMARKS

The dual immunologic role of HSP60 and derived peptides – inflammatory and regulatory – can be viewed as an advantage for multiple clinical applications, either boosting effector responses or controlling inflammation. On the other hand, it also raises concerns, since it is still unclear what determines HSP60's immune-regulatory or inflammatory activities. We believe that critical factors such as route of administration, time course of the disease in which it will be used, dosage, HSP60 peptides chosen, and the combination with other modulatory molecules may circumvent the putative risks posed by its inflammatory potential.

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HSP: bystander antigen in atopic diseases?

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Over the last years insight in the complex interactions between innate and adaptive immunity in the regulation of an inflammatory response has increased enormously. This has revived the interest in stress proteins; proteins that are expressed during cell stress. As these proteins can attract and trigger an immunological response they can act as important mediators in this interaction. In this respect, of special interest are proteins that may act as modulators of both innate and adaptive immunity. Heat shock proteins (HSPs) are stress proteins that have these, and more, characteristics. More than two decades of studies on HSPs has revealed that they are part of intrinsic, "natural" mechanisms that steer inflammation. This has provoked comprehensive explorations of the role of HSPs in various human inflammatory diseases. Most studies have focused on classical autoimmune diseases. This has led to the development of clinical studies with HSPs that have shown promise in Phase II/III clinical trials. Remarkably, only very little is yet known of the role of HSPs in atopic diseases. In allergic disease a number of studies have investigated the possibility that allergen-specific regulatory T cell (Treg) function is defective in individuals with allergic diseases. This raises the question whether methods can be identified to improve the Treg repertoire. Studies from other inflammatory diseases have suggested HSPs may have such a beneficial effect on the T cell repertoire. Based on the immune mechanisms of atopic diseases, in this review we will argue that, as in other human inflammatory conditions, understanding immunity to HSPs is likely also relevant for atopic diseases. Specifically, we will discuss why certain HSPs such as HSP60 connect the immune response to environmental antigens with regulation of the inflammatory response. Thus they provide a molecular link that may eventually even help to better understand the immune pathological basis of the hygiene hypothesis.

Keywords: allergic disease, atopic disease, stress proteins, heat shock protein, HSP60, regulatory T cells, hygiene hypothesis, inflammation

INTRODUCTION

Over the last years insight in the complex interactions between innate and adaptive immunity in the regulation of an inflammatory response has increased enormously. This has revived the interest in stress proteins; proteins that are expressed during cell stress. As these proteins can attract and trigger an immunological response they can act as important mediators in this interaction. In this respect, of special interest are proteins that may act as modulators of both innate and adaptive immunity. Heat shock proteins (HSPs) are stress proteins that have these, and more, characteristics. More than two decades of studies on HSPs has revealed that they are part of intrinsic, "natural" mechanisms that steer an inflammatory response (Pockley, 2003; van Eden et al., 2005). This has provoked comprehensive explorations of the role of HSPs in various human inflammatory diseases. Most of these studies have focused on classical autoimmune diseases such as type I diabetes (IDDM), rheumatoid arthritis (RA), and juvenile idiopathic arthritis (JIA; Abulafia-Lapid et al., 1999; de Kleer et al., 2003, 2004). This has led to the development of clinical studies with HSPs that have shown promise in Phase II/III clinical trials in both IDDM and RA (Albani et al., 1995a; Raz et al., 2001; Prakken et al., 2004).

Remarkably, only very little is yet known of the role of HSPs in atopic diseases. Based on the immune mechanisms of atopic diseases, in this review we will argue that, as in other human inflammatory conditions, understanding immunity to HSPs is likely also relevant for atopic diseases. Specifically, we will discuss why certain HSPs such as HSP60 connect the immune response to environmental antigens with regulation of the inflammatory response. Thus, they provide a molecular link that may eventually even help to better understand the immune pathological basis of the hygiene hypothesis: an important concept relating the increase in the prevalence of atopic disease with exposure to microbes or microbial products early in life.

ALLERGY

Atopy is the predisposition to develop allergic diseases like atopic dermatitis, food allergy, asthma, and allergic rhinitis. It is characterized by a predominant typical T helper 2 (Th2)-like immune response (Umetsu et al., 2003). Firstly, an environmental allergen,

like an inhalant or food allergen interacts with the innate immune system. After uptake by antigen-presenting cells, subsequent T cell priming leads to the stimulation of type 2 cytokines such as interleukin (IL)-4, IL-5, and IL-13. These cytokines interact with their receptors to induce a class switch toward IgE production and to increase the number of eosinophils and mast cells. In immediate hypersensitivity reactions IgE binds to its receptors and causes mast cells to degranulate. Apart from IgE, increased levels of IgG4 are often measured in allergic individuals. IgG4 is considered as an antibody with anti-inflammatory activity (van der Neut et al., 2007). The levels of IgG4 are usually elevated in serum from people that are repeatedly exposed to the same antigen, like beekeepers, or subjects receiving increased doses of antigen during specific immune therapy (SIT). In addition to this humoral immune response, allergy is characterized by a cellular response, mostly mediated by Th2 cells (Del Prete, 1992; Ozdemir et al., 2010), but also by Th1 cells (like in atopic dermatitis) and regulatory T cells.

The combination of these humoral and cellular responses, both innate and adaptive, leads to an allergic inflammatory reaction (Yazdanbakhsh et al., 2002). Here we will discuss various aspects of the allergic immune responses in the context of the possible role of environmental antigens such as HSPs in steering this immune response.

ALLERGY AND THE HYGIENE HYPOTHESIS

The prevalence of atopic disease has increased tremendously in the second half of the twentieth century (Peat et al., 1994). Although the cause of this increase is not known though, a genetic cause is unlikely due to the time span in which the increase took place. Therefore environmental changes are more likely causes of this observed increase. Strachan (1989) first proposed the concept of the hygiene hypothesis. This hypothesis states that the increase in the prevalence of atopic disease is due to a decreased exposure to microbes or microbial products early in life, especially in western societies. The concept was supported by the observation of an inverse association between both atopic dermatitis and hay fever and the number of children in a household. Although the hygiene hypothesis and its proposed immune mechanism are still under debate, in the 1990s numerous of studies have been published supporting this hypothesis. For example, these observations relate the number of siblings, the number of infections and exposure to endotoxins on the farm, with the incidence of atopic diseases (Jarvis et al., 1997; Bodner et al., 1998; von Mutius et al., 1999; Wickens et al., 1999; Braun-Fahrlander et al., 2002).

IMMUNE MECHANISMS OF THE HYGIENE HYPOTHESIS

What could be the immunological cause of this inverse relation between exposure to environmental antigens and the incidence of allergic disease? The explanation takes us back to the original observations in the eighties of Mosmann and Coffman who defined two main subtypes of helper T lymphocytes: Th1 and Th2 cells. Th2 cells are characterized by their production of cytokines like IL-4, IL-5, IL-9, and IL-13 and chemokines like TARC and MDC. They are involved in mediating allergic responses and host defense against parasitic infection (Mosmann and Coffman, 1989).

During fetal life and shortly after birth a predominant Th2 response is present (Wegmann et al., 1993). During life, a shift to a predominant Th1 response occurs in non-allergic subjects, but this shift may be incomplete in allergic individuals. As a consequence, an allergic individual will develop a Th2 response to an allergen leading to IgE production and typical type 2 cytokine production (IL-4, IL-5, and IL-13) and ultimately to a clinical allergic reaction, whereas non-allergic subjects develop a Th1 response leading to a protective IgG response and production of IFN-γ (Larche, 2007). This is the basis for the immunological explanation of the hygiene hypothesis, as exposure to microbes and microbial products would stimulate a Th1 response. Those individuals that were not enough exposed to these stress factors would be more prone to develop predominant Th2 responses, and thus an allergic response. Obviously this is still a simplification of the immune pathogenesis that does not explain all observations. For example, a Th1 response in allergic individuals would implicate the development of late hypersensitivity responses, whereas healthy individuals do not respond to allergens with a clinically noticeable response (Chen et al., 2004).

The hygiene hypothesis also does not explain why not only the incidence of allergic diseases is increasing but also that of auto immune diseases like RA and IDDM (Sheikh et al., 2003). These diseases are obviously known as typical Th1-like diseases.

TRIGGERING AN ALLERGIC IMMUNE RESPONSE

In order to fully assess and better appreciate the value of the hygiene hypothesis it is important to understand which cells are responsible for the initial trigger leading to the characteristic allergic immune response. First, naive T cells have to be activated, in a way that promotes the classical Th2 like response. There is recent evidence that basophils may play a crucial role in antigen recognition and processing. When recruited to lymph nodes they are able to induce Th2 cell differentiation through the release of IL-4 (Sokol et al., 2009; Falcone et al., 2011). Other cells also have the capacity to process and present allergens, including mast cells, macrophages, eosinophils, and natural helper cells. Natural helper cells are innate cell populations that include innate Th2 cells and are activated by IL-25 and IL-33 to secrete Th2 cytokines (Saenz et al., 2010).

There is an increasing realization that Th2 cells are not the only cells involved in the pathophysiology of allergic disease, as particularly in severe disease other cells than Th2 play a role in aggravating and perpetuating the immune response (**Table 1**; Lloyd and Hessel, 2010).

First of all, whereas Th2 cytokines have a clear role in initiating an allergic response, especially Th1 cells have been implicated in more chronic severe allergic disease, both in atopic dermatitis and allergic asthma (Krug et al., 1996; Ong and Leung, 2006; Yamanaka and Mizutani, 2011).

Secondly, in addition to the Th2 cytokines IL-4, IL-5, and IL-13, recently also IL-9 was shown to play an important role in asthma. At first IL-9 was discussed as a new Th2 cytokine. It was shown that Th9 cells are dependent on both IL-4 and transforming growth factor-beta (TGF-β) for their development. Interestingly, like Th2 cells, also Th9 cells are regulated by IL-25, predominantly seen in lung inflammation (Devos et al., 2006; Hauber et al., 2007; Soroosh and Doherty, 2009; Angkasekwinai et al., 2010). IL-25 (like IL-33 and thymic stromal lymphopoietin) is a type II initiating cytokine (Oliphant et al., 2011). In addition to the role of

Table 1 | Different subtypes described to play a role in the pathogenesis of allergic diseases.

Cell	Main transcription factor	Activating cytokines	Effector cytokines	Role in allergic diseases
Th1	T-Bet	IL-12	IFNy	Possible role in chronic asthma or atopic dermatitis
Th2	GATA-3	IL-4, IL-25, IL-33, TSLP	IL-4, IL-5, IL-13, IL-25	Eosinophil production; IgE induction
Th9	PU-1	IL-4, TGFβ, IL-10, IL-25	IL-9, IL-10	Mucus production, lung inflammation, dermatitis
Th17	Batf	TGFβ, IL-1β IL-6, IL-21	IL-6, IL-8, IL-17, IL-22	Possibly related to steroid resistant asthma; related to neutrophil production
Th22	RORγT(?)	?	IL-22	Limiting airway inflammation in mice. Negative association with Th2 cytokines
Tr1	FOXP3	TGFβ	IL-5, IL-10, IL-13, TGFβ	Regulatory function on B cells by suppression of allergen-specific IgE and induction of IgG4 and IgA
Tregs	FOXP3	TGFβ	IL-10	Diminished function and number in allergic individuals

IL-25 in lung inflammation, it is also of importance in atopic dermatitis and food allergy (Aalberse et al. unpublished data; Hvid et al., 2011). Although IL-25 (also known as IL-17E) is a cytokine from the IL-17 family it has opposite effects of IL17A (IL-17).

Thirdly, Th17 cells (named after their main effector cytokine; IL-17) like Th1 cells may be associated with more severe asthma and mediate a more neutrophilic pattern of inflammation.

Fourthly, it was recently shown that IL-22 (also produced by Th17 cells), is produced by a distinct set of CD4+ cells, named Th22-cells. These cells have been implicated as being protective in a mouse model of allergic lung inflammation (Souwer et al., 2010) and may play a role in the severity of atopic dermatitis in humans (Nograles et al., 2009).

Finally, a lot of attention has recently focused on a subset of T cells with the capacity to suppress other T cells, namely regulatory T cells (Treg). Two types of regulatory T cells will be discussed below, namely IL-10 producing regulatory cells and FOXP3 expressing regulatory cells.

REGULATORY T CELLS

Here we will discuss two types of regulatory T cells; FOXP3 expressing Tregs and IL-10 producing regulatory T cells. However, it has to be emphasized that the distinction between these two types is not absolute, as for example FOXP3 expressing Tregs are also capable of producing IL-10. Moreover, many different regulatory T cells, may even act in concert in a single immune response.

IL-10 PRODUCING REGULATORY T CELLS

As discussed above, exposure to an allergen leads to the development of sensitization in a susceptible individual. Upon a next encounter, a typical Th2 reaction will follow, including a humoral (IgE and IgG4) response. In contrast, when healthy (non-allergic) individuals are exposed to an allergen it was long thought that (in line with the Th1/Th2 dichotomy) instead of a Th2 response a Th1 response would develop. However it has become clear that, in fact, this is not the only difference between a normal and an allergic response to an allergen. Though indeed some IFN-γ responses to allergens are present in healthy individuals, a study by Akdis et al. (2004) showed that this is a simplification of the reality. They isolated CD4 T cells specific to several food- and aeroallergens from healthy and allergic individuals according to their IL-4, IFN-γ, and IL-10 secretion profile. Interestingly, allergen-specific T cells that belong to all three secretion profiles were detectable in both healthy and allergic individuals, with allergen-specific IL-10-secreting T cells being the predominant subset in healthy individuals. They regarded these IL-10 producing CD4+ T cells as T regulatory cell type 1 (Tr1 cells). Seemingly, low Tr1 numbers and high Th2 cell numbers resulted in an allergic response, whereas in healthy individuals a mixed Th1/Th2 response is associated with a strong IL-10 response (Larche, 2007). The crucial role for IL-10 in allergy is also suggested by other studies on food and inhalant allergies. In cow's milk allergy, T-cell clones derived from children that are persistently allergic produced Th2 cytokines (IL-4, IL-13), whereas allergic control subjects that are cow milk tolerant produced a mixed Th1/Th2 response associated with markedly elevated IL-10 levels (Tiemessen et al., 2004).

Similar observations of elevated IL-10 levels associated with less allergy symptoms were made in inhalation allergies. Children raised in a house with a cat are less likely to become allergic to cat allergen than those who only get indirect exposure. Many of these highly exposed children had an IgG and IgG4 response to the major cat allergen Fel d 1 without production of specific IgE. This induction of high levels of allergen-specific IgG4 in the relative absence of IgE has been referred to as a modified Th2 response (Platts-Mills et al., 2004). Interestingly also in the peripheral blood of these non-allergic children, T cell response to the allergens are characterized by an elevated level of IL-10. Comparable indications for a role for IL-10-producing Tr1 cells in the maintenance of tolerance to allergens can be found in individuals exposed to relatively high doses of allergen like bee keepers and allergic patients undergoing immunotherapy. Bee keepers with a repeated exposure to bee venom during the bee-keeping season demonstrate a marked increase in allergen-specific IL-10 secretion from peripheral blood T cells as the season progresses, while allergen-specific IgE is seen especially in the beginning of the season. Interestingly, reactions to stings disappear during the season, simultaneously with the increased IL-10 production by T-cells (Akdis and Blaser, 1999). Apart from IL-10, also TGF-β, another cytokine that can be produced by Tr1 cells, is reported to be induced by SIT, while both IL-10 and TGF-β are associated respectively with the blocking antibodies IgG4 and IgA (Larche et al., 2006; Taylor et al., 2006).

FOXP3 REGULATORY T CELLS

Not only IL-10 producing T cells but also FOXP3+ Tregare associated with an allergic response. The important role of the transcription factor FOXP3 for maintaining immune tolerance stems for multiple basic studies, mainly in experimental models. This importance of FOXP3+ Treg in human disease was underscored by the IPEX syndrome (immunodysregulation, polyendocrinopathy, and enteropathy, X-linked). In IPEX patients a genetic mutation causes the FOXP3 transcription factor to be defective. Patients with IPEX have symptoms that fit both generalized autoimmunity and allergy (Bennett et al., 2001; Patel, 2001). The relevance of FOX P3 Treg has been extensively demonstrated in mice models, showing an inverse correlation to Treg and diseases like RA, inflammatory bowel disease, MS, and IDDM (Shevach, 2000; Fontenot et al., 2003; Sakaguchi, 2005). Not only in autoimmune diseases but also in allergic disease various studies have suggested a possible defective suppressive function of Tregs (Viglietta et al., 2004; Sakaguchi et al., 2006). In addition to the bee keeper model also the season-dependent antigen exposure during the pollen season offers a model to study responses in the same individuals with and without exposure. Whereas in several studies it was shown that both non-allergic as allergic individuals have CD4+CD25+ Treg, in the allergic subjects, these cells still produced IL-5 and IL-13 cytokines (Grindebacke et al., 2004; Ling et al., 2004). Further related experiments demonstrated that both dose and type of allergen appear to have effects on the ability of CD4+CD25+ T cells to suppress responses. At the time of these studies $CD25^b$ expression on CD4 T cells was used as a surrogate marker of FOXP3+ Treg. Later studies using FOXP3 as a direct marker seemed to confirm these data. During venom immunotherapy the increased allergen-specific IgG4 and reduced IgE correlated with circulating FOXP3 positive Treg (Pereira-Santos et al., 2008). A recent study even suggested that Treg function may be impaired in patients with allergic diseases and that this function can be enhanced by specific immunotherapy (Palomares et al., 2010).

Thus, altogether, there are ample suggestions that the presence of allergen-specific T cells with a regulatory phenotype (either expressing FOXP3, and/or capable of producing IL-10 and TGF-β) may have a beneficial effect on allergic diseases. However in some atopic diseases, like atopic dermatitis and asthma, the specific trigger is often not known. This raises the question whether methods can be identified to improve the Treg repertoire, when the triggering allergen is unknown. Studies from other inflammatory diseases, like JIA and RA, have suggested that a certain group of antigens, called HSPs, are present at the site of inflammation and may have such a beneficial effect on the T cell repertoire.

HEAT SHOCK PROTEINS

The Treg repertoire is both formed in the thymus (so-called *natural Treg*) and generated in the periphery upon encounter with an antigen (induced or *adaptive Treg*). Both self and non-self antigens can induce Treg in the periphery, although the repertoire of Treg might be biased toward self antigens (Romagnoli et al., 2002).

In 1991, Cohen and Young (1991) proposed that a select group of self antigens is especially important for the maintenance of peripheral tolerance). He described the presence of auto reactive immune responses in a healthy individual to a limited set of self molecules, formed by auto reactive T cells and antibodies, which he called the immunological homunculus. The self antigens of this "homunculus" are all evolutionary highly conserved between the self and the non-self homolog of these proteins. According to Cohen, the immune system utilizes these self antigens to form an immunological picture of self which is crucial for the balance of the immune system. One of these homunculus' self proteins is human HSP60 (van Eden et al., 2005). HSPs are indeed evolutionarily highly conserved proteins and either present constitutively, functioning as chaperones, or induced upon cell stress caused by, for instance, heat, oxidative stress, and hypoxia (Craig et al., 1993). Several HSPs have been identified and, according to their size, organized into six families: HSP100, HSP90, HSP70, HSP60, HSP40, and HSP10. In this review we focus on the immune responses of HSP60 in atopic disease. It has to be stressed that as only very little data are available right now on HSPs and allergic disease, and thus we need to deduce the role of HSP in atopic disorders mostly on what is known about HSP reactivity on other diseases.

IMMUNITY TO HSPs AND INFLAMMATION

Humoral and cellular immune responses to HSP60 have been detected both in patients with an inflammatory disease, such as autoimmune and allergic diseases, as well as in healthy subjects. After these initial discoveries the perception was that HSP's may be involved in the development of autoimmunity through antigenis mimicry: an immune response toward a microbial HSP could lead to a cross-reactive response to a self-HSP and thus cause autoimmunity. However, after further studies it quickly became clear that the immune responses toward self HSP60 were more involved in the regulation and not in the induction of autoimmunity. Indeed, a wealth of data obtained in the last decade both in experimental models and from observations in human diseases point to a regulatory role of immunity to self HSP60 (van Eden et al., 2005).

The presence of self-HSP-reactive cells was first shown in animal studies by immunization of rats with mammalian HSP60 (Lopez-Guerrero et al., 1993). Though immunization could induce self-HSP-specific antibodies and T cells, a self-HSP-reactive immune repertoire was also shown to be present in the absence of immunization. The cause for this could be previous contact with homologous bacterial HSPs, for example in the mucosa of the gastro intestinal tract. The first report that immune responses to self HSP60 may have a regulatory role in inflammatory diseases was in mycobacteria-induced adjuvant arthritis. In this model the induction of a self HSP60, cross-reactive T cells response was responsible for the observed protection of HSP60 peptide immune therapy. After these initial findings, protective effects of various (conserved) microbial HSPs were seen in many other experimental disease models, including arthritis, atherosclerosis, allergic encephalomyelitis, and allergic asthma (Anderton et al., 1995; Birnbaum et al., 1998; Harats et al., 2002; Rha et al., 2002).

