Chapter 4
The Heat Shock Protein-CD91 Pathway and Tumor Immunosurveillance

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Abstract The intracellular functions of HSPs have been well studied and delineate a clear role in the unfolded protein response. The functions of extracellular HSPs are only beginning to be appreciated. Specifically, extracellular localization of HSPs endorses the initiation of immune responses against aberrant cells. This chapter examines the role of extracellular HSPs, and the receptor CD91, in immunosurveillance of cancers. Although the concept of cancer immunosurveillance was described over 100 years ago, a molecular description of how the immune responses is initiated has been lacking. Incorporating the HSP-CD91 pathway into cancer immunosurveillance provides the first mechanism of how immune responses are primed.

Keywords Dendritic cell · Chaperone · Tumor immunity · T regs

4.1 Heat Shock Proteins as Chaperones of Macromolecules

Heat shock proteins have long been known for their function as chaperones within cells, where they assist proteins and polypeptides fold into their native, most stable configurations [1, 2]. Many HSPs are inducible by cellular stress [1], a condition where there is a heightened requirement for chaperone function. However, several other HSPs are constitutively expressed. Recently, the chaperone function of HSPs has been shown to be required for transport of other macromolecules. These macromolecules include peptides derived from homeostatic protein turnover [3–9]. This latter function has been implicated in several immunological processes and pathways. For example, peptides in the MHC I processing and presentation pathway are shuttled by HSPs in the cytosol and endoplasmic reticulum [4–6]. Although the normal expression pattern of HSPs is solely intracellular, under certain pathological conditions, HSPs can be found in the extracellular environment, free as a diffusible

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soluble protein [10, 11], as part of the extracellular matrix [11] or on the membrane of cells [12]. Infection by pathogens, hostile cancer microenvironments, and inflammation associated with these events, very frequently, if not always, include cell death which leads to passive release of these abundant chaperones [10]. As described below, the chaperone function of HSPs is critical to its role in the immune system.

4.2 Immune Responses Elicited by Extracellular HSPs

In the extracellular environment, select HSPs have been shown to elicit immune responses of diverse nature [13–25]. This remarkable property was first observed when gp96 was isolated as the immunogenic entity of tumor cells [13]. In that pioneering study, when mice where immunized with gp96 preparations derived from a tumor, they became resistant to a subsequent challenge of that tumor. This phenomenon has been replicated for hsp70 [14], hsp90 [15], calreticulin [16], grp170 [17], and hsp110 [17], the major chaperones of cells. HSPs chaperone peptides [3–9], and when isolated from tumor cells, the peptide repertoire includes tumor antigens [8, 9, 13–17]. In other words, purified HSP-peptide complexes represent the antigenic fingerprint of the cell from which they are isolated. This has been empirically tested. In several antigenically defined systems, HSPs have been shown to be associated with antigens that ultimately get presented by MHC I and MHC II molecules thereby dictating T cell specificities of the immune response. These systems include HSPs isolated from tumors [8, 9, 26–28], infected cells [29–34], allo-MHC cells [35, 36], and cells expressing model antigens [35–38]. In studies where crystal structures of HSPs have been resolved, peptide binding pockets have been clearly identified [39–41]. Over two decades of work has elucidated the major immunological mechanisms through which HSPs prime immune responses. These mechanisms are dependent on the ability of HSP to bind cell surface receptors on antigen-presenting cells (APC) [42]. In the extracellular environment, HSPs engage a cell surface receptor, CD91, which is expressed by most APCs [20, 43–55]. On conventional dendritic cells, CD91 acts as an endocytic receptor to internalize HSP-peptide complexes [43–46]. Several other cell surface receptors for the immunogenic HSPs have been proposed and are discussed elsewhere [42]. Following CD91-dependent endocytosis, the HSP-peptide complexes are processed, and the peptides enter the pathways for antigen presentation for MHC I [43, 44, 47] or MHC II [45, 48] of the APC. CD91 also acts as a signaling receptor [20]. Upon engagement by HSPs, various signaling and transcription factors are activated following phosphorylation of the CD91 cytoplasmic chain, leading to production and secretion of cytokines and upregulation of co-stimulatory molecules [10, 20, 51]. On conventional dendritic cells, the signaling pathways and outcomes are responsible for and supportive of Th1 responses and subsequent HSP-mediated rejection of tumors and pathogens following vaccination. Interestingly CD91 is expressed by hematopoietic cells of both myeloid and lymphoid origin including macrophages and a variety of DC subsets [52–55]. When HSPs are in the extracellular environment, HSPs can engage