In vitro experiments show that immune responses modulated by HSPs can result in the induction of various cytokines like IFNγ and TNFα, as well as IL-10. HSPs are strong immune modulators and are able to influence the impact and direction of immune responses (Kaufmann et al., 1987). Moreover, HSP60 has the capacity to steer both innate and humoral immunity.

In human diseases, antibody responses to HSP60 were previously described in skin lesions of patients with Behçets disease, whereas HSP60 has been described as a target for T-cells in autoimmune diseases such as IDDM and JIA (Albani et al., 1995b; Ergun et al., 2001; Raz et al., 2001). Data in the experimental models have pointed out that the protective effect of HSP60 was independent of an antigenic relationship (antigen mimicry) between HSP60 and the disease-inducing antigen. Instead it seemed that HSP60 could confer protection through so-called bystander suppression (Horner et al., 2001; Larche et al., 2006). Inflammation causes local damage and cell stress leading to upregulation of stress proteins such as HSP60. Next, these bystander (self) antigens can become the target of subsequent, possibly suppressive immune responses. So, it is possible that immune responses to HSPs could be involved in the control of human chronic inflammatory diseases that have distinct, although as-yet-unknown, initiating auto-antigens, or even allergens.

HSP RESPONSES IN EARLY LIFE

Apparently, responses to HSP60 are important for maintaining peripheral tolerance in the adult immune system. It is unknown when during live these specific T cells arise. It would seem reasonable to expect that they are primed after birth upon encounter with homologous microbial HSP in the gut. However, there are indications that self HSP60 reactivity is present at birth. In a study by Ramage et al. (1999) it was shown that cord blood cells proliferate in response to an *in vitro* challenge with the self HSP60. This supported the hypothesis that this reactivity is part of the normal naive immune repertoire. A more recent study by Merbl et al. (2007) put these findings also in a different perspective. They found that normal cord blood contains IgM and IgA auto-antibodies directed against a relatively uniform set of auto-antigens, such as auto-antigens related to immune regulation such as HSP60. This obviously benign autoimmune self reactivity, present at birth, may have a dual function. On the one hand it may provide the basis for autoimmunity in later life, while on the other hand the "inborn" autoimmunity to regulatory self antigens such as HSP60 may actually serve to protect against autoimmune disease. Intrigued by these studies, we questioned whether CD4+ T cells specific for HSP60 are already detectable at birth before exposure to the microbial flora, and if so, to what type of immune response these "inborn" autoreactive CD4+ T cells have upon exposure to the self antigen HSP60. We found that HSP60 specific T cells are indeed present at birth. Moreover, stimulation of CBMC with HSP60 leads to CD4+ T cell proliferation and cytokine production, and induces T cells with an *in vitro* regulatory phenotype that are functionally suppressive (Aalberse et al., 2011).

HSP60 AND ALLERGY

Thus, HSP60 is a self-protein, recognized already at birth in healthy subjects, but also in individuals with chronic inflammatory diseases. Thereby a correlation between self-HSP reactivity and diminished disease activity is seen both in experimental as in human autoimmunity. This has already led to the successful exploration of HSP60 immune therapy in human disease, firstly in IDDM. It is not known yet if the levels and responses to HSP60 are different in individuals developing chronic inflammatory disease later in life.

For once, priming of HSP60 T cell responsiveness takes places primarily in the gut through contact with microbial HSP60 (van Eden et al., 2005). Thus, priming of HSP60 immunity early in life through contact with microbiota leads to more self HSP60 mediated immune regulation. This could be one of possibly multiple mechanisms that explain why individuals exposed to more microbial triggers early in life, could be less prone to develop immune mediated diseases. Thus it may very well fit the concept of the hygiene hypothesis.

For that reason we set out to study the role of HSP60 immunity in human atopic diseases. Obviously the first pre-requisite for a potential role of self HSP60 in dermatitis is the (preferably increased) expression of HSP60 at the site of inflammation. Indeed we were able to show that self HSP60 is increased expressed in the skin of patients with atopic dermatitis (Kapitein and Aalberse submitted for publication). Moreover, *in vitro* stimulation with self HSP60 induced FOXP3 positive T cells, as well as T cells producing IL-10 and IFN-γ.

It has to be emphasized that this increased expression by no means is very specific for atopic diseases (Seung et al., 2007). Still it could be highly relevant for atopic disease as the characterization of an antigen present at the site of inflammation, without being the disease-causing antigen has therapeutic possibilities through, as previously mentioned, "antigen driven bystander suppression." Bystander suppression as a mechanism is especially important in human autoimmune diseases, because often the immunizing trigger is unknown.

Theoretically, in allergic diseases this could also be an interesting option for intervention. Although often at least one of the allergens triggering the disease is known, mono-sensitization is rare which would imply multi-antigen immune therapy. Immune therapy with a single bystander antigen might undermine this issue.

NOVEL IMMUNE THERAPEUTIC POSSIBILITIES IN ALLERGIC DISEASE

Various novel immune therapeutic approaches to diminish allergic symptoms have been or are being studied. Of these new interventions, blocking of effector cytokines (such as IL-4 and IL-5) and IgEis the most important. As IL-5 is important for the priming and survival of eosinophils and these cells play an important role in the pathophysiology of allergic disease, blocking IL-5 seemed logical. However only a highly selected patient group with severe asthma, shown as sputum eosinophilia, had profit from this therapy (Busse et al., 2010). Also blocking IL-4 and IL-13 has not yet been shown to be of clinical benefit in asthma (Oh et al., 2010). Thus, so far, the experiences with blocking antibodies to effector cytokines suggest that either the cytokines cannot be fully blocked or that not one of these cytokines is solely responsible for the allergic response. As mentioned above in recent years Th2 induction cytokines have been described among which IL-25. IL-25 is strongly elevated in a subgroup of peanut allergic subjects (Aalberse et al., submitted for publication). Moreover, in mouse models blocking IL-25 has a positive effect on bronchial hyperreactivity (Ballantyne et al., 2007). Apart from blocking cytokines that are important in the pathophysiology of allergic disease, in the last decade various studies in asthmatic patients have been

performed in which an IgE-blocker was used (Rodrigo et al., 2011). Although the first results look promising, the effect is not present in all patients, underlining again the complexity of chronic allergic diseases.

Antigen SIT, in which an allergic individual is exposed to increasing doses of the allergen triggering disease, has been used for over a century. It is one of the earliest and most effective forms of human immune therapy. Although for long the mechanisms behind this clearly effective immune intervention was not fully understood, we now know that IgG4, IL-10, and Treg probably play an important role. A disadvantage of this approach in which the eliciting protein is used, is the chance of a severe anaphylactic reaction, as has been described following immune therapy in peanut allergic subjects (Reid et al., 1993).

To tackle this issue, recent work has focused on allergy peptide therapy, showing good results. Mice studies showed that after immunization with Der p 2, the house dust mite allergen, down-regulation of T cell and antibody responses was seen to Der p 2. Similar results were seen using Fel d 1 (cat allergen) and Bet v 1 (birch allergen) peptide therapy. As most patients are not sensitized to just one allergen, it is now studied if combination immune therapy is also effective. Another approach is the use of a peptide of an antigen present at the site of inflammation, without being the triggering antigen. This is the concept of the previous mentioned antigen bystander suppression model (Horner et al., 2001; Larche et al., 2006).

Based on the development of immune therapies usingHSP60 for other human inflammatory diseases and encouraged by recent data showing that HSP60 expression is increased in the skin in patients with atopic dermatitis, it is tempting to suggest that HSP60 may also be a potential candidate for bystander therapy in allergic diseases.

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Chronic inflammation in cancer development

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Chronic inflammatory mediators exert pleiotropic effects in the development of cancer. On the one hand, inflammation favors carcinogenesis, malignant transformation, tumor growth, invasion, and metastatic spread; on the other hand inflammation can stimulate immune effector mechanisms that might limit tumor growth. The link between cancer and inflammation depends on intrinsic and extrinsic pathways. Both pathways result in the activation of transcription factors such as NF-κB, STAT-3, and HIF-1 and in accumulation of tumorigenic factors in tumor and microenvironment. STAT-3 and NF-κB interact at multiple levels and thereby boost tumor-associated inflammation which can suppress anti-tumor immune responses. These factors also promote tumor growth, progression, and metastatic spread. IL-1, IL-6, TNF, and PGHS-2 are key mediators of an inflammatory milieu by modulating the expression of tumor-promoting factors. In this review we concentrate on the crucial role of pro-inflammatory mediators in inflammation-driven carcinogenesis and outline molecular mechanisms of IL-1 signaling in tumors. In addition, we elucidate the dual roles of stress proteins as danger signals in the development of anti-cancer immunity and anti-apoptotic functions.

Keywords: inflammation, carcinogenesis, tumorigenic factors, heat shock proteins, NF-κB, STAT-3, IL-1

INTRODUCTION

Based on the presence of leukocytes in cancerous lesions, Rudolf Virchow, the founder of cellular pathology, speculated about an association between chronic inflammation and development of cancer already in 1863 (Virchow, 1863). In line with this observation, epidemiological studies indicate that apart from hereditary predisposition, inflammation serves as a potential risk factor for the development of cancer. Nowadays it is generally accepted that up to 25% of human malignancies are related to chronic inflammation and to viral and bacterial infections (Hussain and Harris, 2007). **Table 1** provides an overview on inflammatory and pathogenic conditions that are considered to be associated with malignant transformation.

Cancer-related chronic inflammation facilitates unlimited replicative potential, independence of growth factors, resistance to growth inhibition, escape of programmed cell death, enhanced angiogenesis, tumor extravasation, and metastasis (Hanahan and Weinberg, 2000). Cancer-related inflammation represents the seventh hallmark in the development of cancer (Colotta et al., 2009). Persistent microbial infections induced by parasites, bacteria, and viruses and physical and/or chemical stimuli can cause inflammation (Coussens and Werb, 2002). Bacterial infections following surgical removal of primary tumors can promote metastatic growth in mice (Pidgeon et al., 1999) and humans (Taketomi et al., 1997). This process is mediated most likely by endotoxins altering the critical balance between cell growth and angiogenesis (Pidgeon et al., 1999). Moreover, chronic inflammation induced by non-infectious agents can also contribute to carcinogenesis and act as a driving force in tumor development. Apart from toxins, oncoproteins and growth factors can affect the host via an activation of pattern recognition receptors (PRR) that interact with pathogen-associated molecular patterns (PAMP). These receptors comprise to members of the Toll-like receptor (TLR) family, nucleotide-binding oligomerization domain-like (NOD-like) receptors (NLR), C-type lectin receptors (CLR), triggering receptors on myeloid cells (TREM), and retinoic acid inducible gene-I-like receptors (RLR; Kawai and Akira, 2011). Binding of PAMP to these receptors leads to an initiation of the host's immune response by activation of inflammatory cells. The engagement of PRR triggers the induction of intracellular signaling pathways that induce the activation of numerous transcription factors such as NF-κB, STAT, and FOXO. These factors regulate the expression of several genes involved in the innate and adaptive immunity (Akira et al., 2006; Karin et al., 2006). Inadequate pathogen eradication, recurring tissue injury, prolonged inflammatory signaling, and failure of anti-inflammatory mechanisms can cause chronic inflammation which as a result supports tumorigenesis.

IMPACT OF INFLAMMATION IN TUMORIGENESIS

Numerous studies provide evidence that chronic inflammation increases the risk of cancer, promotes tumor progression, and supports metastatic spread (Mantovani et al., 2008; Aggarwal and Gehlot, 2009). In the initial phase of tumor development, inflammatory mediators such as cytokines, reactive oxygen species (ROS), and reactive nitrogen species (RNS) derived from tumor-infiltrating immune cells induce epigenetic alterations in pre-malignant lesions and silence tumor suppressor genes (Grivennikov and Karin, 2010). During tumor promotion, immune cells secrete cytokines and chemokines that act as survival and proliferation factors for malignant cells. The angiogenic switch is critical for

Table 1 | Inflammation and their related cancers.

Inductor	Inflammation	Cancer
Gut pathogens	Inflammatory bowel disease	Colorectal cancer
Tobacco smoke	Bronchitis	Bronchial lung cancer
Helicobacter pylori	Gastritis	Gastric cancer
Human papilloma virus	Cervicitis	Cervical cancer
Hepatitic B/C virus	Hepatitis	Hepatocellular carcinoma
Bacteria, gall bladder stones	Cholecystitis	Gall bladder cancer
Tobacco, genetics, alcohol	Pancreatitis	Pancreatic cancer
Epstein-Barr virus	Mononucleosis	Burkitt's lymphoma
Ultraviolet light	Sunburn	Melanoma
Asbestos fibers	Asbestosis	Mesothelioma
Gram-uropathogens	Schistosomiasis (Bilharzia)	Bladder cancer
Gastric acid, alcohol, tobacco	Esophagitis	Esophageal adenocarcinoma

an adequate supply of tumor cells with oxygen, nutrition, growth, and survival factors (Zumsteg and Christofori, 2009). During tumor progression and metastasis, both tumor and immune cells produce cytokines and chemokines leading to an increase in cell survival, motility, and invasiveness (DeNardo et al., 2008). Epithelial–mesenchymal transition (EMT), a crucial process in tumor invasiveness and metastasis, is also promoted (Yang and Weinberg, 2008). EMT refers to the loss of carcinoma epithelial phenotype and the acquisition of mesenchymal features (Zeisberg and Neilson, 2009). The group of Mehta recently found that aberrant tissue transglutaminase (TG2) expression induces EMT in epithelial cells (Kumar et al., 2010). This finding, in conjunction with the observation that inflammatory signals (e.g., TGF-β, TNF, and NF-κB) which induce EMT, also induce TG2 expression (Kawata et al., 2011), suggests a possible link between TG2, inflammation, and cancer progression presumably yielding novel therapeutic targets for improved patient outcomes. Other typical markers of EMT are cadherin-11 and fibroblast-specific protein (FSP)-1 which are associated with an increased motility (Zeisberg and Neilson, 2009). Twist is necessary to repress the transcription of E-cadherin (Thiery et al., 2009; Zeisberg and Neilson, 2009).

PATHWAYS CONNECTING INFLAMMATION AND CANCER

According to Mantovani et al. (2008), the connection between tumorigenesis and inflammation is mediated via intrinsic and extrinsic pathways. The intrinsic pathway is activated by genetic alterations causing inflammation and neoplasia. These alterations comprise mutation-driven proto-oncogene activation, chromosomal rearrangement/amplification, and inactivation of tumor suppressor genes. Transformed cells secrete inflammatory mediators and thus generate an inflammatory microenvironment. The extrinsic pathway is driven by inflammation or infections that increase the risk for the development of cancer in organs at risk such as the prostate, pancreas, colon, lung, and skin. Both pathways interfere in tumor cells and induce the activation of several transcription factors such as NF-κB, STAT-3, and HIF-1 that result in the formation of pro-inflammatory factors including chemokines, cytokines, and PGHS-2. These molecules recruit and activate various leukocyte populations such as macrophages, mast cells, eosinophils, and neutrophils into the tumor microenvironment like stromal and endothelial cells as well as infiltrating cells. This concerted action of tumor and micromilieu results in a more pronounced generation of inflammatory mediators that drives the progression of a positive amplification loop which further triggers tumor growth and invasiveness.

Proto-oncogene activation represents a critical component in the intrinsic pathway of cancer-related inflammation. In this context, mutations in *RAS* genes play an important role in tumorigenesis. Overall, up to 30% of all human tumors harbor mutations in canonical *RAS* genes (*KRAS*, *HRAS*, *NRAS*). Remarkably, these oncogenic mutations predominantly affect the *KRAS* locus, with oncogenic *KRAS* mutations being detected in 25–30% of all screened tumor samples (Forbes et al., 2011). The high frequency of *KRAS* mutations and their appearance in early tumor stages argue for a causative role of the K-Ras protein in human tumorigenesis (Fernandez-Medarde and Santos, 2011). More than 30 years ago the founding members of the *RAS* gene superfamily (*HRAS*, *NRAS*, *KRAS)* were discovered in human tumors as the first proto-oncogenes. Members of the RAS family are crucial for the connection of up-stream signals to down-stream effector pathways that are functionally related to cell cycle progression, growth, migration, cytoskeletal changes, apoptosis, and senescence. In tumor cells, activation of mutated RAS is followed by the induction of several intracellular signaling pathways. Signaling cascades induced by mutated RAS comprise the RAF/MEK/ERK kinase cascade, the PI3K/AKT pathway, and RalGDS proteins (Downward, 2009), the latter belonging to the family of nucleotide-exchange factors activating small GTPases such as RalB. Via the exocyst complex, an octameric protein complex implicated in tethering of vesicles to membranes (Yamashita et al., 2010), RalB stimulates the TANK-binding kinase-1 (TBK-1) resulting in NF-κB activation by IκBα phosphorylation. In cancer cells, a constitutive activation of this pathway, via chronic RalB activation, restricts the initiation of apoptosis after oncogenic stress (Chien et al., 2006). Beside NF-κB activation, TBK-1 activates the transcription factors IRF-3 and IRF-7 (Hacker and Karin, 2006) leading to the production of growth and inflammatory mediators. Previously it has been shown that K-Ras is a direct inducer of pro-inflammatory IL-6 and pro-angiogenic IL-8 required for the initiation of tumor-associated inflammation and neovascularization and promoting tumor growth. In these studies knock-down of *IL6*, genetic ablation of the *IL6* gene, or treatment with a neutralizing IL-6 antibody retarded K-Ras-driven tumorigenesis (Ancrile et al., 2007). Overexpression of oncogenic K-Ras in tumorigenic HeLa cells induced IL-8 secretion, while IL-8 inhibition reduced growth of these cells and the number of CD31+ cells in a xenograft tumor model (Sparmann and Bar-Sagi, 2004). Moreover, TBK-1 and NF-κB signaling have been identified as being essential in K-Ras mutant tumors (Barbie et al., 2009). Regarding these observations it was assumed that targeting the NF-κB signaling pathway might be effective in treating RAS-mutated tumors (Downward, 2009). Meylan et al. (2009) demonstrated that inhibition of the NF-κB pathway in lung tumors resulted in significantly reduced tumor growth.

CRITICAL MOLECULES IN CANCER-RELATED INFLAMMATION

Tumor-associated inflammation requires the presence and activation of inflammatory cells such as macrophages and granulocytes in the tumor microenvironment, formation of inflammatory mediators by tumor and stromal cells, tumor remodeling, and angiogenesis (Kundu and Surh, 2008; Colotta et al., 2009). Accumulation of microbial pathogens and tissue necrosis activate transcription factors that are necessary for the expression of, e.g., pro-angiogenic factors (IL-8, VEGF), growth factors (IL-6, GM-CSF), anti-apoptotic factors (Bcl-X_L, c-FLIP), invasion-promoting factors (MMP-2, MMP-7, MMP-9, uPA), inflammatory enzymes (PGHS-2, LOX), prostaglandins, iNOS, chemokines (CCL2, CCL20, IL-8), and pro-inflammatory cytokines (IL-1, IL-6, IL-23, TNF, TGF-β, EGF) that support the malignant phenotype. All molecules mentioned above are regulated by the transcription factor NF-κB, a key orchestrator in innate immunity and inflammation that has emerged as a crucial tumor promoter (Karin, 2006). The presence of constitutively active NF-κB was found to be associated with poor clinical outcome (for an overview see Aggarwal and Gehlot, 2009). NF-κB activation in inflammatory cells in response to infectious pathogens, pro-inflammatory mediators as well as necrotic cell products results in the generation of secretable factors that support growth, survival, and vascularization of pre-malignant and malignant cells (Karin, 2006). Activation of NF-κB up-regulates cell cycle mediators (cyclin D1, c-Myc), anti-apoptotic (c-FLIP, survivin, Bcl-X_L) and adhesion molecules (ICAM-1, ELAM-1, VCAM-17), proteolytic enzymes (e.g., MMP, uPA), and pro-inflammatory factors (PGHS-2, cytokines) that promote an invasive phenotype (Aggarwal and Gehlot, 2009). iNOS is another important inflammatory mediator that causes the production of NO by macrophages that links chronic inflammation and tumorigenesis. Elevated levels of NO have been found in numerous pre-cancerous and malignant lesions such as Barrett's mucosa (Wilson et al., 1998), prostate cancer (Aaltoma et al., 2001), breast cancer (De Paepe et al., 2002), and gastrointestinal carcinomas (Wink et al., 1998; Jaiswal et al., 2001). The iNOS product NO contributes to inflammation-associated tumorigenesis by inducing DNA damage, suppression of DNA repair, modification of oncoproteins, inhibition of apoptosis, promotion of tumor growth, angiogenesis, and metastasis as well as suppression of anti-tumor immunity (De Paepe et al., 2002). The NO-mediated inhibition of DNA repair enables cells harboring epigenetic alterations to escape from apoptosis. This results in clonal expansion of pre-malignant cells and subsequently to carcinogenesis (Sawa and Ohshima, 2006). Furthermore, NO promotes tumor growth by a transactivation of HIF-1α (Sandau et al., 2000), induces the expression of pro-angiogenic VEGF (Ravi et al., 2000), and down-regulates the tumor suppressor protein p53 (Ambs et al., 1998).

RANKL

RANKL, a member of the TNF superfamily of cytokines, was originally found in T and dendritic cells (DC). RANKL supports differentiation and survival of effector cells (Anderson et al., 1997). Moreover, it is essential for the differentiation of bone-resorbing osteoclasts derived from monocyte–macrophage precursors, and enables survival and function of mature osteoclasts (Li et al., 2000; Teitelbaum, 2000). Recent studies documented an expression of RANKL in a variety of other cell types, including tumor cells. Breast cancer cells are able to produce RANKL (Park et al., 2003; Cross et al., 2006) and stimulate osteoclast differentiation when co-cultured with bone marrow stromal cells (Park et al., 2003). HIF-1α-induced expression of RANKL initiates an increased migration of breast cancer cells via the PI3K/AKT pathway (Tang et al., 2011). The expression of RANKL in prostate cancer cells was found to be associated with an increased appearance of bone metastases (Brown et al., 2001; Chen et al., 2006). In head and neck squamous cell carcinoma RANKL expression promotes EMT and tumor progression by inducing VEGF-independent angiogenesis (Yamada et al., 2011). Moreover, the activity of RANKL was found to be involved in the pathophysiology of osteosarcoma (Mori et al., 2007a,b), giant cell tumors of the bone (Ng et al., 2010), Paget's sarcomas (Sun et al., 2006), and vascular diseases (Hofbauer and Schoppet, 2004). The expression of RANKL increases in response to pro-inflammatory mediators, such as IL-1 (Fernandez et al., 2010; Jurado et al., 2010). In fibroblast-like synoviocytes, murine osteoblastic, and fibroblastic cells, IL-23 was found to induce an up-regulation of RANKL via STAT-3 and NF-κB signaling pathways (Li et al., 2010; Mori et al., 2011). An exposure of these cells to pro-inflammatory cytokines such as IL-1, TNF, and IL-6 resulted into a direct or indirect activation of STAT-3 in a feed-forward loop. Further evidence for a crucial role of STAT-3 in the regulation of RANKL is shown by Schulze et al. (2010) who found that osteolytic prostate cancer cells induce the expression of RANKL in a STAT-3/5-dependent manner. Together these data highlight the significance of a STAT-3/5-mediated cytokine production in tumor cell migration and the formation of distant metastases.

IL-1 AND TNF

Elevated levels of IL-1 have been identified in several human tumor entities such as melanoma, head and neck, colon, lung, and breast cancer. Overall, patients harboring IL-1-positive tumors have markedly worse prognoses (Lewis et al., 2006). Due to its pleiotropic nature, IL-1 promotes tumor growth and metastasis in an autocrine/paracrine manner. IL-1 is produced by tumor, stromal and endothelial cells, and the host's infiltrating immune cells (Lewis et al., 2006). Depending on its subcellular location, different IL-1 isoforms mediate different functions. Membrane-bound IL-1α which is expressed on malignant cells induces anti-tumor immune responses, whereas, intracellular residing precursors of IL-1α control homeostatic functions including gene expression, differentiation, and cell growth. In contrast, low concentrations of secreted IL-1β down-regulate inflammatory responses and immune mechanisms, whereas high concentrations promote inflammation-associated tissue damage and tumor invasiveness (Apte et al., 2006). IL-1 can stimulate other cell types to produce pro-angiogenic and pro-metastatic mediators and thus plays an important role in inflammation-associated carcinogenesis (Lin and Karin, 2007; Voronov et al., 2007). In pancreatic cancer IL-1 confers chemoresistance via an up-regulation of PGHS-2 (Angst et al., 2008) and promotes angiogenesis during tumor progression (Shchors et al., 2006).

IL-1α and IL-1β exert identical agonist actions by binding to the IL-1 receptor type I (IL-1RI). After ligation, IL-1/IL-1RI associates with the IL-1 receptor accessory protein (IL-1RAcP) leading to

activation of intracellular signal transduction cascades. This complex recruits a number of intracellular adapter molecules including MyD88 (Watters et al., 2007; Gay et al., 2011) to activate signal transduction pathways such as AP-1, p38MAPK, JNK, and NF-κB (**Figure 1**). In particular NF-κB provides a mechanistic link between inflammation and tumorigenesis. NF-κB is a major factor which controls apoptosis-based tumor immune surveillance mechanisms of pre-neoplastic and malignant cells. NF-κB also regulates tumor angiogenesis and invasiveness (Karin, 2006), and may contribute to chemoresistance of tumor cells (Fahy et al., 2004). A detailed description of the IL-1 signaling pathway is visualized schematically in **Figure 1**.

A third ligand, the naturally occurring IL-1 receptor antagonist (IL-1Ra), also binds to IL-1RI and acts as a true receptor antagonist. Because of its collagenase and prostaglandin-inhibiting properties, IL-1Ra (anakinra™) is approved for the treatment of chronic inflammatory diseases including rheumatoid arthritis (Dinarello, 1996) and systemic onset juvenile idiopathic arthritis (Hedrich et al., 2011). It has also been identified as being powerful in reverting IL-1 effects in numerous pathological settings (Dinarello, 1996). Actually, anakinra was successfully used in treating the rare lymphoproliferative disorder Castleman's disease (El-Osta et al., 2010) as well as in myeloma (Lust et al., 2009) rendering the use of anakinra and other IL-1-blocking agents such as canakinumab™(anti-IL-1β antibody) or rilonacept™(construct of the two extracellular chains of IL-1RI/IL-1RAcP complex fused to the Fc segment of IgG) promising therapeutic approaches in human metastatic diseases. The last two agents have been approved for the treatment of the cryopyrin-associated periodic syndrome (CAPS; Hoffman et al., 2008; Lachmann et al., 2009a), a grouping of familial cold auto-inflammatory syndrome, Muckle–Wells syndrome, and neonatal onset multi-inflammatory disease. As summarized by Dinarello (2010), there are two meaningful reasons for the use of IL-1-blocking agents in the treatment of metastatic diseases. On the one hand, none of the above mentioned agents have been found as being associated with any organ toxicities, gastrointestinal, or hematological abnormalities. On the other hand, unlike TNF-blocking agents IL-1-inhibiting treatments lack opportunistic infections although routine bacterial and upper airway infections are observed. Due to the safety of IL-1 blockage and the availability of the three therapeutics in limiting IL-1 actions, clinical trials are encouraged. An NIH trial of anakinra in the treatment of cutaneous melanoma is ongoing because IL-1 plays a pivotal role in angiogenesis by inducing/up-regulating pro-angiogenic IL-8 and VEGF contributing to the pathogenesis of, e.g., multiple melanoma (Dinarello, 2010).

As a pleiotropic cytokine, IL-1 harbors numerous intensifying effects on the physiological functions of diverse innate and immunocompetent cells (Mizel, 1982), IL-12-mediated induction of Th1 development (Weaver et al., 1988), and induction of Th17 cells (Sutton et al., 2006). IL-1 effects on immune tolerance are also reported (Nakata et al., 1995). For instance, IL-1β stimulates function of memory T cells and impairs that of Treg cells (O'Sullivan et al., 2006). This brief overview highlights the complexity of the mechanisms by which IL-1 regulates all types of immune responses including tumor cell eradication.

IL-1β is first synthesized as biologically inactive precursor (pro-IL-1β) that is further processed by caspase-1, also known as IL-1-converting enzyme (ICE), to the mature form, while pro-IL-1α is cleaved by calpain. Although IL-1β contributes to growth and metastatic spread in experimental and human cancers, the molecular mechanisms regulating the conversion of pro-IL-1β to the secreted and active cytokine remains to be elucidated.

An elaborate multi-protein complex, the so-called "inflammasome," is responsible for the recruitment and activation of caspase-1 (Martinon et al., 2002). Each inflammasome consists of different members of the nucleotide oligomerization domain-like receptor (NLR) family of proteins. Two of the best characterized human inflammasomes are NALP (NACHT, LRR, and pyrin domain-containing protein) 1 inflammasome and NALP2/3 inflammasome (Franchi et al., 2009). It was shown previously that in several auto-inflammatory diseases constitutive activation of NALP3 inflammasome leads to sustained local and systemic inflammation mediated by IL-1β (Goldbach-Mansky et al., 2006; Lachmann et al., 2009b). Recently, constitutively activated inflammasome was found in human melanoma cells (Okamoto et al., 2010). In this study human melanoma cells from the late stage of the disease spontaneously secrete biologically active IL-1β in the absence of exogenous stimuli because of constitutive activation of the inflammasome and IL-1 receptor (IL-1R) signaling. From these findings it can be concluded that IL-1-mediated autoinflammation contributes to the development and progression of human melanoma suggesting that inhibiting the inflammasome pathway or reducing IL-1 activity can be a therapeutic option for melanoma patients. The inflammasome also plays a substantial role in environmental cancer. It has been shown previously that silica and asbestos both activate the NALP3 inflammasome resulting in an increased IL-1β production and causing lung inflammation (Dostert et al., 2008). Chronic exposure to asbestos has been identified as being a high-risk factor for the development of mesothelioma implying a crucial contribution of inflammasome-mediated inflammation to the pathogenesis of mesothelioma. Controversely, in an animal model of colitis-associated cancer (CAC) the NALP3 inflammasome was found to be protective against CAC (Allen et al., 2010). The NALP3 inflammasome in DC obviously plays a crucial role by linking innate and adaptive immune responses against dying tumors (Ghiringhelli et al., 2009). Based on these observations one can hypothesize that constitutively active NALP3 inflammasome as can be found in certain tumors produces large amounts of IL-1 contributing to cancer-related inflammation and thus promoting tumor growth and invasiveness, whereas activation of the inflammasome in tumor-infiltrating immune cells might be beneficial in inducing anti-tumor immunity. According to Menu and Vince (2011), the NALP3 inflammasome can be considered as a triple-function agent ("the good, the bad, and the ugly") in human malignancies.

The pleiotropic cytokine TNF plays a dual role in tumorigenesis. At high concentrations TNF is destructive to tumor vasculature and induces necrosis. On the other hand, its critical role in chronic inflammation and its tumor-promoting capacity are well documented (Lin and Yeh, 2005; Mocellin et al., 2005). An increased expression of TNF was found in human bladder, breast, colorectal,

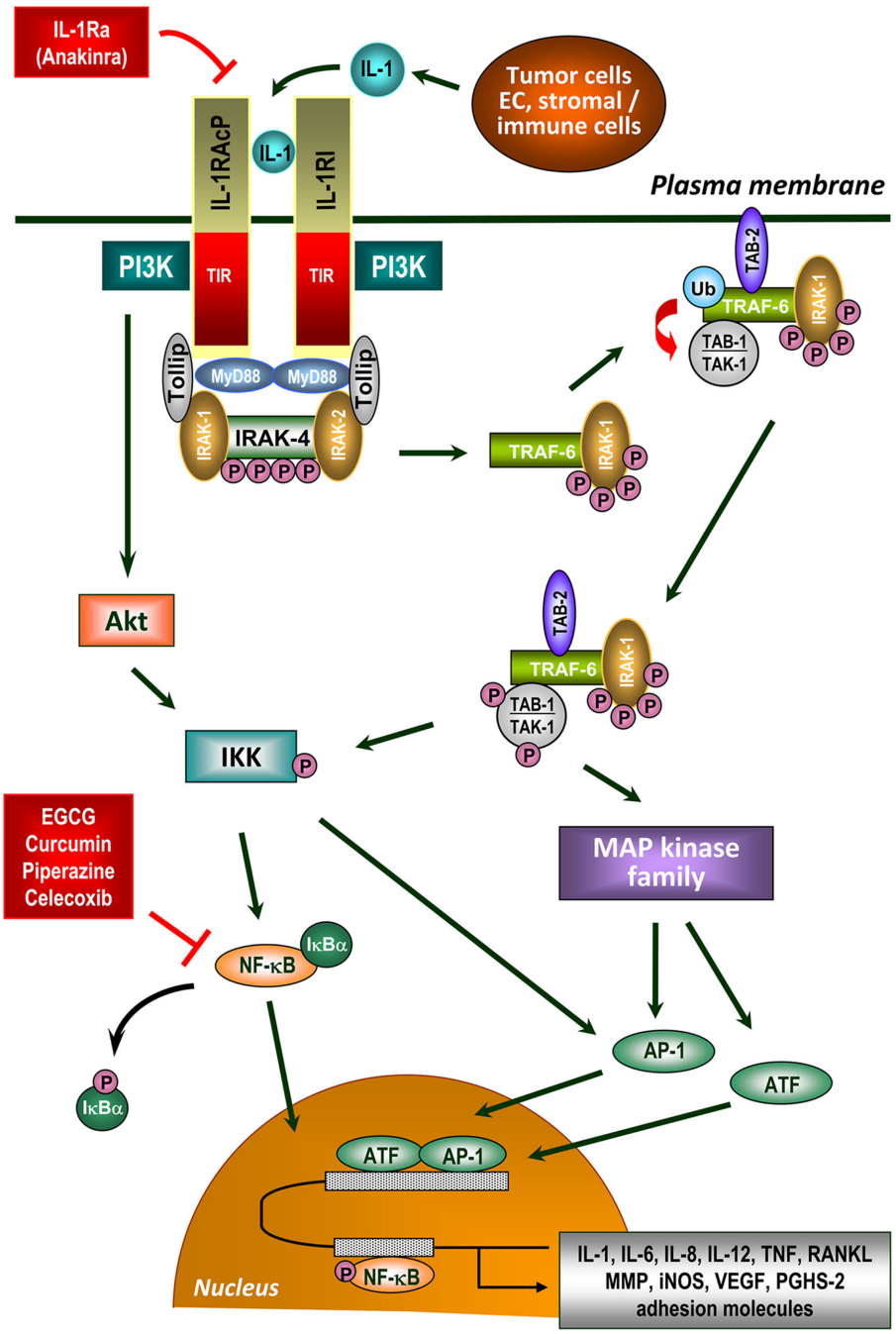

FIGURE 1 | IL-1 signaling in the tumor microenvironment. IL-1 is a critical molecule in inflammation-associated carcinogenesis produced directly by tumor cells or cells of the tumor microenvironment. IL-1 signal transduction is initiated by binding of either form of IL-1 to IL-1 receptor type I (IL-1RI), which undergoes a conformational change allowing the IL-1 receptor accessory protein (IL-1RAcP) to recognize the ligated IL-1RI. IL-1RAcP does not recognize IL-1 but represents an essential component in the IL-1 signaling pathway (Wesche et al., 1997b; Radons et al., 2002). The naturally occurring IL-1 receptor antagonist (IL-1Ra) also binds to IL-1RI without leading to its activation. Ligand-mediated heterodimerization of the receptor complex leads to recruitment of dimeric myeloid differentiation protein 88 (MyD88) via its TIR domain (Muzio et al., 1997; Wesche et al., 1997a; Radons et al., 2003) followed by complex formation between IRAK-4, MyD88, and IL-1RAcP and subsequent phosphorylation of IRAK-4 (Cahill and Rogers, 2008). After recruitment of IRAK-1/Tollip to the complex, IRAK-1 is initially phosphorylated by IRAK-4 (Born et al., 1998; Dunne and O'Neill, 2003). Subsequently, IRAK-1 (and possibly IRAK-2) becomes hyperphosphorylated and dissociates into the cytoplasm where it binds TNF receptor-associated factor 6 (TRAF-6; Cao et al., 1996). IRAK-1 interacts with membrane-bound TAK-binding protein 2 (TAB-2) as well as TAK-1/TAB-1 complex (Dower and Qwarnstrom, 2003) followed by translocation of TAB-2 from the plasma membrane to the signalosome and subsequent partial activation of TAK-1 by TAB-2. IRAK-1, presumably as dimer or oligomer, enables dimerization of TRAF-6 resulting in its ubiquitination and activation. In close proximity to TAB-2, TAK-1 is partially activated followed by complete activation through polyubiquitinated TRAF-6 (Kishimoto et al., 2000; *(continued)*

FIGURE 1 | Continued
Martin and Wesche, 2002) enabling activation of numerous signaling cascades. Polyubiquitination of TRAF-6 obviously occurs through IRAK-2 (Keating et al., 2007). On the one hand, TAK-1 activates certain members of the MAP kinase family leading to activation of AP-1 and ATF (Ninomiya-Tsuji et al., 1999; O'Neill, 2000; Hefler et al., 2005; Blanco et al., 2008) the latter augmenting NF-κB-mediated transcription via transactivation (Jefferies and O'Neill, 2000; Cahill and Rogers, 2008). On the other hand, TAK-1 phosphorylates and activates IKK resulting in phosphorylation and inactivation of IκBα (Wang et al., 2001). Afterward, IκBα dissociates from the complex with NF-κB and undergoes proteasomal degradation. After phosphorylation, NF-κB translocates to the nucleus and activates NF-κB-dependent gene transcription (Chen and Greene, 2004). Inhibitors of NF-κB activation are indicated that suppress the inflammatory network in cancer development. IL-1 signaling also involves recruitment of PI3-kinase (PI3K) to the IL-1 receptor complex via the p85 regulatory subunit of PI3K (Reddy et al., 1997) and subsequent activation of AKT/PKB leading to IKK-dependent activation of NF-κB and AP-1 (Cahill and Rogers, 2008). Receptor ligation can also activate numerous G proteins resulting in activation of AP-1 and ATF mediated by several MAP kinases and an IκBα-independent transactivation of NF-κB (Singh et al., 1999; Jefferies and O'Neill, 2000). IL-1 signaling finally regulates gene expression of a great variety of tumorigenic factors including pro-angiogenic factors (IL-8, VEGF), growth factors (IL-6, GM-CSF), anti-apoptotic factors (Bcl-X_L, c-FLIP), invasion-promoting factors (MMP-2, MMP-7, MMP-9, uPA), inflammatory enzymes (PGHS-2, LOX), prostaglandins, iNOS, chemokines (CCL2, CCL20, IL-8), and pro-inflammatory cytokines (IL-1, IL-6, IL-23, TNF, TGF-β, EGF, RANKL).

and prostate cancer as well as in leukemia and lymphoma (Balkwill and Mantovani, 2001). TNF is also produced by cells of the tumor microenvironment. Binding of TNF to the TNF receptor 1 (TNFR1) activates signaling cascades of NF-κB and c-Jun N-terminal kinase (JNK) which lead to an up-regulation of several pro-inflammatory, pro-angiogenic and invasiveness-promoting factors, and to the induction of anti-apoptotic molecules such as the caspase-8 inhibitor c-FLIP. Activation of NF-κB in response to TNFR1 terminates the activity of JNK (Kamata et al., 2005). The ubiquitin ligase Itch is a substrate of JNK that enables the degradation of c-FLIP (Chang et al., 2006). Inhibition of JNK via NF-κB-mediated blockage leads to an inactivation of Itch. This prevents degradation of c-FLIP and ensures tumor cell survival. Apart from its role in tumor initiation, TNF promotes angiogenesis and impairs immune surveillance by affecting T cell responses and the activity of macrophages (Elgert et al., 1998). The tumor-promoting role of TNF was confirmed in animal models. In the absence of TNF mice do not develop hepatocellular carcinoma in response to cholestatic hepatitis (Pikarsky et al., 2004). TNF and TNFR-deficient mice were also found to be resistant to chemically induced carcinogenesis of the skin (Arnott et al., 2004). These findings indicate that pro-inflammatory activity of TNF functions as a pivotal mediator in tumorigenesis (Lin and Karin, 2007).

IL-6 AND PGHS-2 (COX-2)

IL-6 is another NF-κB-regulated pleiotropic pro-inflammatory mediator that enables tumor growth and inhibits apoptosis in a variety of human tumors (Rose-John and Schooltink, 2007). In contrast, IL-6 has also been reported as playing a crucial role in terminating inflammation (Hudson et al., 2008). IL-6 signaling via the membrane-bound receptor IL-6Ra is linked to the JAK/STAT pathway (predominantly through activation of STAT-3) and leads to the expression of genes encoding for anti-apoptotic cell cycle progression molecules (Lin and Karin, 2007). In contrast, a soluble form of the IL-6R can bind IL-6 with the same affinity as the membrane-bound form and the complex of IL-6 and the soluble IL-6R (sIL6R) can induce signaling in a process called IL-6 trans-signaling (Peters et al., 1998). Because the IL-6R is only sparely expressed, IL-6 trans-signaling dramatically increases the number of potential IL-6 target cells (Rose-John et al., 2006). Animal models of inflammatory colon cancer suggest that IL-6 trans-signaling serves as the major pro-inflammatory paradigm of IL-6 signaling under pathophysiologic conditions (Becker et al., 2004). It turns out that regenerative or anti-inflammatory activities of IL-6 are mediated by classic signaling whereas pro-inflammatory responses of interleukin-6 are rather mediated by trans-signaling (Rabe et al., 2008). This is important since therapeutic blockade of IL-6 by the neutralizing anti-IL-6 receptor monoclonal antibody tocilizumab™ has recently been approved for the treatment of inflammatory diseases. A recently performed clinical trial revealed that IL-6 inhibition by tocilizumab retards joint damage progression in patients with rheumatoid arthritis (Smolen et al., 2011). Interestingly, inhibition of IL-6R-mediated signaling using tocilizumab in a xenograft model of oral squamous cell carcinoma (OSCC) suppressed tumor growth and angiogenesis by down-regulating VEGF mRNA expression (Shinriki et al., 2009). Clinical studies inclusive those mentioned above have shown that inhibition of IL-6 signaling by tocilizumab is therapeutically effective not only in chronic inflammatory diseases such as rheumatoid arthritis (Nishimoto et al., 2004), juvenile idiopathic arthritis (Yokota et al., 2004), and Crohn's disease (Ito et al., 2004) but also in Castleman's disease (Nishimoto et al., 2005). In all of these diseases, tocilizumab ameliorates inflammatory manifestations, and normalizes acute phase protein levels. Given its success in treating these diseases, tocilizumab may also prove useful in treating IL-6-related cancers.