CD91 on any cell in that microenvironment or can drain to lymph nodes and engage (additional) cells at this distal site [52]. Using fluorescent tags, HSPs were shown to engage cDCs in vivo at doses capable of priming Th1 responses [52]. However, increasing amounts of HSPs will engage additional cells, including pDCs [53, 91]. The exact phenotype of the immunological responses is determined by the $CD91^+$ APC engaged by the extracellular HSP. For example, pDCs engage extracellular HSPs but do not cross-present HSP-chaperoned peptides nor upregulate B7 or CD40 [53], promoting an immune-regulatory phenotype characterized by T reg [91]. These responses have been harnessed for immunotherapy of autoimmune disease and amelioration of tissue allograft acceptance [21–23]. Engagement of cDCs by the same HSPs promote Th1 response that reject tumors [43, 44, 47, 52]. The influence of other tumor-secreted molecules, besides HSPs, in the immediate microenvironment potentially also plays a role in the resulting immune response [20]. Molecules like HMGB1 [56], dsDNA [57], and cytokines [20] have been shown to be immunologically important and could complement or antagonize the responses emanating from the HSP-APC interaction. For example, tumor-secreted TGF-β synergizes with HSP/CD91-dependent IL-6 and TNF-α released from APCs to prime Th17 responses [20]. The resulting immunological response elicited by extracellular HSPs will be dependent on the influence of local APCs on cross-priming by cDCs in the draining lymph node. Many of these mechanisms, while demonstrated in murine models, also hold true in the human setting [58, 59].

4.3 Extracellular HSPs as the Molecular Signature for Immunological Responsiveness

A majority of the findings described above have been performed in a vaccination setting where purified HSPs are administered to rodents or humans [13–19, 21–24]. However in studies examining HSPs released from cells in situ, the same stimulation of APCs can be observed [52, 60]. Under pathological conditions and cell death, HSPs are released from cells and delivered to the extracellular environment [10–12]. Mechanisms of active secretion of HSPs have also been put forward to explain the extracellular presence of HSPs, but these are not fully elucidated [61]. Since HSPs contain no consensus sequences for such cellular trafficking and secretion, it is hard to conceive the cell biology comprising such a pathway, especially for the cytosolic HSPs. Thus, a passive release mechanism, when membrane integrity is compromised, appears more likely. Examples of these pathological conditions leading to HSP release include cellular infection by bacteria and viruses, cancer, trauma, and associated inflammation. Collectively, HSPs are the most abundant proteins in cells accounting for >5% of the proteome [1]. Thus, they are ideal indicators to the immune system of cellular aberrancy. There are now six key HSPs known to be rapidly recognized by the APCs [13–17] via cell surface receptor(s). The surprising discovery of the HSP receptor expressed on APCs afforded a molecular

description of these immunological mechanisms [43]. Since the receptor(s) offers a significant degree of specificity for recognition of intracellular content, they become key players in the immune system, allowing HSPs to be critical initiators *and* mediators of resulting immune responses. Following the initiation of antigen-specific immune responses against cancers or pathogen-infected cells, extracellular HSPs exacerbate existing inflammatory conditions or suppress ongoing immunity [60]. There is currently a well-developed picture on the cross-presentation of HSP-chaperoned peptides to which T cells are primed and pathways which lead to the release of cytokines, including the pro-inflammatory IL-1, IL-6, and TNF-α [10, 20]. Thus, extracellular HSPs have been implicated in the etiology, progression, and/or resolution of several diseases including cancer and rheumatoid arthritis [60, 62, 63]. In rheumatoid arthritis, the presence of extracellular hsp70 and gp96 in synovial fluid of inflamed joints has been shown to stimulate local APCs which release pro-inflammatory cytokines. These events constitute a cycle of tissue destruction, increased release of HSPs, and increased inflammation [62, 63]. Recognition of endogenous molecules (HSPs) by their respective receptors can be compared on many levels to the recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) [64].