Elevated IL-6 levels are found in numerous tumors such as multiple myeloma (Klein et al., 1992), colorectal cancer (Chung and Chang, 2003), gastric carcinoma (Kai et al., 2005), and Hodgkin lymphoma (Cozen et al., 2004). Moreover, malignant ascites from patients with epithelial ovarian cancer was found to contain high levels of IL-6 (Offner et al., 1995). In breast cancer patients high IL-6 concentrations induced by an *IL6* gene polymorphism correlate with poor prognosis (Berger, 2004). A comparison of non-metastasizing pancreatic cancer, benign prostatic hyperplasia, and metastasized pancreatic cancer revealed elevated levels of IL-6 in the latter, more aggressive tumor (Weiss et al., 2011). In this study it was shown that IL-6 leads to an increased expression of uPA and VEGF which implies a crucial role of IL-6 in angiogenesis of pancreatic tumors. In OSCC lysophosphatidic acid (LPA), a bioactive lipid with a growth factor-like activity induces the secretion of IL-6 and IL-8 in an NF-κB- and AP-1-dependent manner (Hwang et al., 2011). Direct stimulation of human osteoblasts with IL-6 and IL-8 induced the expression of RANKL and thereby promotes osteoclast formation. From these findings it can be concluded that IL-6 and IL-8 derived from LPA-stimulated OSCC play a crucial role in

osteogenesis and bone resorption. Inhibition of IL-6 signaling in colon cancer resulted in a reduced tumor growth in mice (Becker et al., 2004; Greten et al., 2004). Incubation of cholangiocarcinoma cells with an anti-IL-6-neutralizing antiserum reduced AKT phosphorylation and down-regulated the expression of Mcl-1. This indicates a contribution of IL-6 in the AKT-mediated survival mechanisms (Kobayashi et al., 2005).

PGHS-2, formerly termed as COX-2, has emerged as another pro-inflammatory mediator in tumorigenesis whose expression is mediated by NF-κB. The expression of PGHS-2 is inducible in response to stimuli such as mitogens, cytokines, growth factors, or hormones. PGHS-2 is the rate-limiting enzyme involved in the conversion of arachidonic acid to prostanoids acting as key mediators of inflammation. Aberrant or increased expression of PGHS-2 has been shown to be involved in the pathogenesis of breast, gastric, colorectal, lung, prostate, head/neck, and pancreatic cancer. PGHS-2 affects cell proliferation, apoptosis, angiogenesis, and metastasis (Lu et al., 2006; Aggarwal and Gehlot, 2009). Overexpression of PGHS-2 results in the secretion of large amounts of VEGF and therefore, is associated with increased tumor cell invasion and poor prognosis (Raut et al., 2004; Ladetto et al., 2005). In human basal cell carcinoma cells elevated levels of PGHS-2 led to an up-regulated expression of the anti-apoptotic molecules Mcl-1 and Bcl-2, VEGF and basic fibroblast growth factor (bFGF; Tjiu et al., 2006). In chronic inflammation, endothelial cells express both, acute phase genes and adhesion molecules that enable recruitment of leukocytes to the site of tissue damage. Moreover, an enhanced production of prostaglandins mediated by PGHS-2 augments vasopermeability leading to a more pronounced recruitment of leukocytes (Jura et al., 2005). Leukocytes are the main source of RNS and ROS acting as chemical effectors in inflammation-driven carcinogenesis (Kundu and Surh, 2008). We identified a constitutively enhanced expression of PGHS-2 in human pancreatic adenocarcinoma cells that is further increased in the presence of IL-1 (Bauer et al., 2009; Hoffmann et al., 2011). The constitutive production of PGHS-2 and its key product PGE_2 in the microenvironment of pancreatic carcinomas accounts for an enhanced malignancy of pancreatic tumor cells which is caused by inhibition of apoptosis, increase in cell proliferation, induction of angiogenesis, and invasion of malignant cells into surrounding tissue (Merati et al., 2001; Kong et al., 2002; Garcea et al., 2005). PGHS-2 also induces the expression of MMP-2 (Surh et al., 2001; Sansone et al., 2009; Wang et al., 2009b) and pro-inflammatory IL-6 and IL-1 via PGE_2 (Takahashi et al., 2008). PGHS-2-mediated effects on growth, angiogenesis, invasiveness, and metastasis are augmented by the IL-1-induced up-regulation of the enzyme by forcing the progression of a positive amplification loop triggered by PGE_2 and IL-6. Of note, it was demonstrated that PGE_2 contributes to cancer progression by inhibiting DC differentiation and function, acting paradoxically as an immunosuppressive factor (Muthuswamy et al., 2010; Stock et al., 2011). In cervical cancer, PGE_2 was found to induce a cytokine production profile and phenotypical features of tolerogenic DC suggesting that the altered expression of PGE_2 might promote carcinogenesis by favoring (pre)cancer immunotolerance (Herfs et al., 2009). In addition, IL-6 derived from tumor cells or cells of the tumor microenvironment was shown to polarize DC toward immune tolerance through the induction of STAT-3 activation (Alshamsan, 2011). Therefore, tumor-induced p-STAT-3 in DC can be seen as a promising target for colon cancer immunotherapy. In this context, knocking-down the IL-6 receptor α-chain of DC vaccines significantly enhanced the frequency of tumor-specific CD8+ CTL-producing effector molecules such as IFN-γ, TNF, FasL, perforin, and granzyme B, and generated more CD8+ memory T cells, leading to the substantially prolonged survival of cytotoxic lymphocytes (Tc1) tumor-bearing mice (Hwang et al., 2010).

Most of the PGHS-2-induced effects are mediated through its product PGE_2 (Yoshimatsu et al., 2001a,b). Thus, down-regulation of prostaglandins in tumor tissues by PGHS-2 inhibition blocks several neoplastic pathways leading to the suppression of tumor growth (Maier et al., 2004). In this context, PGHS-2 inhibitors hold promise for cancer chemoprevention. Among them, the non-steroidal anti-inflammatory drug (NSAID) celecoxib constitutes a potent and specific inhibitor of the inducible human PGHS-2. Celecoxib interferes with tumor initiation and tumor cell growth *in vitro* and *in vivo*. Preclinical studies demonstrate promising anti-cancer effects of celecoxib in colorectal, pancreatic as well as head and neck carcinomas. Additionally, celecoxib was found to increase tumor cell sensitivity toward radiochemotherapy (reviewed by Jendrossek, 2011). Celecoxib has also been found to impair tissue expression of VEGF, tumor angiogenesis, and metastasis in an experimental model of pancreatic cancer (Wei et al., 2004). Thus, modulation of PGHS-2 expression may be a promising approach in cancer therapy (Jimeno et al., 2006). However, due to the high toxicity of PGHS-2 inhibitors, the development of novel components is necessary (Spektor and Fuster, 2005; Lee et al., 2007). Randomized clinical trials and meta-analyses reported on an increased risk for cardiovascular diseases in patients receiving long-term treatment with PGHS-2 inhibitors. These cardiovascular adverse effects include myocardial infarction, stroke, and cardiovascular death/heart failure (summarized by Trelle et al., 2011). This increased rate of life-threatening cardiovascular side effects led to the withdrawal of the PGHS-2 inhibitors valdecoxib and rofecoxib from the market that had been approved by the United States Food and Drug Association. A patient-pooled analysis of adjudicated data from 7,950 patients in six placebo-controlled trials demonstrated a dose regimen-related increase in the risk of serious cardiovascular events after a daily administration of 400 and 800 mg celecoxib (Solomon et al., 2008). Although long-term treatment with high-dose celecoxib can enhance the risk for cardiovascular diseases, the drug is still used at lower doses in the United States since it is less toxic compared to other PGHS-2 inhibitors (Solomon et al., 2008; Trelle et al., 2011).

A novel approach to overcome the limitations associated with the toxicity of PGHS-2 inhibitors is to combine chemical PGHS-2 inhibitors at low doses with naturally occurring compounds such as the catechin EGCG which is a promising chemopreventive agent derived from green tea (summarized by Cerella et al., 2010). Our group investigated the effects of a combinatorial treatment with celecoxib and EGCG on the expression of IL-1-induced tumorigenic factors in human pancreatic adenocarcinoma cells. We found that the combined administration of celecoxib and EGCG can induce synergistic cancer preventive effects in pancreatic cancer cells by down-regulating tumorigenic factors and

inducing apoptosis (Härdtner et al., 2009). Previous investigations of our group and others revealed anti-proliferative and apoptosis-inducing effects of EGCG and celecoxib in pancreatic cancer cells (Chen and Zhang, 2007; Inaba et al., 2008; Xu et al., 2008; Hoffmann et al., 2011). The anti-proliferative properties of NSAID such as celecoxib are related to effects on the cell cycle (Xiong, 2004) including changes in gene expression that favor cell cycle arrest (Yip-Schneider et al., 2001; Tseng et al., 2002). Interestingly, several studies revealed an anti-proliferative effect of PGHS-2-selective inhibitors not only in PGHS-2-positive but also in PGHS-2-negative pancreatic tumor cells implying that the inhibitory action of NSAID on cell proliferation can affect both, PGHS-2-dependent and -independent pathways (Molina et al., 1999; Yip-Schneider et al., 2001). Numerous investigations also documented an apoptosis-inducing potential of NSAID (Maier et al., 2004; Suganuma et al., 2011). Celecoxib targets several proteins distinct from PGHS-2 that are involved in the control of cell survival and cell death including the anti-apoptotic proteins survivin, Mcl-1, and Bcl-2 (Sakoguchi-Okada et al., 2007; Rudner et al., 2010). Further, PGHS-2-independent molecular targets of celecoxib comprise the survival kinase AKT/PKB and its up-stream regulator 3-phosphoinositide-dependent kinase-1 (PDK-1; Belham et al., 1999; Kulp et al., 2004), cyclin-dependent kinase inhibitors, and cyclins (Grosch et al., 2006), as well as the sarcoplasmic/endoplasmic reticulum calcium ATPase SERCA (Johnson et al., 2002). By counteracting these molecules celecoxib interferes with the activation status of caspases and finally induces apoptosis.

EGCG-mediated effects on apoptosis include caspase-3/-9 activation, PARP cleavage, Bax oligomerization, mitochondrial membrane depolarization, direct interaction with anti-apoptotic members of the Bcl-2 family as well as NF-κB inhibition (Lambert et al., 2005; Shimizu et al., 2005; Inaba et al., 2008). Celecoxib is reported to interfere, among others, with the NF-κB signaling pathway (Niederberger et al., 2001; Shirode and Sylvester, 2010) providing the basis for the synergism with EGCG. Based on these findings one can hypothesize that celecoxib in combination with EGCG may promote apoptosis directly or indirectly thus altering the cellular death threshold in tumor cells (Jendrossek, 2011). In previous studies EGCG has been shown to synergistically enhance the effects of TRAIL (Siddiqui et al., 2008) and PGHS-2 inhibitors NS-398 (Adhami et al., 2007) and celecoxib (Basu and Haldar, 2009). In a human lung (Suganuma et al., 2011) and prostate cancer (Adhami et al., 2007) model the combination of celecoxib and EGCG increased tumor cell apoptosis and decreased inflammation. Other natural compounds affecting the PGHS-2 expression include the non-flavonoid polyphenols curcumin from turmeric *Curcuma longa,* resveratrol from red wine, isoflavone genistein from lupin as well as omega-3 fatty acids from oily fish flaxseeds. Such a combinatory administration might have future clinical implications with respect to an adjuvant therapy in cancer patients, since it might reduce the adverse effects of high-dose celecoxib as a monotherapy.

INTERACTIONS OF NF-κB AND STAT-3

Both, STAT-3 and NF-κB are crucial for cancer-related inflammation. NF-κB does not only mediate tumorigenesis but also exerts anti-tumorigenic effects in tumor cells and in the tumor microenvironment (for a review see Ben-Neriah and Karin, 2011). Evidence for a positive association of NF-κB activation with tumor-associated inflammation came from colitis-associated colon cancer (Greten et al., 2004; Pikarsky et al., 2004) and hepatitis-associated hepatocellular carcinoma (Pikarsky et al., 2004). Colitis-associated colon cancer represents a classical example for an inflammation-triggered malignancy. NF-κB activation in intestinal epithelial cells of this cancer model was found to enhance the survival of pre-malignant progenitor cells by inducing anti-apoptotic Bcl-X_L (Greten et al., 2004; Pikarsky et al., 2004). NF-κB activation in cancer seems to be related, at least in part, by mutations in components of the signaling cascade or effects of inflammatory factors in the tumor microenvironment that accumulate after NF-κB activation (Karin et al., 2002). Transcriptional activation of NF-κB leads to the induction of pro-inflammatory cytokines (e.g., IL-1, IL-6, TNF), chemokines (IL-8), PGHS-2, MMP, and adhesion molecules (ICAM-1, VCAM-1). The presence of constitutively active NF-κB in most tumors correlate with a poor clinical outcome (Weichert et al., 2007). Moreover, most chemopreventive agents including nutraceuticals derived from different sources have the potential to suppress constitutive and inducible NF-κB activation pathways (Aggarwal and Gehlot, 2009) in order to block chronic inflammation.

Whilst NF-κB signaling contributes to both, inflammation-driven carcinogenesis and anti-tumor immunity, STAT-3 induces cancer-promoting inflammation and restrains anti-tumor immune responses by counteracting NF-κB-induced expression of anti-tumor Th1 cytokines (IL-12, IFN-γ; Kortylewski et al., 2005; Yu et al., 2007). Furthermore, STAT-3 contributes to the expansion and development of Treg and Th17 cells (Wang et al., 2009a; Wu et al., 2009). STAT-3 also induces the expression of tumorigenic mediators (cytokines, pro-angiogenic, and growth factors) and their corresponding receptors that in turn activate a STAT-3 mediated immunoregulatory circuit in the tumor microenvironment (Yu et al., 2007). Thus, the constitutive activation of STAT-3, does not only promote cancer-related inflammation but also suppresses anti-tumor immune responses (Yu et al., 2009).

As already mentioned, the association of cancer with chronic inflammation is related to intrinsic and extrinsic pathways, both leading to activation of NF-κB and STAT-3. Similarly to NF-κB, constitutively active STAT-3 is found in breast, ovarian, prostate, and brain tumors, leukemia, lymphoma, and multiple myeloma. STAT-3 activation results in the modulation of the expression of numerous genes that are crucial for maintaining/amplifying tumor-associated inflammation and promoting tumor growth and progression (Yu et al., 2009). The NF-κB family comprises homo- and heterodimeric transcription factors consisting of RelA, c-Rel, RelB, NF-κB1 (p50 and its precursor p105), and NF-κB2 (p52 and its precursor p100) with RelA-p50 as being the most prominent NF-κB transcription factor (Vallabhapurapu and Karin, 2009). Physiologically, NF-κB is sequestered in the cytosol by its inhibitory component, IκBα. Upon phosphorylation by the IκB kinase complex (IKK), IκBα is degraded in an ubiquitin-dependent manner in the proteasome. Then NF-κB translocates into the nucleus where predominantly RelA-p50 up-regulates the expression of Th1 immunostimulatory genes (IL-12, CD40, CD80)

that are important for the control of microbial infections and tumor cells (Yu et al., 2007, 2009). STAT-3 opposes the anti-tumor immune responses mediated by NF-κB within a cell. On the one hand, STAT-3 is able to inhibit IKK during acute inflammation and thus attenuates Th1 immune responses (Lee et al., 2009). On the other hand, STAT-3 prolongs the nuclear retention of RelA during oncogenic and chronic inflammation by acting as a co-transcription factor for RelA thus contributing to the persistent activation of NF-κB during chronic inflammation and the malignant process (Yu et al., 2009). It has been shown previously that STAT-3 promotes nuclear localization of RelA by acetyltransferase p300-mediated acetylation affecting the NF-κB/IκBα interaction and avoiding its nuclear export (Chen and Greene, 2004). Since STAT-3 is a prerequisite for p300-mediated acetylation of RelA, constitutive activity of RelA in tumors requires continuous STAT-3 signaling (Lee et al., 2009). Accordingly, increased STAT-3 activity found in tumors preferentially leads to an association of NF-κB with STAT-3 via p300. Due to the NF-κB activating capacity of STAT-3 in malignancies constitutive activity of STAT-3 found in tumors preferentially requires RelA (Yu et al., 2009). As stated by the authors, this reciprocal relationship is related to the fact that numerous RelA-encoded target gene products function as STAT-3 activators (e.g., IL-6, IL-11, IL-17, IL-21, IL-23, PGHS-2). Remarkably, expression of IL-6, IL-17, IL-23, and PGHS-2 (all of them activating STAT-3) depends on STAT-3 as co-transcription factor for NF-κB. As a consequence, STAT-3 and NF-κB interact at multiple levels and thereby boost tumor-associated inflammation.

HEAT SHOCK PROTEINS AND TUMORIGENESIS

Heat shock proteins (HSP) are highly conserved proteins expressed in a wide range of species where they inhabit nearly all cellular and subcellular compartments. Environmental stress (e.g., heat, hypoxia, bacterial infections, heavy metals, oxidative stress, inflammation) as well as physiological processes (differentiation, proliferation, maturation) result in an increased HSP synthesis (Lindquist and Craig, 1988; DeNagel and Pierce, 1992). Intracellular residing HSP protect cells against lethal damage induced by environmental stress, and support folding and transport of newly produced polypeptides and aberrant proteins (Hartl, 1996). Depending on their intra-/extracellular localization HSP mediate different functions. On the one hand, up-regulated intracellular HSP levels protect tumor cells from lethal damage induced by environmental stress. On the other hand, membrane-bound and extracellular residing HSP with molecular weights of 70 and 90 kDa were identified as key regulators of the host's immune system.

A variety of HSP were found on the plasma membrane of tumor cell lines as determined by selective cell surface protein profiling (Shin et al., 2003). These findings were confirmed by a broad screening program of human tumor biopsies in our laboratory using cmHsp70.1 mAb (Stangl et al., 2011). Phenotypic analyses revealed that Hsp70, the major stress-inducible member of the HSP70 group, is found on the plasma membrane in 50–70% of colon, lung, pancreas, mammary, head and neck, lung, and urogenital carcinomas (Multhoff et al., 1995a,b; Chen et al., 2002). Metastases exhibit an elevated Hsp70 membrane density compared to primary tumors in humans (unpublished observation). These data were confirmed in a xenograft tumor mouse model. After orthotopic injection of human tumor cells into immunodeficient animals the cell surface density of Hsp70 was greater on metastases than on primary tumors (Multhoff et al., 2000; Stangl et al., 2006). Interestingly, the corresponding normal tissue of the mice was always found to be membrane Hsp70-negative (Stangl et al., 2011). These findings might be explained by the fact that membrane Hsp70 might facilitate metastases, support adherence of tumor cells to endothelial cells and organs, or might confer resistance to an unfavorable milieu during metastasis. In line with these findings we could show that overall survival of patients with membrane Hsp70-positive squamous cell carcinomas of the lung and lower rectal carcinomas was significantly reduced compared to those patients with membrane Hsp70-negative tumors (Pfister et al., 2007). Apart from solid tumors also bone marrow samples of patients suffering from acute (AML) and chronic (CML) myeloid leukemia are frequently membrane Hsp70-positive (Gehrmann et al., 2003). Quantitative analysis revealed that ∼15–20% of the total Hsp70 is present in tumor cell membranes (Gehrmann et al., 2008). The anchorage of Hsp70 within the plasma membrane is most likely mediated by the tumor-specific glycosphingolipid Gb3 (Gehrmann et al., 2008). This led us to the hypothesis that membrane Hsp70 might provide an ideal tumor-specific molecule for a targeted immunotherapeutic approach.

Even in the absence of immunogenic peptides, Hsp70, or a peptide derived thereof in combination with pro-inflammatory cytokines such as IL-2 and IL-15 has the capacity to stimulate the cytolytic activity of NK cells against membrane Hsp70-positive tumor cells (Multhoff et al., 1997, 2001). The mechanism of tumor cell killing has been identified as perforin-independent granzyme B-mediated apoptosis (Gross et al., 2003b). Granzyme B derived from activated NK cells specifically binds to membrane Hsp70 on tumor cells and following Hsp70-mediated endocytosis, apoptosis is induced (Gross et al., 2003a,b). Hsp70 also has been detected on tumor-derived exosomes of membrane Hsp70-positive tumors (Gastpar et al., 2005). These data suggest that NK cells might be attracted to membrane Hsp70-positive tumors *in vivo* via the secretion of Hsp70 surface-positive exosomes. Incubation of NK cells with Hsp70 protein or a 14mer-peptide derived from the C-terminus of Hsp70 is accompanied by an up-regulation of activating receptors on NK cells such as CD94/NKG2C, NKG2D, NKp30, NKp44, and NKp46 (Gross et al., 2003b,c). Hsp70 membrane-positive tumors are thus efficiently eliminated by NK cells that had been pre-stimulated with low dose IL-2 plus Hsp70 peptide (Multhoff et al., 1999). Adoptive transfer of these TKD-stimulated NK cells in tumor-bearing mice revealed identical results *in vivo* (Botzler et al., 1998; Multhoff et al., 2000; Moser et al., 2002). It is known that IL-2-activated NK cells are able to induce regression of established lung and liver tumors (Schwarz et al., 1989; Yasumura et al., 1994; Vujanovic et al., 1995; Whiteside et al., 1998). Our group identified a specific migratory capacity of NK cells toward Hsp70-postive tumor cells and supernatants derived thereof. The same effect could be observed for the Hsp70 peptide TKD (Gastpar et al., 2004). From these results we speculated that killing of Hsp70-positive tumors *in vivo* might be related to an enhanced migratory and cytolytic capacity of pre-activated NK cells.

The rapid induction of HSP in response to environmental stress is based on a variety of genetic and biochemical processes referred to as the heat shock response (HSR; Shamovsky and Nudler, 2008). The link between HSR and cancer development has been emerging since more than 20 years. HSR is regulated mainly at the transcription level by heat shock factors (HSF). Among them, HSF-1 is considered as being the key transcription factor of stress-inducible HSP (Pirkkala et al., 2001; Akerfelt et al., 2010). As a consequence, HSP are over-expressed in a wide spectrum of human malignancies contributing to tumor growth, differentiation, invasiveness, and metastasis and being associated with poor prognosis in certain cancer types (Ciocca and Calderwood, 2005). HSP over-expression in tumor cells plays a pivotal role in tumorigenesis by inhibiting apoptosis and senescence. In breast cancer, transformation-induced activation of HSF-1 results in an up-regulated expression of Hsp27 and Hsp70 which in turn results in protection against apoptosis (Calderwood, 2010). HSF-1 also triggers expression of Hsp90, an essential factor in tumor growth due to its ability to chaperone a variety of oncogenic signaling proteins including Her-2/neu and c-Src (Kamal et al., 2003; Neckers and Lee, 2003; Calderwood, 2010). Several studies have shown that Her-2/neu (c-ErbB-2) is amplified and over-expressed in many tumors such as breast, ovarian, and gastric adenocarcinoma (Hynes and Stern, 1994). Since HSP over-expression also protects from drug-related apoptosis (Khaleque et al., 2005), these mechanisms highlight the role of HSP in tumor progression and therapy resistance.

Recent studies indicate an involvement of HSP such as Hsp70/Hsp72 and Hsp90 in the recognition of PAMP by binding to TLR-4 within lipid rafts (Triantafilou and Triantafilou, 2004; Wheeler et al., 2009). Since extracellular residing Hsp70 acts as a danger signal for the immune system (Matzinger, 1998), this stress protein has been added to the list of "alarmins." Endogenous alarmins and exogenous PAMP both comprise the group of danger-associated molecular patterns (DAMP; Bianchi, 2007). Hsp70, added exogenously to cells stimulates the production of pro-inflammatory cytokines TNF, IL-1β, and IL-6 by antigen presenting cells (Asea et al., 2000a,b, 2002). Extracellular Hsp70 has also been found to induce IL-8 production in human bronchial epithelial cells (Chase et al., 2007). *In vitro* co-culturing of colon tumor cell spheroids with normal cells caused a significant tumor grade-dependent increase in IL-6 production thereby altering Hsp70 expression (Paduch et al., 2009). From these observations it can be concluded that Hsp70 may enhance the impact of tumorigenic mediators in the tumor microenvironment.

CONCLUDING REMARKS

Cancer-related inflammation has emerged as one of the hallmarks of cancer (Hanahan and Weinberg, 2011). In the last two decades, several tumorigenic factors have been identified as being implicated in inflammation-associated carcinogenesis. These factors are released by tumor cells or cells of the tumor microenvironment such as stromal cells, endothelial cells, or host infiltrating cells, respectively, and include pro-inflammatory cytokines, pro-angiogenic and growth-promoting factors, anti-apoptotic and invasion-promoting factors, inflammatory enzymes, prostaglandins, iNOS as well as chemokines. Among them, IL-1, TNF, and IL-6 act as crucial mediators of inflammation-driven tumorigenesis forming an inflammatory network in cancer as outlined in **Figure 2**. These mediators activate the key transcription factors in tumor-associated inflammation: NF-κB, STAT-3, and HIF-1 impacting any stage of tumorigenesis such as initiation, promotion as well as progression, and metastasis. It is noteworthy that NF-κB might be the central player in tumorigenesis. NF-κB is activated by a great variety of lifestyle-related factors including infectious agents, irradiation, environmental stimuli, tobacco, stress, dietary agents, obesity, and alcohol accounting for almost 95% of all cancers (Aggarwal and Gehlot, 2009). Modern anti-tumor therapies thus aim to suppress NF-κB activation. Most of the chemopreventive agents have been found as being able to suppress NF-κB activation like the selective PGHS-2 inhibitor celecoxib. Moreover, lifestyle-related agents derived from different sources including fruits, legumes, vegetables, grains, spices, and exercise are also able to inhibit NF-κB leading to suppression of the inflammatory network (Aggarwal and Gehlot, 2009). Clinical and preclinical studies are conducted to suppress the inflammatory network by the use of, e.g., steroids (dexamethasone, prednisolone), TNF inhibitors (thalidomide = thalomid™, anti-TNF antibodies such as infliximab = remicade™, etanercept = enbrel™, adalimumab = humira™), IL-1 inhibitors (anakinra™ = IL-1 receptor antagonist), PGHS-2 inhibitors (celecoxib), NF-κB inhibitors (curcumin, EGCG, piperazine), and RANKL inhibitors (denosumab™ = fully human monoclonal anti-RANKL antibody). A promising approach in cancer therapy also might be targeting HSP, because up-regulated Hsp90 and Hsp70 in cancer cells have

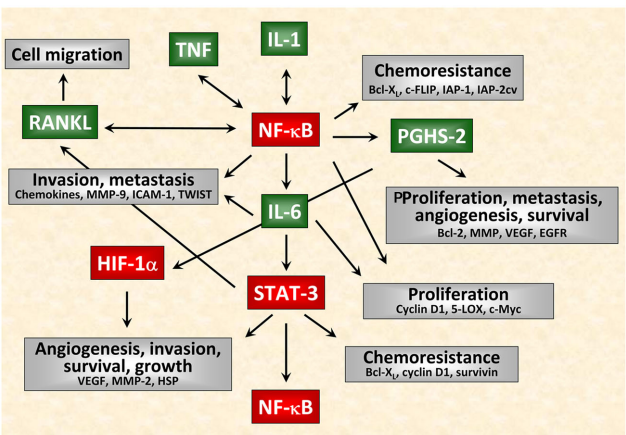

FIGURE 2 | Interactions of inflammatory mediators in tumor cells. Schematical simplified representation of the complex intracellular signaling network in cancer. Among the tumorigenic factors produced by tumor cells or cells of the tumor microenvironment, IL-1, TNF, and IL-6 act as crucial mediators of inflammation-driven tumorigenesis. In particular IL-1 and TNF are major pleiotropic cytokines involved in tumor/host interactions. Nevertheless, these cytokines function as autocrine growth factors and modulate the expression of several tumorigenic factors at any stage of tumorigenesis not only affecting proliferation, migration, and survival of tumor cells but also angiogenesis, invasiveness, metastasis as well as chemoresistance of tumors.

been recognized as important drug targets and are under intensive studies in recent years. HSP are currently being targeted in the therapy of breast cancer and other carcinomas and effective drugs for Hsp90 (e.g., geldanamycin and its analogs) have been synthesized and evaluated in clinical trials (Calderwood and Gong, 2011; Kim and Kim, 2011). HSP vaccines have been intensively studied in the preceding two decades, proving to be safe and effective in treating a number of malignancies (Murshid et al., 2011). However, therapeutical approaches that completely block one tumorigenic factor should be avoided, since it might also interfere with the physiological anti-tumor immune response and could therefore prove to be harmful. Instead, partial inhibition of numerous factors is preferred resulting in an enhanced efficacy thereby being less toxic to the patient.

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ER stress proteins in autoimmune and inflammatory diseases

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Over the past two decades, heat shock proteins (HSPs) have been implicated in inflammatory responses and autoimmunity. HSPs were originally believed to maintain protein quality control in the cytosol. However, they also exist extracellularly and appear to act as inflammatory factors. Recently, a growing body of evidence suggested that the other class of stress proteins such as, endoplasmic reticulum (ER) stress proteins, which originally act as protein quality control factors in the secretory pathway and are induced by ER stress in inflammatory lesions, also participate in inflammation and autoimmunity. The immunoglobulin heavy-chain binding protein (Bip)/glucose-regulated protein 78 (GRP78), calnexin, calreticulin, glucose-regulated protein 94 (GRP94)/gp96, oxygen regulated protein 150 (ORP150)/glucose-regulated protein 170 (GRP170), homocysteine-induced ER protein (Herp) and heat shock protein 47 (hsp47)/Serpin H1, which are expressed not only in the ER but also occasionally at the cell surface play pathophysiological roles in autoimmune and inflammatory diseases as pro- or anti-inflammatory factors. Here we describe the accumulating evidence of the participation of ER stress proteins in autoimmunity and inflammation and discuss the critical differences between the two classes of stress proteins.

Keywords: autoimmunity, inflammation, ER stress, ERAD, molecular chaperone

INTRODUCTION

Inflammation is a typical sign of autoimmune diseases. Misdirected immune responses target self-antigens and induce severe inflammatory responses, which sometimes cause death. In addition to numerous components in autoimmune responses, heat shock proteins (HSPs) have been implicated in autoimmune and inflammatory diseases over the past two decades (Hauet-Broere et al., 2006; Van Eden et al., 2007). Many HSPs are so-called molecular chaperones and folding enzymes, which maintain protein folding in a cell (Bukau et al., 2006; Hartl, 2011). Because protein folding is easily impaired by various cytotoxic stresses, such as heat shock, cytotoxic chemicals, hypoxia, and inflammation, prokaryotic to higher eukaryotic organisms have evolved stress responses and HSPs. The transcription of HSPs and their subsequent protein expression are stimulated by cytotoxic stresses, and they immediately restore protein folding and cellular homeostasis to counter toxic stresses (Morimoto, 1998). Thus, HSPs could be postulated to act as intracellular protein homeostasis maintenance factors. However, HSPs have also been reported to be observed in the extracellular fluid (Njemini et al., 2011), and act as pro- and anti-inflammatory factors especially in autoimmune and inflammatory diseases in diverse manners. In inflammatory lesions, HSPs are upregulated by inflammatory stress and are released into the extracellular fluid. Then, the extracellular HSPs specifically induce proinflammatory cytokines and enhance the antigenicity of autoantigens through modulations of antigen presentation (Multhoff, 2006; Yokota and Fujii, 2010). However, the extracellular HSPs can also stimulate anti-inflammatory regulatory T cell responses, thereby inducing the negative feedback control of inflammation (Hauet-Broere et al., 2006; Van Eden et al., 2007). Indeed, immunization with HSP peptides prevents disease development in autoimmune model animals, such as adjuvant arthritis and collagen-induced arthritis (CIA; van Eden et al., 1988; Jorgensen et al., 1998; Wendling et al., 2000). Presently, besides HSPs, another class of stress proteins is also known to exist in eukaryotic cells. Endoplasmic reticulum (ER) stress proteins, the specialized factors involved in protein quality control in the secretory pathway (Hoseki et al., 2010; Araki and Nagata, 2011; Smith et al., 2011), are induced by ER stress, which is very different from cytosolic stress (Mori, 2000; Walter and Ron, 2011). However, similar to HSPs, ER stress proteins are also induced by inflammatory stress (Yoshida, 2007), suggesting that ER stress proteins might also participate in autoimmune and inflammatory responses. Here, we discuss the accumulating evidence that ER stress proteins and their autoantibodies play roles in autoimmune and inflammatory diseases.

REGULAR FUNCTIONS OF ER STRESS PROTEINS

Before discussing the relevance of ER stress proteins in autoimmune diseases, we have briefly sketched the regular functions of ER stress proteins in a cell. Although the boundary between the two is ambiguous, ER stress proteins can be classified into two groups. The first group consists of molecular chaperones and folding enzymes, which assist the folding and assembly of newly synthesized proteins and prevent misfolding and aggregation of preexisting proteins. The other group consists of protein degradation factors, which mediate the clearance of proteins that should be degraded, such as misfolded proteins. The latter protein clearance

pathway is called the ER-associated degradation (ERAD) pathway (McCracken and Brodsky, 1996; Brodsky and McCracken, 1997).

The ER is located at the starting point of the protein secretory pathway. Secretory or membrane proteins are first cotranslationally transported into the ER from cytosolic ribosomes, modified and properly folded, and then translocated to the next step of the secretory pathway (**Figures 1A,B**; Ni and Lee, 2007; Araki and Nagata, 2011). After the ER, proteins pass through the Golgi apparatus and are finally secreted to the cell surface through a secretory vesicle. In the ER, most proteins are glycosylated and covalently crosslinked with disulfide bonds. Such protein modifications are believed necessary for the structural stabilization of the proteins destined to be secreted into the extracellular environment (Ni and Lee, 2007; Araki and Nagata, 2011). Calnexin and calreticulin are lectin-like chaperones that specifically maintain the folding of glycosylated proteins (Williams, 2006). Protein disulfide isomerase (PDI) is an oxidoreductase that mediates protein disulfide bond formation and isomerization (Tu and Weissman, 2004; Ellgaard and Ruddock, 2005). The immunoglobulin heavy-chain binding protein (Bip)/glucose-regulated protein 78 (GRP78) is a hsp70-type molecular chaperone that maintains protein folding (Ni and Lee, 2007; Araki and Nagata, 2011). Glucose-regulated protein 94 (GRP94)/gp96 and oxygen regulated protein 150 (ORP150) are hsp90 and hsp70 family molecular chaperones, respectively; their functions remain unclear (Ni and Lee, 2007; Araki and Nagata, 2011). Another characteristic stress protein in the ER is heat shock protein 47 (hsp47)/Serpin H1, the only heat shock regulated protein in the ER, is the collagen-specific molecular chaperone (Nagata, 2003; Ishida and Nagata, 2011). Using these molecular chaperones and folding enzymes, secretory and membrane proteins are properly folded in the ER and then transported to the Golgi apparatus. Other chaperones and enzymes were precisely described in a recent article (Araki and Nagata, 2011).

Improperly folded proteins are strictly retained in the ER via anchoring by ER chaperones and enter the ERAD pathway (Araki and Nagata, 2011; Smith et al., 2011). ERAD is a multi-step mechanism, which can be divided into the following three steps: (1) the substrate is recognized and isolated, (2) the substrate is dislocated from the ER into the cytosol, and (3) the substrate is then degraded by the ubiquitin proteasome system in the cytosol (**Figures 1C–E**; Hershko and Ciechanover, 1998). Thereby, the clearance of the proteins from the ER is completed. Among these steps, the first step is mediated by ERAD-enhancing mannosidase-like protein 1 (EDEM-1), ERdj5, Bip, Osteosarcoma 9 (OS9), XTP3 transactivating gene B (XTP3B), and SEL1L (Hosokawa et al., 2001; Ushioda et al., 2008; Hagiwara et al., 2011; Ushioda and Nagata, 2011). The substrate is directly transferred from the calnexin/calreticulin-mediated folding cycle to the EDEM-1-containing degradation recognition complex (Molinari et al., 2003; Oda et al., 2003). The substrate is then transferred from EDEM-1 and SEL1L to the so-called ERAD complex, postulated to form a protein dislocation channel (Mueller et al., 2006, 2008; Christianson et al.,

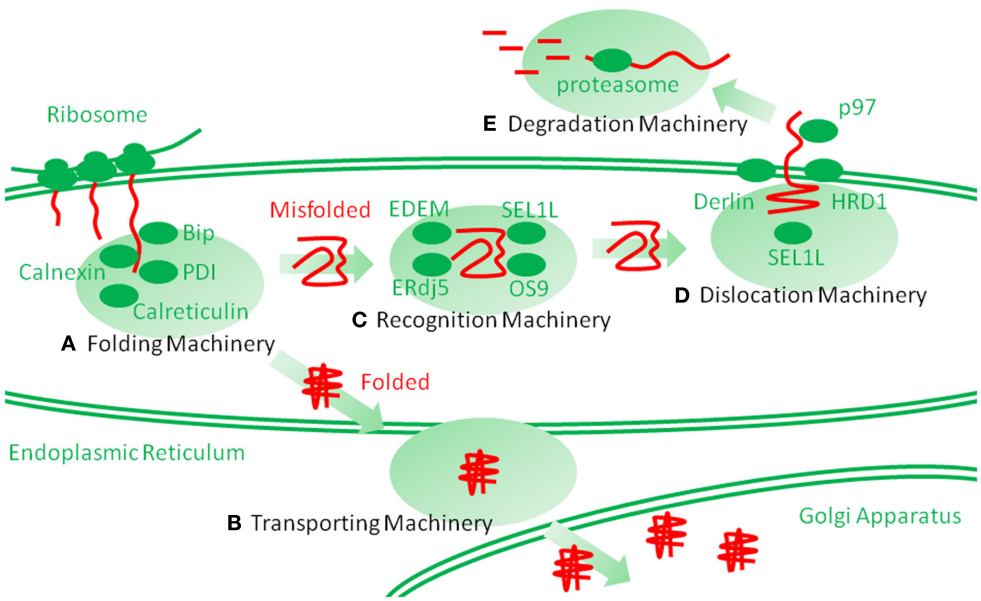

FIGURE 1 | Schematic representation of endoplasmic reticulum (ER) protein folding and ER-associated degradation (ERAD). Secretory and membrane proteins are cotranslationally transported into the ER by cytosolic ribosomes. Newly synthesized unfolded polypeptide are then captured by molecular chaperones and protein folding enzymes **(A)**, which involve immunoglobulin heavy-chain binding protein (Bip), calnexin, calreticulin, protein disulfide isomerase (PDI) and heat shock protein 47 (hsp47), and are properly folded. The folded polypeptides are transported into the Golgi apparatus through transporting machinery **(B)**. However, the proteins that are improperly folded are specifically recognized and isolated by ERAD recognition machinery **(C)**, which involves ERAD-enhancing mannosidase-like protein (EDEM), ERdj5, Bip, SEL1L, osteosarcoma 9 (OS9), and XBP1. The misfolded proteins are then transferred to the dislocation machinery **(D)**, which involves HRD1, Derlin, Herp, and p97. Then, the proteins are dislocated into the cytosol through a putative narrow pore. The dislocated proteins are ubiquitinated by ERAD ubiquitin ligases, such as HRD1, and are finally degraded by huge cytosolic protease complexes, the proteasome **(E)**, into peptides.

2008). ER stress proteins, such as HRD1/synoviolin, Derlin, and homocysteine-induced ER protein (Herp), are involved in this supramolecular complex (Lilley and Ploegh, 2004, 2005; Oda et al., 2006). The substrate is then dislocated into the cytosol from the ER. During dislocation, the substrate is ubiquitinated by the ER ubiquitin ligase and pulled by p97/VCP from the cytosolic side (Ye et al., 2001, 2003; Tsai et al., 2002). Finally, in the third step, the ubiquitinated substrate is degraded by the cytosolic complex protease, the proteasome. Detailed comprehensive descriptions of the ERAD pathways can be found elsewhere (Araki and Nagata, 2011; Smith et al., 2011).

ER STRESS

The stress response for HSPs, called heat shock response, is mediated by transcription factors called heat shock factors (HSFs; Morimoto, 1998). Similarly, the stress response for ER stress proteins, called the unfolded protein response (UPR), is mediated by the unconventional transcription factors, ATF6 and XBP1, and a translation and apoptosis regulating factor, PERK (**Figure 2**; Mori, 2000; Walter and Ron, 2011). The UPR can be experimentally stimulated by chemical compounds that specifically interfere with protein glycosylation and disulfide bond formation because these protein modifications are necessary for the structural stability of secretory and membrane proteins. In addition, perturbation of calcium storage in the ER causes impairments in the quality of ER proteins because the ER has a high calcium concentration, and many of the ER proteins need calcium ions. Alternatively, the UPR is physiologically activated during plasma cell differentiation in order to expand the protein folding capacity for massive production of immunoglobulins. The activation of the UPR has been observed in various neurodegenerative diseases, inflammatory diseases, and viral infections (Yoshida, 2007). Thus, the UPR can be postulated as a completely independent pathway from cytosolic stresses. Both cytosolic and ER stresses are commonly stimulated by inflammation. Although several studies suggest that ER stress protein levels correlate with inflammatory pathogenesis, the roles of ER stress and ER stress proteins in autoimmune and inflammatory diseases remain unclear. Thus, in addition to the heat shock response, the other axis of the cellular stress response might provide new insights into disease mechanisms and clinical treatments.