4.4 The Role of Extracellular HSPs in Tumor Immunosurveillance

The original concept of immunosurveillance was that the immune system recognized aberrant cells and eliminated them before progression to cancer occurred [65–67]. We now know that priming of T cell and NK cell immunity is necessary for rejection of aberrant cells. In the absence of such immunity in mice [68, 69] or in humans [70], achieved by the loss of these immune cells themselves or their effector molecules, multiple and frequent tumors arise. The tumors that arise under these immune-compromised conditions are less edited compared to tumors from wild-type mice [68, 71]. The literature, however, until recently, failed to reconcile two issues. The first pertains to the miniscule amount of antigen available for priming T cell responses at the very earliest stages of nascent tumor development [72, 73]. The realization that most tumor *rejection* antigens are unique and derived from mutated proteins [74–77] predicts that antigen levels in (emerging) tumors (and the quantity available for cross-presentation) are minute and, as a soluble protein, have indeed been shown to be insufficient for cross-priming of T cell responses [72, 73]. Yet, T cell responses are easily measurable at these early time points of tumorigenesis (e.g. [78, 79]). Mechanisms of antigen transfer and cross-presentation described for other systems [80–87] where antigen is abundant or supraphysiological are not justifiable for nascent emerging tumors. Thus, a super-efficient mechanism must exist for antigen cross-presentation in this setting [88]. Experimental evidence shows that these quantitative restrictions are satisfied if one invokes the HSP-peptide complexes

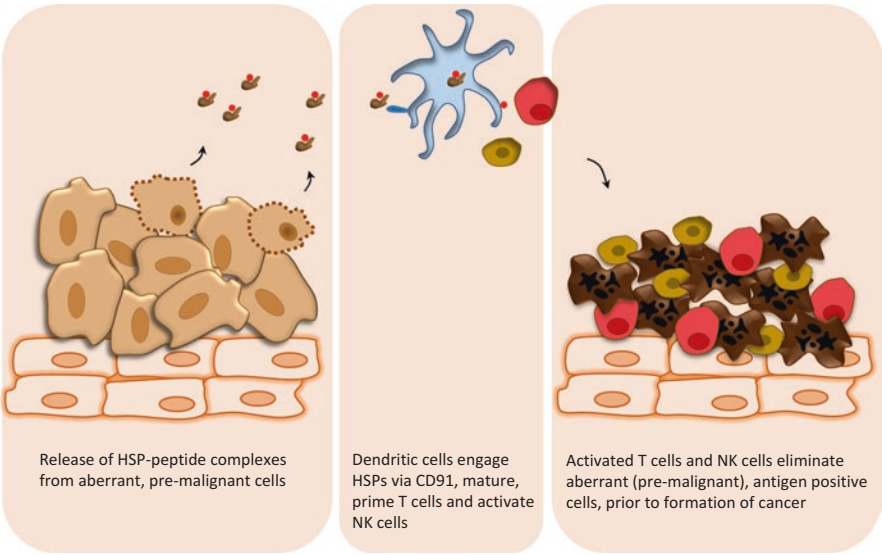

Fig. 4.1 HSPs prime immune responses responsible for eradication of premalignant cells. HSPs released from aberrant, membrane-compromised cells engage dendritic cells in the draining lymph nodes via the receptor CD91. Dendritic cells mature and cross-present HSP-chaperoned antigens to T cells. T cells are primed and NK cells are activated by these DCs. Activated effector cells eliminate aberrant, premalignant cells prior to formation of cancer

released by tumor cells as a mechanism of antigen transfer [60, 88]. When tumor antigen levels are low, peptides derived from tumor antigens and chaperoned by HSP are efficiently cross-presented by APCs, a system that is dependent on CD91 expressed on APCs [43, 44]. One microgram of total immunogenic HSP (an amount that will be present in <10,000 cells) will chaperone approximately a nanogram of a specific antigenic/mutated peptide. This amount of antigen is sufficient for cross-priming only when chaperoned by the HSP (Fig. 4.1).