BIP/GRP78

The 68-kDa glycoprotein was identified through exploration of autoantigens that predominantly immunoreact with sera from rheumatoid arthritis (RA) patients and thus expected to be associated with RA pathogenesis (Blass et al., 1995, 1997, 1998). Subsequently, the 68-kDa antigen was demonstrated to be identical to the ER resident hsp70 family chaperone Bip (Blass et al., 2001; Corrigall et al., 2001). This crucial ER resident stress protein appears to be expressed at the cell surface and acts as an autoantigen in RA. Indeed, 30–75% of RA patients exhibit Bip overexpression in synovial fluid or autoantibodies to Bip in serum (Blass et al., 2001; Corrigall et al., 2001; Shoda et al., 2011). Although the exact roles of extracellular Bip and anti-Bip autoantibodies in RA pathogenesis remain debatable, three possible functions can be considered. First, extracellular Bip and its antibody may result

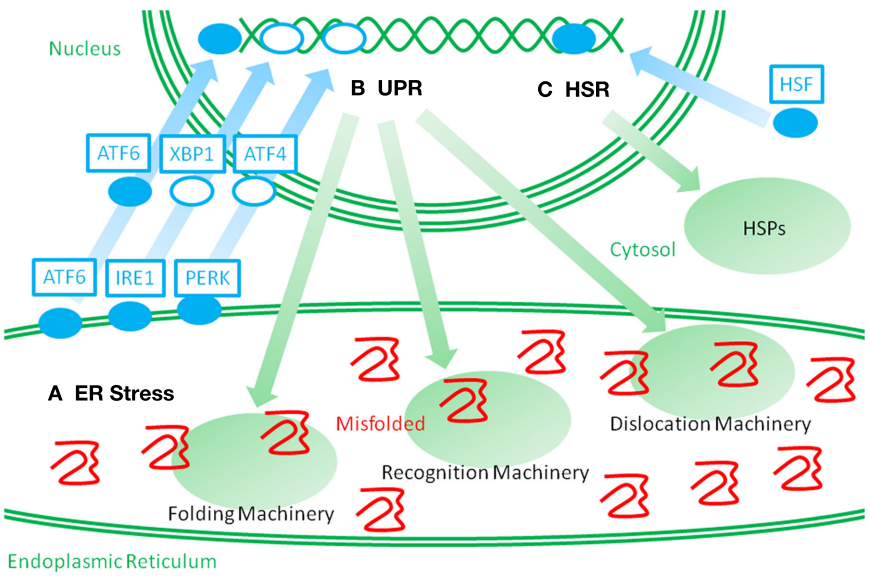

FIGURE 2 | Schematic representation of two distinct stress responses. When misfolded proteins accumulate in the ER, they perturb ER protein homeostasis and organelle functions. Such perturbations are called ER stressors **(A)**. ER stress leads to the activation of membrane spanning stress sensors. ATF6 is a membrane spanning transcription factor that is processed and released from the ER membrane under ER stress, and the stress signal is then transmitted to the nucleus. IRE1 and PERK activate the transcription factors, XBP1 and ATF4, and stimulate a stress response. Such stress responses are called an unfolded protein response (UPR) **(B)**. UPR leads to an upregulation of ER stress proteins, which consist of molecular chaperones and folding enzymes, and ERAD recognition and dislocation factors. Such cross-membrane signal transduction is very different from the heat shock response (HSR) **(C)**. When the cytosolic stresses perturb the cytosolic protein homeostasis, heat shock factor (HSF) is activated and transmits the signal into the nucleus. Then, an upregulation of HSPs is induced. It is a challenge to recover cytosolic protein homeostasis.

from inflammatory stress during RA progression, and they might have no pathophysiological roles. Because the UPR is stimulated through inflammation and ER stress proteins, including Bip, are subsequently induced (Yoshida, 2007), Bip could be simply a specific and abundant autoantigen in the inflammatory lesion that is unrelated to RA pathogenesis. Second, the autoantigen might act as an anti-inflammatory factor, similar to HSPs. Several studies suggested that extracellular Bip stimulates the production of the anti-inflammatory cytokines, IL-4 and IL-10, through specific T lymphocytes (Blass et al., 2001; Corrigall et al., 2001, 2004; Bodman-Smith et al., 2003; Brownlie et al., 2006; Panayi and Corrigall, 2006). Furthermore, the pre-administration of Bip protein to mice prevents the *in vivo* induction of adjuvant arthritis or CIA, both of which are well-known artificial models of autoimmune diseases (Corrigall et al., 2001; Brownlie et al., 2006). Third, the Bip antigen could act as a proinflammatory factor. Bip positively causes immunological responses and inflammation. Recently, Shoda et al. (2011) reported that in addition to the intact Bip antibody, anti-citrullinated Bip (ctBip) antibody is frequently detected in RA patients. The ctBip protein, but not intact Bip, enhances anti-citrullin antibodies and worsens arthritis symptoms in a mouse model of adjuvant arthritis. Citrullination is protein modification in which an arginine residue is converted into a citrulline residue by a specific intracellular enzyme, peptidylarginine deiminase (PAD; Vossenaar et al., 2003). The anti-citrullinated peptide/protein antibody (ACPA) is frequently detected in RA patients (Vincent et al., 2005; Suzuki et al., 2007; van Venrooij et al., 2011). Although the reasons why citrullination is frequently observed and how it participates in RA pathogenesis remains unclear, the relationship between stress proteins and this specific protein modification suggests an undescribed crosstalk between inflammatory stress and disease-specific protein modifications in RA pathogenesis. In addition to RA, the anti-Bip autoantibody is also detected in another autoimmune and inflammatory disease, systemic lupus erythematosus (SLE; Casciola-Rosen et al., 1994; Weber et al., 2010), in which its pathophysiological role remains unknown.

HSP47

Hsp47 is an ER resident molecular chaperone; it is the only HSP in the ER. Hsp47 specifically maintains collagen biosynthesis (Nagata, 2003; Ishida and Nagata, 2011). Its gene disruption in mice causes significant reductions in mature collagens in connective tissues, resulting in embryonic lethality (Nagai et al., 2000; Marutani et al., 2004; Matsuoka et al., 2004). Several studies showed that the levels of anti-hsp47 autoantibody are specifically increased in RA patients (Hattori et al., 1998, 2000, 2001, 2003, 2005). However, little is known about how hsp47 and its autoantibody correlate with RA pathogenesis. In addition to RA, the levels of the autoantibody to hsp47 are also increased in other autoimmune diseases, such as SLE, Sjögren's syndrome (SjS), mixed connective tissue disease (MCTD), systemic sclerosis (SSc), and non-specific idiopathic pneumonia (Yokota et al., 2003; Fujimoto et al., 2004; Kakugawa et al., 2008). Most of these diseases can be considered connective tissue diseases in which an upregulation of various types of collagen is observed. The expression profiles of collagens and hsp47 are fully consistent in both healthy (Masuda et al., 1998; Yamamura et al., 1998; Hirata et al., 1999; Yasuda et al., 2002) and diseased conditions (Masuda et al., 1994; Naitoh et al., 2001; Sato et al., 2008). Hsp47 might be the protein that stands at the junction of stress, the extracellular matrix (ECM) biogenesis, and autoimmune/connective tissue diseases.

HERP

Lupus nephritis, which is a kidney inflammatory disorder, is one of the manifestations of SLE, a complex autoimmune disease. Among the variety of autoantibodies that are detected in SLE patients, the anti-double-stranded DNA (dsDNA) antibody, which is a type of anti-nuclear antibody (ANA), is most characteristic of SLE and appears to significantly contribute to the pathogenesis of lupus nephritis (Isenberg et al., 2007). Although administration of dsDNA failed to initiate antibody production (Madaio et al., 1984), nucleosome-forming dsDNA elicited the anti-dsDNA antibody production (Rumore and Steinman, 1990; Casciola-Rosen et al., 1994; Voynova et al., 2005), suggesting that proteins like histone can work as an adjuvant for enhancing the antigenicity of dsDNA. Another possibility for anti-dsDNA antibody production is elicitation by cross-reactive protein antigens. Several proteins have been reported to cross-react with the anti-dsDNA antibody (Isenberg et al., 2007). Among them, α-actinin, which is an actin-associated protein, might be a potent candidate for the original antigen, which evokes anti-dsDNA antibody production (Mostoslavsky et al., 2001; Deocharan et al., 2002). However, a dysregulation of the negative selection of B-cell clones, which produce autoantibodies, occurs in these diseases.

Recently, Herp was suggested as a possible cross-reactive antigen of the anti-dsDNA antibody (Hirabayashi et al., 2010). Herp is an ER resident membrane protein that is involved in the ERAD complex and is induced by UPR (Kokame et al., 2000, 2001; Okuda-Shimizu and Hendershot, 2007; Kny et al., 2011; Marutani et al., 2011). Thus, Herp is an ER stress protein, can be induced by inflammatory ER stress. Among the known candidates for the original antigen of the anti-dsDNA antibody including α-actinin, only Herp can be induced by inflammatory stress. Thus, Herp can be the key component that connects inflammatory stress responses and anti-dsDNA antibody production in SLE.

CALRETICULIN

The anti-Ro/SS-A antibody, which is also considered an ANA, is one of the most studied and crucial markers in many autoimmune diseases, such as SLE, Sjs, SSc, and RA (Anderson et al., 1962; Clark et al., 1969; Alspaugh and Tan, 1975; Schulte-Pelkum et al., 2009; Defendenti et al., 2011). Although it has a strong, well-established association with autoimmune diseases, especially with neonatal lupus, it is unclear how it participates in autoimmune diseases (Schulte-Pelkum et al., 2009; Defendenti et al., 2011). Recently, it was shown that the anti-Ro/SS-A antibody can be categorized into two types, each of which specifically recognizes the 52-kDa (Ro52) and 60-kDa (Ro60) antigens. Ro52 and Ro60 form a Ro ribonucleoprotein (RNP) complex with a couple of other proteins, while the intracellular functions and pathogenic relevancies of those antigens remain unclear (Schulte-Pelkum et al., 2009; Defendenti et al., 2011).

Calreticulin is an essential ER resident lectin-like chaperone, which is induced by ER stress (Yoshida et al., 1998; Mesaeli et al.,

1999; Williams, 2006). Calreticulin was first postulated as the Ro/SS-A autoantigen (Collins et al., 1989; McCauliffe et al., 1990). However, it was later demonstrated that calreticulin itself was not an antigen (Lu et al., 1993; Boehm et al., 1994), but it maintained Ro RNP complex formation through its chaperone activity and modulated the antigenicity of the complex (Cheng et al., 1996; Staikou et al., 2003). Although it was shown that calreticulin was not the autoantigen for the Ro/SS-A autoantibody, the anti-calreticulin autoantibody has been independently observed in SLE patients (Boehm et al., 1994), complete congenital heart block (CCHB; Orth et al., 1996), which is one of the manifestations of SLE, and inflammatory bowel disease (Watanabe et al., 2006).

CALNEXIN, GRP94, AND ORP150

Calnexin is an ER resident lectin-like chaperone (Wada et al., 1991, 1997; Hebert et al., 1995; Deprez et al., 2005), which is not induced or is only mildly induced by ER stress (Kamauchi et al., 2005). An autoantibody to calnexin is observed in SLE patients, while its biological relevance remains unknown (Weber et al., 2010).

GRP94 is an essential ER resident hsp90 family chaperone, which is induced by cytosolic and ER stress (Wanderling et al., 2007; Eletto et al., 2010). Anti-GRP94 autoantibodies have been observed in SLE patients (Boehm et al., 1994), RA (Weber et al., 2010), and myasthenia gravis (MG; Suzuki et al., 2011). In addition, cell surface expression of GRP94 itself is detected in patients with type I diabetes (Pagetta et al., 2003) and in GRP94 transgenic mice (Liu et al., 2003). In GRP94 transgenic mice, lupus-like autoimmune disorder and systemic inflammation are induced, suggesting that extracellular GRP94 and its autoantibody have proinflammatory effects in autoimmune diseases.

ORP150 is an essential ER resident hsp70 chaperone that is induced by ER stress and hypoxia (Kitao et al., 2001; Ni and Lee, 2007). An autoantibody to ORP150 is detected in patients with atherosclerosis and type I diabetes (Tsukamoto et al., 1996; Nakatani et al., 2006), but it remains unclear if they have a pathophysiological role in autoimmune responses.

REGULATION OF ER STRESS PROTEIN DISTRIBUTION

As discussed above, the critical difference between HSPs and ER stress proteins is based on the stress pathway they are induced through. One is through a heat shock response, and the other is through the UPR. However, another striking difference exists, i.e., HSPs are originally distributed in the cytosol. In patients with inflammatory diseases, HSPs are observed in extracellular fluid; this could be due to the destruction of the plasma membrane that accompanies stress-induced apoptosis, necrosis, and phagocytosis or alternative active secretion through exosomes. On the other hand, ER stress proteins are originally located in the secretory pathway. Thus, ER stress proteins can potentially be secreted into the cell surface, after which they escape from the ER retention mechanism. Indeed, some ER stress proteins are expressed on the cell surface without any cell destruction, e.g., calnexin (Okazaki et al., 2000), calreticulin (Jeffery et al., 2011), Bip (Delpino and Castelli, 2002), GRP94 (Altmeyer et al., 1996), and hsp47 (Hebert et al., 1999). How they escape, how they are retained, and whether this leaky expression correlates with autoimmune pathogenesis remains unknown. However, this apparent difference between the two types of stress proteins might cause physiological differences, therefore, this should be examined in autoimmune and inflammatory diseases.

CONCLUSION

Several studies suggested that ER stress proteins are potent immunomodulating components in autoimmune and inflammatory diseases. Although their pathogenic relevancies remain unclear, some of them appear to participate in disease progression. Their inducibility by inflammatory stress and their original distribution in the secretory pathway could be advantageous for participation in autoimmune and inflammatory responses. Until now, the major evidence of the involvement of ER stress proteins in autoimmune diseases appears to be autoantigens. This situation can be compared to that of HSPs two decades ago. Since then, much has been revealed about the functions of HSPs in addition to being autoantigens. Because HSPs and ER stress proteins share several properties as molecular chaperones, we may be able to expect unidentified and important roles of ER stress proteins in autoimmune response as well as HSPs. Indeed, another issue of the stress proteins in autoimmune responses is their potential immunomodulating abilities. Because chaperones can broadly associate with other proteins, including autoantigens and recruit antigen presentation pathway, they can work as endogenous adjuvants in immune responses. In this review, we focused on the roles of ER stress proteins as autoantigens. However, several other issues that are related to ER stress are also important, e.g., HLA-B27 misfolding in ankylosing spondylitis and anti-apoptotic function of HRD1/synoviolin in synovial cells in RA (Yoshida, 2007; Todd et al., 2008; Yagishita et al., 2008; Colbert et al., 2010). At the moment, the features of ER stress proteins in autoimmunity remain largely unclear. The potential relevance of ER stress proteins in many autoimmune and inflammatory diseases makes them potential clinical targets.

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Role of heat shock protein 70 in innate alloimmunity

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This article briefly describes our own experience with the proven demonstration of heat shock protein 70 (HSP70) in reperfused renal allografts from brain-dead donors and reflects about its potential role as a typical damage-associated molecular pattern (DAMP) in the setting of innate alloimmunity. In fact, our group was able to demonstrate a dramatic up-regulation of HSP70 expression after postischemic reperfusion of renal allografts. Of note, up-regulation of this stress protein expression, although to a lesser extent, was already observed after cold storage of the organ indicating that this molecule is already induced in the stressed organism of a brain-dead donor. However, whether or not the dramatic up-regulation of HSP70 expression contributes to mounting an innate alloimmune response cannot be judged in view of these clinical findings. Nevertheless, HSP70, since generated in association with postischemic reperfusion-induced allograft injury, can be called a typical DAMP – as can every molecule be termed a DAMP that is generated in association with any stressful tissue injury regardless of its final positive or negative regulatory function within the innate immune response elicited by it. In fact, as we discuss in this article, the context-dependent, even contradistinctive activities of HSP70 reflect the biological phenomenon that, throughout evolution, mammals have developed an elaborate network of positive and negative regulatory mechanisms, which provide balance between defensive and protective measures against unwarranted destruction of the host. In this sense, up-regulated expression of HSP70 in an injured allograft might reflect a pure protective response against the severe oxidative injury of a reperfused donor organ. On the other hand, up-regulated expression of this stress protein in an injured allograft might reflect a (futile) attempt of the innate immune system to restore homeostasis with the aim to eliminate the "unwanted foreign allograft invader" by contributing to development of an adaptive alloimmune response. However, this adaptive immune response against donor histocompatibility alloantigens – in its evolutionary sense aimed to restore homeostasis – is by no means protective from a recipient's view point but tragically ends up with allograft rejection. Indeed: in this sense, allograft rejection is the result of a fateful confusion by the immune system of danger and benefit!

Keywords: injury hypothesis, innate alloimmunity, DAMPs, HSP72

INTRODUCTION: ALLOGRAFT INJURY-INDUCED INNATE ALLOIMMUNITY

FIRST CLUE TO THE EXISTENCE OF INNATE ALLOIMMUNITY: THE INJURY HYPOTHESIS (1994)

Originally, the immune system was regarded as a host defense system that is primarily directed against invading pathogens with the aim to prevent/cure infection. Following the re-discovery of innate immunity in the mid/late 1990s, this concept was confirmed and extended: Infection of cells by microorganisms was seen to activate the innate immune system that elicits an inflammatory response merging into an adaptive immune response. The initial sensing of infection was recognized to be mediated by pattern recognition receptors (PRRs) in/on innate immune cells that play a pivotal role in the first line of this host defense system. PRRs were found to recognize distinct pathogen-derived motifs, the pathogen-associated molecular patterns (PAMPs) to initiate and regulate innate and adaptive immune responses (for review, see Kumar et al., 2011).

First notions that the immune system has evolved the capacity to detect *any* tissue injury in ways that stimulate the initiation and generation of adaptive immune responses to antigens were addressed and discussed by two hypotheses nearly simultaneously published early in 1994 (Land et al., 1994; Matzinger, 1994). These two hypotheses postulated that the adaptive immune system evolved to respond not only to pathogen-mediated "infectious" tissue injury *per se* but also to non-physiological cell death, tissue injury, or stress, as, for example, mediated by postischemic reperfusion injury (IRI). As a matter of fact, it was Matzinger (1994) who proposed her famous "Danger Hypothesis." Her model – proposed on theoretical grounds – suggested that the primary driving force of the immune system is the need to detect and protect against danger. Danger, however, equals tissue destruction, that is, tissue injury. Our "Injury Hypothesis" – proposed on statistically significant data from a prospective clinical trial in kidney transplant patients (the "Munich SOD Trial") – discussed the possibility that it is the primary injury to an allograft

that – via activation of antigen-presenting cells – induces pathways leading to an adaptive alloimmune response (Land et al., 1994). In fact, this hypothesis was based on our pivotal clinical observation that (antioxidative) treatment of a non-specific tissue injury [here: the reactive oxygen species (ROS)-mediated IRI to allografts] leads to a significant reduction in subsequent specific, adaptive immunity-mediated processes (here: reduction of alloimmune-mediated allograft rejection).

Under the line, the two hypotheses postulated the same scenario: The initial tissue injury, that is, the injurious inflammatory tissue environment, alerts the immune system, and is a mandatory prerequisite to mount an efficient adaptive immune response against foreign antigens.

ROLE OF DAMAGE-ASSOCIATED MOLECULAR PATTERNS AND PATTERN RECOGNITION RECEPTORS IN OXIDATIVE INJURY – INDUCED ALLOGRAFT ("STERILE") INFLAMMATION

Over the past decade, increasing evidence has been published in support of the notion that PRRs recognize non-infectious but injurious agents that can cause tissue damage (for reviews, see: Beutler, 2007; Manfredi et al., 2009; Chen and Nuñez, 2010; Bauernfeind et al., 2011; Jaeschke, 2011; Yanai et al., 2011). In this scenario, PRRs sense injury-induced, host-derived endogenous molecules in terms of damage-associated molecular patterns, that is, DAMPs, an acronym that was coined by us in analogy to PAMPs 8 years ago (Land, 2003a). These DAMPs are released following tissue injury or cell death and have similar functions as PAMPs in terms of their ability to activate pro-inflammatory pathways in innate immune cells.

Of note, ROS-mediated IRI to allografts can be regarded as a model of a non-pathogen-induced oxidative tissue injury that activates the innate immune system. In fact, an emerging role of innate immune events in adaptive alloimmunity-mediated allograft rejection (= innate alloimmunity) has been noted (for reviews, see Land, 2011a,b). However, the situation in the allograft setting is more complex as two individually different categories of DCs are involved: (1) donor-derived PRR-bearing innate immune cells such as DCs, vascular, and epithelial cells already residing in the transplant and (2) recipient-derived PRR-bearing innate immune cells such as DCs and neutrophils invading the allograft during reperfusion in the recipient. Moreover, various DAMPs such as high mobility group box 1 (HMGB1) and heat shock protein 70 (HSP70) are generated already in the brain-dead organ donor (Arbogast et al., 2002; Krüger et al., 2009; Kaminska et al., 2011), however, their up-regulated expression culminate during allograft reperfusion in the recipient (Arbogast et al., 2002; Kaczorowski et al., 2009; Klune and Tsung, 2010). Indeed, according to new insights into mechanisms of innate immunity, a brain-dead organism, characterized by the demonstration of DAMPs, PRRs, and circulating cytokines, may be defined as an acute systemic innate immunity-mediated autoinflammatory syndrome (Land, 2011b). To add yet another level of complexity in the allograft setting, it can be assumed that further DAMPs are released from necrotic cells caused by allograft reperfusion injury which may include nucleic acids, oxidation-specific DAMPs such as thioredoxin (TRX)-interacting protein (TXNIP) and purine metabolites such as ATP (Schröder et al., 2010; Bours et al., 2011; Kis-Toth et al., 2011). In addition to those DAMPs derived from an intracellular source, IRI leads to release of extracellular located DAMPs such as extracellular matrix fragments including hyaluronan and heparan sulfate. The prototypical PRRs recognizing all those various DAMPs include Toll-like receptors (TLRs) such as TLR2 and TLR4 and the receptor for advanced glycation end-products (RAGE) as well as PRRs able to recognize nucleic acids such as RIG-I-like receptors (RLRs) and AIM2-like receptors (for review, see Land, 2011e).

There is a special role for the cytosolic nucleotide-binding domain leucine-rich repeats (NLR) receptors because one of its members represents the core structure of the NOD, LRR, and pyrin domain-containing 3 (NLRP3) inflammasome, the most widely studied inflammasome now known to be activated by various DAMPs (for review, see Bauernfeind et al., 2011). In fact, the well-known creation of "sterile" inflammation in reperfused organs (and solid allografts have to be included here) is now believed to be induced by inflammasomes. Most important in regard to the topic of IRI are recent studies in both *Nlrp3*- and *ASC*-deficient mice clearly demonstrating that the NLRP3 inflammasome contributes to the IRI-mediated acute inflammatory response (Iyer et al., 2009). These studies revealed that non-lethal renal IRI results in a significant up-regulation of *Nlrp3* gene expression, which is accompanied by pronounced acute tubular necrosis that is similar between wild-type (WT) and *Nlrp3*-deficient animals. Importantly, the studies showed that *Nlrp3*-deficiency protected animals from lethal renal IRI. Similarly, *ASC*-deficiency protected animals from lethal renal IRI, although the difference, compared to WT mice, was less pronounced than in *Nlrp3*-deficient mice.

Activation of the NLRP3 inflammasome is currently thought to consist of two steps: a first priming step that consists of stimulation of PRRs which leads to the up-regulation of NLRP3 expression and also induces pro-IL-1β expression. Stimuli priming NLRP3 appear to include all ligands for TLRs, RLRs, and NLRs, that lead to enhanced NLRP3 expression (Bauernfeind et al., 2011). Thus, theoretically, HSP70, as a ligand of TLR4 and TLR2, can be counted to those priming DAMPs (The LPS-contamination debate in regard to TLR2 and TLR4 is not dealt with here but discussed elsewhere; Land, 2011e). The second step consists of the activation of NLRP3 itself which is distinct from this initial priming step. Three presumably distinct pathways have been postulated that can lead to the activation of NLRP3 (activation step): (1) extracellular ATP that is often seen to be largely increased during cell death, can bind to purinergic receptor P2X7 and leads to NLRP3 activation via ion flux effects (including intracellular K^+ depletion) mediated by the P2X7-receptor-associated hemi-channel pannexin-1; (2) endocytosis of sterile particulates, such as cholesterol crystals, resulting in lysosomal disintegration which leads to the leakage of lysosomal enzymes into the cytosol (activation of the protease cathepsin B); and (3) generation of ROS during cellular stress or death leading to the activation of NLRP3 through the release of TXNIP from thioredoxin and then binds to NLRP3 (for review, see Bauernfeind et al., 2011). Although the role of ROS in NLRP3 activation is still not quite clear, this potential activation mechanism is of considerable attraction in regard to ROS-mediated reperfusion injury to allografts.

ALLOGRAFT INJURY-INDUCED, DAMPs-MEDIATED GENERATION OF DONOR- AND RECIPIENT-DERIVED IMMUNOSTIMULATORY DCs

In fact, growing evidence suggests that allograft injury induces innate immune events which precede adaptive alloimmunity. Within an innate immune-mediated intragraft inflammatory milieu, donor- and recipient-derived DCs get activated, migrate to the secondary lymphoid tissue of the recipient to translate innate immunity to an adaptive alloimmune response. The generation of these immunostimulatory DCs is the result of innate immune pathways initiated and induced by various DAMPs which, as reviewed (Land, 2011b), may be divided into four different classes of DAMPs: (1) class I DAMPs such as HMGB1 and HSP70 that, when recognized by PRRs of immature DCs (iDCs), trigger their activation to immunostimulatory DCs; (2) class II DAMPs such as MHC class I chain-related molecules A and B that, when recognized by special activating receptors (NKG2D) on innate lymphocytes may contribute to generation of immunostimulatory DCs in terms of a cross-talk; (3) class III DAMPs such as TXNIP and extracellular ATP that are recognized by PRRs involved in the activation of the NLRP3 inflammasome, and (4) class IV DAMPs in terms of neoantigens that are recognized by pre-existing natural immunoglobulin M antibodies, which – via complement activation – are able to aggravate the oxidative tissue injury and, thereby, may indirectly promote metamorphosis of iDCs into immunostimulatory DCs.

In fact, there seems to be a complex collaboration between various membrane-bound and cytosolic PRRs on one side and their different cognate DAMPs on the other side which results in those well-known vigorous innate alloimmune responses. Most likely, this complex orchestration of intragraft DAMP–PRR interactions may finely regulate the maturation process of donor- and recipient-derived iDCs into immunostimulatory DCs, that, via processes of direct and indirect allorecognition, may stimulate naïve T cells of the recipient, thereby mounting, and fine-tuning adaptive alloimmune responses of various intensities.

ROLE OF HEAT SHOCK PROTEIN 70 IN INNATE ALLOIMMUNITY

DONOR BRAIN DEATH-MEDIATED AND REPERFUSION INJURY-INDUCED UP-REGULATION OF HSP70 EXPRESSION IN HUMAN ALLOGRAFTS

The first DAMP shown to be involved in the setting of clinical organ transplantation was the inducible HSP70 that was up-regulated following renal allograft reperfusion injury, that is, a condition in which contamination with pathogen-derived exogenous ligands of PRRs can be totally ruled out (Arbogast et al., 2002). In fact, our group was able to demonstrate a dramatic up-regulation of HSP70 expression after postischemic reperfusion of renal allografts from deceased (brain-dead) donors (**Figure 1**). Interestingly, up-regulation of HSP70 expression, although to a lesser extent, was already observed after cold storage of the organ indicating that this stress protein is already induced in the stressed organism of a brain-dead donor (**Figure 1**). These findings prompted us to discuss a role of HSP70 in innate alloimmunity by proposing that HSPs (1) in their function as endogenous ligands of TLRs may lead to DC maturation and (2) in their function as chaperokines may facilitate cross-presentation of HSP-chaperoned allopeptides on MHC class I molecules of recipient-derived DCs (Land, 2002, 2003b). Whether or not dramatic up-regulation of HSP70 expression in an allograft contributes to mounting an innate alloimmune response cannot be judged in view of these clinical findings alone, but a brief look at the more recent literature may help in interpreting our earlier findings.

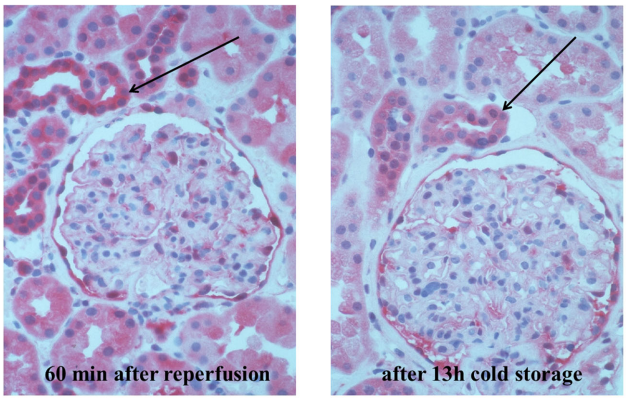

FIGURE 1 | Immunohistochemical demonstration of expression of the inducible heat shock protein 70. The renal biopsy specimen shows a human kidney removed from a brain-dead donor, perfused with the University Wisconsin preservation solution, and cold-stored over 13 h. The biopsy was taken before (right) and 60 min after reperfusion (left) following implantation into the recipient. Demonstration was performed using the monoclonal antibody HSP 6B3 (GSF, non-commercially available), specific for inducible heat shock component HSP 70 (formerly HSP72c) in a dilution of 1:150, at room temperature, for 1 h. A detection system APAAP (alkaline phosphatase–antialkaline phosphatase) from the rat (Fa Dako) is used, by incubation with linking antibody and staining with fast red. Sources: Further properties (e.g., specificity) of the monoclonal antibodies are described in: Milani et al. (2002) and Arbogast et al. (2002). Permission: Reproduced with permission of: Land WG. *Innate Alloimmunity, Part 2. Innate Immunity and Allograft Rejection*, and Başkent University, Pabst Science Publishers, 2011.

ROLE OF HSP70 FAMILY MEMBERS IN ENHANCING ADAPTIVE IMMUNE RESPONSES

In general, extracellular HSPs released from damaged cells are immunogenic and have been shown to enhance TH1 adaptive immune responses and, therefore, have been used for improved vaccination procedures against infectious diseases and cancer (for review, see Binder, 2006). For example, a novel HSP70 family member, termed Hsp70-like protein 1 (Hsp70L1) was recently identified as a potent TH1 polarizing adjuvant that contributes to antitumor immune responses (Fang et al., 2011). In another line of experiments on a fusion cell model (DC and tumor cell fusion), efficient cytotoxic T lymphocyte productivity of modified fusion cells was demonstrated and supposed to be due to an up-regulation of HSP70 (Koide et al., 2009).

Such enhancement of adaptive immune responses by HSP70 is thought to be preferentially mediated by its capacity to promote maturation of immunostimulatory DCs as well as to enhance cross-presentation of peptide antigens, that is, a crucial pathway of exogenous antigen presentation on the HLA class I molecules by DCs, enabling adaptive antiviral or antitumor responses.

In fact, in earlier studies, it was already shown that human HSP70 preparations are able to induce DC maturation (Kuppner et al., 2001; Bethke et al., 2002). In a more recent study on an *in vitro*-induced ischemia model, bone marrow–derived DCs were shown to augment allogeneic T-cell proliferation as well as the interferon-gamma response (Jurewicz et al., 2010). In this study, TLR4 ligation was also shown to occur in ischemic DCs, most likely – as concluded by the authors – in response to HSP70, which was found to be elevated in DCs after ischemic injury.

In addition to contributing to the generation of immunostimulatory DCs in terms of a ligand of TLR2/4, extracellular HSP70 has been shown in models of antitumor T-cell responses to promote the cross-presentation of peptide antigens to MHC class I molecules in DCs, leading to efficient induction of antigen-specific cytotoxic T-lymphocytes (Castelli et al., 2001; Srivastava, 2002; Ueda et al., 2004). Of particular importance for the discussion of a role of cross-presentation in innate alloimmunity are recent studies demonstrating that HSP70 binds HLA class I and class II epitope precursors, which further strengthens the role of HSP70 in the HLA class I and class II antigen presentation process (Stocki et al., 2010). In fact, these studies lent support to a more general characteristic of the HSP70-chaperoned peptides and suggest involvement of HSP70 as a more general phenomenon in the antigen presentation process.

HEAT SHOCK PROTEINS AND ALLOGRAFT SURVIVAL

Most interesting for our early assumption in 2002/2003 that HSP70 may be involved in innate alloimmunity would be the provision of data from experimental studies on allograft models. However, there are only a few such data published so far and, unfortunately, they are even controversial. Thus, it could be shown that targeted gene disruption of the *hsp72* gene in donor tissue is associated with significantly but modestly prolonged rejection-free survival in a murine skin allograft model, the difference between *hsp72*-deficient mice and controls being 3 days only (Oh et al., 2004). Nonetheless, these experimental data suggest that HSP70 in terms of a pro-inflammatory acting DAMP contributes to an innate alloimmune response although only in a moderate way.

On the other hand, data from another experimental allograft model indicate that HSP70 in terms of an anti-inflammatory acting DAMP may even exert a beneficial effect on allograft survival, although again quite modestly (Borges et al., 2010). In this study, *Mycobacterium tuberculosis* HSP70 (TBHSP70) was shown to inhibit allograft rejection in a skin allograft model in which the donor skin was immersed in a PBS solution containing TBHSP70 before transplantation. Interestingly, however, TBHSP70-induced prolongation of skin allograft survival was associated with the demonstration of T regulatory cells (Tregs), a finding supposed by the authors to mediate the graft-prolonging effect. Interestingly, the authors discuss (although not conclusive from their observations) whether or not the Tregs observed in their studies are specific for TBHSP70, by referring to growing evidence in support of the notion that HSPs possess inherent immunoregulatory properties (van Eden et al., 2005).

SYNOPSIS

In my opinion, HSPs, since generated in *association* with any stressful tissue injury, can be called typical damage-*associated* molecular patterns, that is, DAMPs. Their evolutionarily determined, inherent protective role in concert with a large panoply of other molecules in commission of the innate immune system lies in defense against any cell/tissue injury (including elimination of the injurious agent concerned, e.g., viruses, tumor cells).

In regard to innate alloimmunity, it can be concluded from our own experience that the expression of HSP70 is moderately increased in kidneys from brain-dead donors but dramatically up-regulated after IRI to human renal allografts, thus, fulfilling the criteria of a DAMP. No more, no less. However, the exact biological activity of HSP70 in innate alloimmunity remains elusive. Without addressing the "contamination debate" here, one may discuss that, in the excessively inflammatory milieu of a postischemically reperfused allograft, this DAMP – in concert and collaboration with a variety of other DAMPs such as HMGB1 and nucleic acids released from necrotic graft cells – may operate as an immunity-initiating, -promoting, and -facilitating stress protein to allograft rejection [the originally protective role of HSPs here is being converted to a harmful response from a recipient's point of view, caused by a fateful confusion of "invading" foreign antigens (alloantigens) within an injured organ and foreign antigens derived from invading dangerous pathogens]. Potential pro-inflammatory properties of HSP70 may lie in its capacities (1) to act as a "priming" DAMP of the NLRP3 inflammasome contributing to the typical inflammatory environment of a reperfused allograft, (2) to contribute to maturation of donor- and recipient-derived DCs by engagement with TLR4/2, and (3) to intensify re-presentation of allopeptides within donor MHC class II molecules on donor- and recipient-derived DCs as well as promote cross-presentation of allopeptides within donor MHC class I molecules on recipient-derived DCs.

In contrast, in a non-injurious or weakly injurious tissue microenvironment, for example, when HSP70 is added to skin grafts immersed in culture prior to transplantation (Borges et al., 2010), this DAMP may elicit negative regulation mechanisms in graft-containing antigen-presenting cells on the level of TLR signaling. As known, in order to prevent catastrophic host immune overreactions, such negative feedback regulations exist that control the intensity and duration of TLR-triggered innate immune responses – best known as the classic negative regulation in response to LPS, the "LPS or endotoxin tolerance." In fact, molecular feedback inhibitors operating at different levels of TLR-signaling pathways can be divided into three major groups: extracellular regulators such as soluble decoy TLRs, transmembrane protein regulators such as the *suppressor of tumorigenicity 2*, and intracellular negative regulators such *as IL-1 receptor-associated kinase-M* (IRAK-M) and members of the *suppressors of cytokine signaling* (SOCS) family (for review, see Land, 2011d).

For example, the well-known anti-inflammatory effect of preconditioning procedures including heat preconditioning associated with the expression of HSP70 (Jo et al., 2006) has been thought to be due to elicitation of regulatory feedback inhibitors of TLR signaling (for review, see also Karikó et al., 2004). In fact, in earlier studies, it was demonstrated that prior exposure to HSP70, here the *Toxoplasma gondii*-derived HSP70,

induces a hyporesponse in macrophages to subsequent stimulation with this HSP70 by expression of SOCS1 via TLR4 (Mun et al., 2005). Of note, as shown in more recent studies on a model of hepatic IRI, preconditioning using the TLR4 agonist LPS elicited the up-regulation of specific negative regulators including SOCS1 and IRAK-M in the TLR4-signaling pathway (Sano et al., 2011). Impressively, molecules operating in those regulatory feedback loops have been shown to also prevent DC maturation, that is, to promote generation of tolerogenic DCs known to induce Tregs (for reviews, see Maldonado and von Andrian, 2010; Land, 2011c). Thus, data from an experimental model of SOCS1-gene overexpression in bone marrow-derived DCs showed that SOCS1 inhibits DC maturation and induces generation of regulatory DCs, which – via generation of $CD4^+CD25^+$Tregs – resulted in prolongation of allograft survival (Fu et al., 2009). First evidence of a similar feedback inhibiting effect of SOCS2 on TLR-induced activation in human monocyte-derived DCs has also been recently reported (Posselt et al., 2011).

Altogether, the context-dependent, even contradistinctive activities of HSP70 reflect the biological phenomenon that, throughout evolution, mammals have developed an elaborate network of positive and negative regulatory mechanisms, which provide balance between defensive measures against dangerous bacterial and viral pathogens and protective measures against unwarranted destruction of the host by the activated immune system. Fine-tuning of TLR signaling in amplitude, space, time, and character is a key aspect of inflammatory reactions in health, homeostasis, and pathology. What is becoming more and more apparent is that positive and negative regulators within immune responses do not work as a single entity, but rather, similar to an orchestral score, each component is reliant on its other tools such as HSP70 to produce a harmonious melody instead of a crashing cacophony.

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Opposing roles for heat and heat shock proteins in macrophage functions during inflammation: a function of cell activation state?

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Macrophages function both under normothermia and during periods of body temperature elevation (fever). Whether macrophages sense and respond to thermal signals in a manner which regulates their function in a specific manner is still not clear. In this brief review, we highlight recent studies which have analyzed the effects of mild heating on macrophage cytokine production, and summarize thermally sensitive molecular mechanisms, such as heat shock protein (HSP) expression, which have been identified. Mild, physiologically achievable, hyperthermia has been shown to have *both pro- and anti-inflammatory effects on macrophage inflammatory cytokine production* and overall it is not clear how hyperthermia or HSPs can exert opposing roles on macrophage function. We propose here that the stage of activation of macrophages predicts how they respond to mild heating and the specific manner in which HSPs function. Continuing research in this area is needed which will help us to better understand the immunological role of body temperature shifts. Such studies could provide a scientific basis for the use of heat in treatment of inflammatory diseases.

Keywords: inflammation, hyperthermia, heat shock protein, fever, heat shock factor, cytokines, arthritis

INTRODUCTION

Inflammation is usually considered a beneficial event which helps to heighten the immune response initiated following tissue injury or infection. Molecular alarm signals generated from the damaged tissues or invading pathogens are recognized by antigen-presenting cells (APCs) such as macrophages. Macrophages are key players in innate immunity and respond rapidly to danger signals generated from inflamed sites. Recognition of these danger signals through specific cell surface pattern recognition receptors (PRRs) on macrophages results in the production of anti-microbial products as well as pro-inflammatory mediators (Depraetere, 2000; Fujiwara and Kobayashi, 2005).

Inflammation is a tightly regulated process with production of alarm signals sharply diminishing after the pathogens or cellular debris have been eliminated and tissue homeostasis restored. Many "stop signals" at appropriate checkpoints prevent further leukocyte infiltration into tissues if they are no longer needed (Serhan et al., 2007). If the balance between inflammation and resolution becomes dysregulated, excess macrophage responses will lead to chronic inflammation, and resultant disease states, which include atherosclerosis, rheumatoid arthritis (RA), asthma (Nathan, 2002), and cancer (Grivennikov et al., 2010). Therefore, understanding the regulation of inflammation and its resolution could help in the development of new therapeutic approaches for inflammatory disease management.

Macrophages are optimally activated by a combination of two macromolecular signals in their environment: antigen recognition by PRPs and IFN-γ (Mosser, 2003; O'Shea and Murray, 2008) and produce pro-inflammatory cytokines, TNF-α, IL-6, and IL-1β. These pro-inflammatory cytokines are important pyrogens capable of inducing the formation of the fever response and elevation of body temperature through complex neurological and behavioral changes (Kluger, 1991a,b; Cooper et al., 1994; Leon et al., 1997; Romanovsky et al., 1998, 2005). Fever is a highly conserved phenomenon in evolution (Kluger, 1979a, 1986; Mackowiak, 1981; Hasday et al., 2000). Although numerous human and animal studies reveal a positive correlation between the febrile response and a better survival rate, the mechanism by which increased core temperature improves host defense is still not clear. In addition, new studies suggest additional levels of complex interaction between macrophage function and body temperature. Nguyen et al. (2011) have shown that exposure to a cold environment induces alternative activation of adipose tissue macrophages. Thus, while it is likely that their function is responsive to thermal signals, the specific mechanisms by which macrophages sense and respond to temperature change in a way that specifically regulates their function has not been clarified.