The second issue relates to the stimuli in the setting of nascent, emerging tumors that results in co-stimulation for T cell priming. Over the millennia, the immune system has evolved to recognize PAMPs associated with pathogens but are necessarily absent in the host [64]. PAMPs generate co-stimulation and cytokines for T cell priming through well-defined pathways. Nascent tumors lack PAMPs and so will be unable to elicit co-stimulation via PRRs. Interestingly, a very short list of molecules of *host* origin, typically called DAMPs, can do so [10, 20, 56, 57]. HSPs are the prototypical DAMPs, the first group of host molecules found to stimulate DCs to release cytokines, upregulate co-stimulatory molecule expression [10], and prime immune responses [13]. The HSP/DAMP receptor, CD91, channels intracellular signals to achieve this, and the co-stimulation provided by APCs has been well defined [10, 20]. Thus, tumor-derived HSP-peptide complexes are a single entity with the capacity of priming robust antigen-specific T cell responses, without the requirement of additional adjuvanticity or antigen.

HSPs have been known to require NK cell activity for effective antitumor immunity [89]. Immunization with tumor-derived HSPs does not lead to tumor rejection in mice devoid of NK cells. NK cell activity in mice immunized with HSPs has recently been examined and showed that HSPs activate NK cells indirectly via the stimulated DC. NK cells are preferentially required for their helper rather than their cytotoxic function [92]. Thus, HSPs have the capacity of priming T cell and NK cell activity which coordinately and cooperatively reject established or nascent emerging tumors. We present a new picture of tumor immunosurveillance, one that has the HSP-CD91 pathway at the center of cross-priming T cell and activation of NK cell responses (Fig. 4.1).

The requirement for T cells or NK cells in tumor immunosurveillance has been shown by their selective deficiency which effectively renders the host susceptible to multiple and frequent cancers as they are unable to eliminate nascent, emerging tumor cells [68, 69]. One would therefore predict that deficiencies in HSPs, CD91, or components of this pathway would similarly abrogate T and NK cell immunity and lead to enhancement of tumor growth. Several of these aspects have been tested empirically to date. In genetically engineered mice with selective deficiency of CD91 in APCs, HSPs are unable to cross-present chaperoned peptides and stimulate co-stimulation [60]. These mice thus fail to mount tumor-specific T cells and control tumor growth. The immunogenic HSPs play redundant roles in cross-priming, and since their collective deletion in mice is not feasible, the alternative experiment with deficiencies in HSPs is technically challenging. However, when HSPs are collectively deleted in tumor cell lysates, the resulting lysates are incapable of priming tumor-specific immunity, even though they contain soluble tumor antigen [72]. These results cumulatively point to the HSP-CD91 pathway as essential for priming immune responses against tumors and for tumor immunosurveillance. While other DAMPs such as HMGB1 and dsDNA may contribute additional cytokines or co-stimulation through APCs, they do not appear to be essential for tumor immunity, but may influence ongoing responses.

4.5 Conclusion

Defining the role of tumor-derived HSPs and CD91 in tumor immunosurveillance is still in its infancy, but the current experimental evidence supporting this premise is significant. There is now an original molecular mechanism as to how immune response, constituting CTL and NK cell activity, is initiated against a nascent, emerging tumor and how this leads to rejection of tumors. The evidence supporting this model also fulfils the quantitative restrictions defined by the scarcity of the tumor antigens. In a tumor microenvironment, with release of multiple HSPs and in the presence of several different APC populations, the immune response is fluid but can be of the Th1 type for tumor rejection. This response may also be fine-tuned by other factors such as additional DAMPs or molecules associated with DNA damage

[90]. The clinical implications of HSP-mediated immunogenicity are currently being investigated.

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