To investigate the effect of febrile temperatures on macrophage activity, researchers often simply increase temperature in cell cultures, or elevate body temperature by external heat application in murine models. While these procedures do not reproduce critical features of actual fevers, several studies reveal both *positive* (Jiang et al., 2000b; Ostberg et al., 2000; Lee et al., 2012) and *negative* (Ensor et al., 1994; Fairchild et al., 2000; Hagiwara et al., 2007) effects on macrophage pro-inflammatory cytokine production. Both positive and negative effects of hyperthermia

have been correlated with an induction of heat shock proteins (HSPs). The overall aim of this brief review is to present previous studies that have studied the impact of hyperthermia and HSPs on macrophage functions and to summarize available information regarding the underlying molecular mechanisms which may mediate this complex interaction of thermal signals.

FEVER-RANGE TEMPERATURES CAN ENHANCE MACROPHAGE PRO-INFLAMMATORY CYTOKINE PRODUCTION

Inducing hyperthermia has been used to study the role of febrile temperatures on the host defense system (Hasday et al., 2000; Jiang et al., 2000a). It was shown that increasing core body temperature has a protective role in the outcome of infection; the improved survival seen was determined not to be simply due to thermal suppression of bacterial growth. Data published by our laboratory (Ostberg et al., 2000) as well as by others (Jiang et al., 1999b) shows that mild, systemic heating at a target temperature of 39.5°C significantly enhances the concentration of TNF-α (3-fold increase in sera; 2.5-fold increase in liver) and IL-6 (4-fold increase in sera; 2.6-fold increase in spleen, 3.4-fold in lung, and 15-fold in liver) in the blood and tissues of BALB/c mice challenged with bacteria endotoxin (LPS). In addition, Jiang et al. (1999a) identified hepatic Kupffer cells as the predominant source of excess TNF-α secretion while multiple organs including lung, spleen, and liver could produce excess IL-6 in the warmer animals. These temperature-induced changes in cytokine expression are associated with an induction of HSP72 in the liver.

Our laboratory has recently observed that mild elevation of body temperature not only significantly enhances subsequent LPS-induced release of TNF-α (3-fold increase), but also reprograms macrophages, resulting in sustained subsequent responsiveness to LPS, i.e., this treatment reduces "endotoxin tolerance" *in vitro* and *in vivo* (Lee et al., 2012). Heat treatment results in an increase in LPS-induced downstream signaling including enhanced phosphorylation of IκB kinase (IKK) and IκB, NF-κB nuclear translocation and binding to the TNF-α promoter in macrophages upon secondary stimulation. The induction of HSP70 is important for mediation of thermal effects on macrophage function (Lee et al., 2012). Our *in vitro* experiments also show that the production of nitric oxide (NO) and inducible NO synthase (iNOS) by peritoneal macrophages is increased by exposure to febrile temperature together with LPS and IFN-γ stimulation. This result is correlated with the presence of HSP70 in the heat-treated macrophages (Pritchard et al., 2005). Collectively, these data suggest that fever-range hyperthermia can enhance macrophage cytokine expression and HSP70 expression, which in turn may help to improve host defense in response to infection.

FEVER-RANGE TEMPERATURES CAN ALSO SUPPRESS MACROPHAGE PRO-INFLAMMATORY CYTOKINE PRODUCTION

In contrast to the research summarized above, other studies using the macrophage cell lines RAW264.7 or human monocyte-derived macrophages have shown that hyperthermia has anti-inflammatory effects and suppresses activated macrophage pro-inflammatory cytokine expression (TNF-α, reduced by 50–98%; IL-6, reduced by 83–87%; and IL-1β, reduced by 50–94%; Ensor et al., 1994; Fairchild et al., 2000; Hagiwara et al., 2007). This inhibition is linked to a marked reduction in cytokine gene transcription and mRNA stability (Ensor et al., 1995). In addition, this thermally suppressed cytokine production is mediated by the binding of heat shock factor (HSF)-1, a transcriptional repressor, to the heat shock response element in the cytokine promoter region, including IL-1β and TNF-α (Cahill et al., 1996; Singh et al., 2002). Recently, Cooper et al. (2010a,b) have shown that fever-range temperatures selectively reduce LPS-induced recruitment of NF-κB and Sp-1 transcription factors to the TNF-α promoter regions.

Heat-induced suppression may involve high mobility group box 1 (HMGB1), an intra-nuclear protein that can be released by activated macrophages, necrotic or damaged cells during inflammation. HMGB1 helps immune cells to recognize damaged tissues and initiates intracellular signaling to activate NF-κB and pro-inflammatory cytokine production (Fiuza et al., 2003). Hyperthermia has been shown to inhibit macrophage HMGB1 secretion following LPS stimulation (Fairchild et al., 2000) and Hagiwara et al. (2007) found that high fever temperature (40°C) enhances HSF-1 and HSP70 expression. These increased levels of HSF-1 and HSP70 may reduce HMGB1 secretion and subsequent NF-κB activation and cytokine production. In general, these data support the concept that febrile temperatures can also have inhibitory effects on macrophage cytokine gene expression, an effect correlated with the activation of HSF-1 or induction of HSP70.

REGULATION OF HEAT SHOCK PROTEINS

Heat shock proteins are evolutionarily conserved proteins that can be induced by stress signals, including environmental stresses (e.g., heat shock), and pathophysiological states (e.g., fever, inflammation, and infection) as well as those induced by normal development stresses (Morimoto, 1993, 1998; Wu, 1995).

Heat shock proteins function as chaperones to assist with protein folding in order to protect cells from protein denaturation or cell death under stress conditions (Fink, 1999; Jaattela, 1999). Although HSPs are considered to be intracellular proteins, they can be mobilized to the plasma membrane or released into the extracellular environment and have immunomodulatory functions (Johnson and Fleshner, 2006). HSPs (e.g., HSP60, HSP70, HSP90, gp96, etc.) can be released from various cells through either a passive (during cell injury) or an active (translocation to the plasma membrane and then secretion) pathway. Previous studies have shown that HSP70 is released into the extracellular environment in a membrane-associated form after heat stress (Multhoff, 2007; Vega et al., 2008). HSPs are known to have both positive and negative effects in regulating macrophage function *and this may depend on the cellular location of these HSPs.* It is proposed that extracellular HSPs might serve as a danger signal to stimulate the immune response, whereas intracellular HSPs could serve as a negative regulator to control the inflammation (Schmitt et al., 2007).

PRO-INFLAMMATORY ROLE OF HEAT SHOCK PROTEINS ON MACROPHAGE FUNCTION

Previous studies have shown that extracellular HSPs exert immunostimulatory effects (Johnson and Fleshner, 2006). Wang et al. (2006) have shown that extracellular HSP70 binds to the lipid raft microdomain on the plasma membrane of macrophages and enhances their phagocytic ability. This HSP70-mediated phagocytosis enhances the processing and presentation of internalized antigens to CD4 T cells. Furthermore, extracellular HSPs can robustly stimulate the release of TNF-α, IL-6, IL-1β, IL-12, NO, as well as chemokines by monocytes/macrophages (Lehner et al., 2000; Asea et al., 2002; Panjwani et al., 2002; Vega et al., 2008). This effect is mediated through the CD14/TLR (both TLR2 and TLR4) complexes which lead to the activation of downstream NF-κB and MAPK pathway (Asea et al., 2000; Kol et al., 2000; Vabulas et al., 2002). In addition, HSPs are actively synthesized in peritoneal macrophages in mice which are administrated with LPS (Zhang et al., 1994). HSP70 and HSP90 have been shown to be involved in the innate recognition of bacterial products. These HSPs are able to bind LPS and form a cluster with TLR4–MD2 within lipid raft to deliver LPS to the complex. Following stimulation, these HSPs further assist the trafficking and targeting of this complex to the Golgi apparatus (Triantafilou et al., 2001; Triantafilou and Triantafilou, 2004). These results indicate that elevation of extracellular HSPs may serve as endogenous danger signals to alert the host defense system through their cytokine-like function.

ANTI-INFLAMMATORY ROLE OF HEAT SHOCK PROTEINS ON MACROPHAGE CYTOKINE EXPRESSION

On the other hand, intracellular HSPs have been shown to have anti-inflammatory roles in suppressing macrophage cytokine production. Intracellular HSPs are involved in protecting the organism from a variety of insults by directly interfering with cell death pathway and suppressing the expression of inflammatory genes (Yenari et al., 2005). The protect roles of stress-inducible intracellular HSPs (such as HSP72) in lethal sepsis and infection are well known. Induction of HSP70 *in vitro* by heat shock response or through overexpression can reduce mortality in experimental models of septic shock and endotoxemia as well as down-regulate inflammatory gene expression (Snyder et al., 1992; Hotchkiss et al., 1993; Villar et al., 1994; Van Molle et al., 2002; Shi et al., 2006). It has been shown that intracellular HSP70 can interact with IKK, prevent IκB phosphorylation, and NF-κB activation (Ran et al., 2004). In addition, HSP70 is actively synthesized in macrophages after exposure to endotoxin. Intracellular HSP70 inhibits LPS-induced NF-κB activation by binding with TRAF6, the important adaptor protein downstream of TLR4 and preventing its ubiquitination (Chen et al., 2006). These results suggest that intracellular HSP70 may act as a suppressor to interfere NF-κB signaling and downstream inflammatory cytokine production. Furthermore, HSP72 has been shown to inhibit LPS- and TNF-α-induced HMGB1 release and subsequent pro-inflammatory cytokine production in macrophages (Tang et al., 2007).

On the other hand, there is only one study by Bouchama et al. (2000) showing that hyperthermia (using target temperature 39, 41, and 43°C) inhibits LPS-stimulated IL-10 production by mononuclear cells isolated from healthy donor as compared to cells maintained at 43°C. In addition, by over-expressing HSP70 in human monocyte-derived macrophages, Ding et al. (2001) found that LPS-induced IL-10 production was significantly inhibited. In our unpublished data, we tried to address whether fever-range temperature affects macrophage IL-10 production at different activation stages. However, we found that temperature did not have any effect on IL-10 expression by either naïve macrophages or previously activated macrophages. Taken together, these studies suggest that intracellular HSPs may exert negative regulatory effects to dampen the inflammatory response in order to prevent tissue damage.

HEAT SHOCK PROTEINS AND INFLAMMATORY DISORDERS

There is some evidence that HSPs may play a role as immunological targets in chronic inflammatory diseases, such as RA (van Eden et al., 2005) and atherosclerosis (Xu, 2002). Increased HSP60 and HSP70 expression have been identified in the synovial tissues from animals with experimental induced arthritis and patients with RA (de Graeff-Meeder et al., 1990; Boog et al., 1992; Martin et al., 2003). Importantly, arthritis disease severity depends on the ability of human HSP60 and HSP70 to induce IL-10 production by monocytes/macrophages and fibroblast-like synoviocytes (MacHt et al., 2000; Luo et al., 2008). The production of IL-10 is negatively correlated with arthritis progression and joint destruction (Isomaki et al., 1996; Tanaka et al., 1996). Several clinical trials in RA patients using immunotherapies involving HSPs have shown to promote anti-inflammatory cytokine production and ameliorate disease severity, indicating that HSPs have immunoregulatory potential (Vischer, 1990; Rosenthal et al., 1991; Prakken et al., 2004).

On the other hand, HSP60 and HSP70 are also found in atherosclerotic lesions (Berberian et al., 1990; Kleindienst et al., 1993; Johnson et al., 1995). During the past decade, it has been noted that infections (e.g., chlamydiae infection) might contribute to the pathogenesis of atherosclerosis to directly stimulate cells of the arterial wall and/or other tissues to express high levels of HSPs (Kiechl et al., 2001). Chlamydial HSP60 (cHSP60) colocalizes with human HSP60 within macrophages in atherosclerotic lesions (Kol et al., 1998). Both chlamydial and human HSPs stimulate the expression of pro-inflammatory cytokines and adhesion molecules by macrophages through TLR4/MD2-dependent pathway (Bulut et al., 2002). Because of the high homology between chlamydial and human HSPs, it is possible that cross-reactions of antibodies and T cells against HSPs between microbes and humans contribute to the development of atherosclerosis (Xu, 2002). Taken together, a better understanding of the role of HSPs in the inflammatory process may lead to the development of new therapies to modify aberrant immune responses.

DOES THE MATURATION STATE OF MACROPHAGES INFLUENCE THE EFFECT OF HEAT AND HEAT SHOCK PROTEINS?

In this paper, we described previous studies revealing contrasting effects of febrile temperatures and HSPs on macrophage functions. Mild hyperthermia *enhances* macrophage TNF-α IL-6 as well as NO production in a HSP70/72-dependent mechanism.

On the other hand, mild hyperthermia *suppresses* macrophage pro-inflammatory cytokine production and this may involve an increase in HSF-1 and HSP70 expression. Thermally induced HSF-1 and HSP70 also play a role in blocking the transcription factors NF-κB and Sp-1 recruitment to the cytokine promoter and subsequent cytokine secretion (see **Figure 1**, for possible pathways by which thermal stress may result in both pro- and anti-inflammatory effects).

One possible explanation for these opposing results is that the resultant effect of heat is determined by the specific activation state of macrophages used for the analysis. For example, enhancing effects of heating were observed using cells freshly isolated from mice early after LPS injection (Jiang et al., 1999a,b; Lee et al., 2012). These cells express a naïve phenotype. On the other hand, the suppressing effects were obtained using RAW264.7 cells which express an activated phenotype (Ensor et al., 1994; Fairchild et al., 2000; Hagiwara et al., 2007). Therefore, we propose that in cells associated with the early activation stage of inflammation, febrile temperature may stimulate macrophage cytokine production, which helps to eliminate invading pathogens, whereas if heat is applied to cells associated with the resolution phase, a suppression of macrophage cytokine production occurs to prevent further tissue damage. This idea, if correct, would suggest that one of the functions of natural fever may be to help resolve inflammation. Furthermore, to our knowledge, there is no previous study addressing the role of heat/HSP on alternatively activated macrophage functions. This indicates that more research is needed to identify the mechanistic

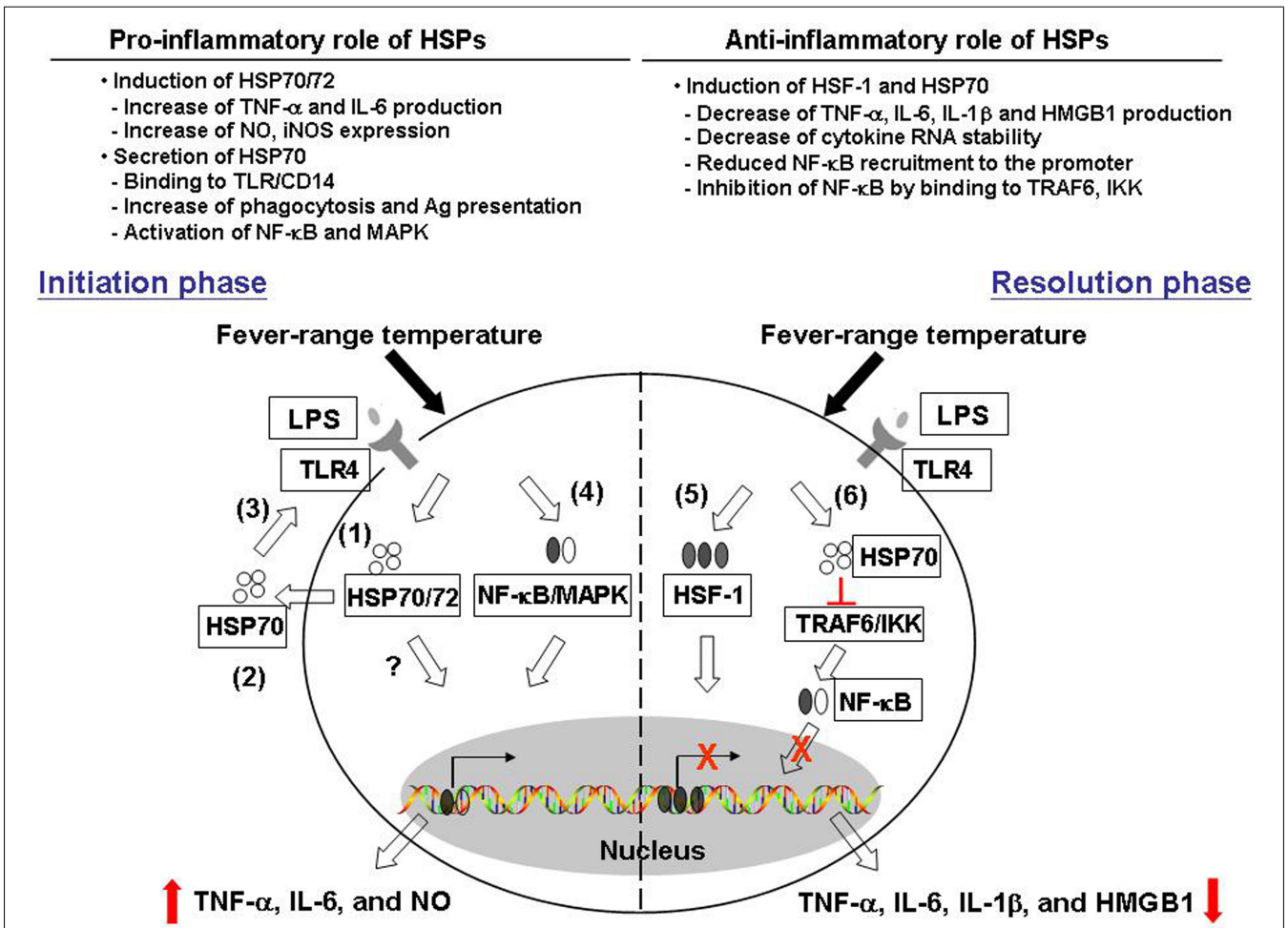

FIGURE 1 | Pro- and anti-inflammatory effects of heat and heat shock proteins. Fever range temperature exerts both positive and negative effects on macrophage pro-inflammatory cytokine production. We proposed that these opposing effects may depend on the activation stage of macrophages as well as the cellular location of heat shock proteins. First, in the initiation phase of macrophage activation, fever range temperature enhances the production of LPS-induced pro-inflammatory cytokines TNF-α, IL-6, and NO. (1) This thermally enhanced cytokine production is associated with an induction of HSP70/72 in these cells. (2) HSP70 can then be released into the extracellular environment, and (3) exerts its cytokine effect through the CD14/TLR pathway. (4) This leads to the activation of NF-κB and MAPK pathway and subsequent cytokine gene transcription. On the other hand, fever-range temperature can also suppress LPS-induced TNF-α, IL-6, and IL-1β production and HMGB-1 release. (5) This is due to thermally induced HSF-1 binding directly to the heat shock response element in the cytokine promoter region and functions as a transcriptional repressor. (6) In addition, LPS and heat treatment can also induce HSP70 expression. HSP70 then binds to TRAF6 or IKK and inhibits subsequent NF-κB activation and cytokine production.

basis of the contrasting effects of hyperthermia on macrophage function.

CONCLUSION

The host inflammatory response to injury or infection is clearly a complex process. Pro-inflammatory cytokines secreted by activated macrophages are essential for successful host defense. However, uncontrolled inflammatory cytokine production will also cause tissue damage. The febrile response is a well recognized component of inflammation and has been shown to provide a beneficial effect for the host but whether the thermal element of fever has a direct role in regulating macrophage function is still not clear. By using hyperthermia, many studies have reported that febrile temperatures can regulate macrophage pro-inflammatory cytokine production both *in vitro* and *in vivo*. These effects are associated with the induction of HSP. However, these effects can be either positive or negative and much more research is needed to understand how these two contrasting effects coincide.

In addition, we also summarized research which shows both the pro- and anti-inflammatory roles of HSPs. Extracellular HSPs might serve as a danger signal to stimulate the immune response, whereas intracellular HSPs could serve as a negative regulator to control the inflammation. Studies of the roles and mechanisms of heat as well as HSPs in inflammatory disorders could provide more information for therapeutic strategies targeting the molecular levels.

Finally, it is important to remember that most of the research on the role of elevated temperature on macrophage function does not actually use a physiological fever (i.e., induced by endoxin or other pyrogens), but instead, simply forces the elevation of body temperature using external heating. While some studies described above do include the addition of LPS, or use heat in the presence of infectious antigens, fever involves many biochemical, neurological, and vascular changes not seen in conditions of forced hyperthermia (Kluger, 1979b, 1991a; Gordon, 1993; Romanovsky et al., 1998, 2005). Therefore, it is possible that the elevated temperature associated with actual fever may have additional and more complex regulatory effects on macrophage function.

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