Immunology
The Journal of cells, molecules, systems and technologies

British Society for immunology

IMMUNOLOGY REVIEW ARTICLE

Heat-shock proteins as dendritic cell-targeting vaccines – getting warmer

Shaun McNulty,[1] Camilo A. Colaco,[1] Lucy E. Blandford,[1] Christopher R. Bailey,[1] Selene Baschieri[2] and Stephen Todryk[3]

[1]*ImmunoBiology Ltd., Babraham Research Campus, Babraham, Cambridge, UK,* [2]*UT-BIORAD-FARM, ENEA C.R. Casaccia, Rome, Italy and* [3]*Faculty of Health and Life Sciences, Northumbria University, Newcastle Upon Tyne, UK*

doi:10.1111/imm.12104
Received 18 January 2013; revised 08 February 2013; accepted 15 February 2013.
Correspondence: Dr S. McNulty, ImmunoBiology Ltd., Babraham Research Campus, Babraham, Cambridge, CB22 3AT, UK.
Email: shaun.mcnulty@immbio.com
Senior author: Dr S. McNulty

Summary

Heat-shock proteins (hsp) provide a natural link between innate and adaptive immune responses by combining the ideal properties of antigen carriage (chaperoning), targeting and activation of antigen-presenting cells (APC), including dendritic cells (DC). Targeting is achieved through binding of hsp to distinct cell surface receptors and is followed by antigen internalization, processing and presentation. An improved understanding of the interaction of hsp with DC has driven the development of numerous hsp-containing vaccines, designed to deliver antigens directly to DC. Studies in mice have shown that for cancers, such vaccines generate impressive immune responses and protection from tumour challenge. However, translation to human use, as for many experimental immunotherapies, has been slow partly because of the need to perform trials in patients with advanced cancers, where demonstration of efficacy is challenging. Recently, the properties of hsp have been used for development of prophylactic vaccines against infectious diseases including tuberculosis and meningitis. These hsp-based vaccines, in the form of pathogen-derived hsp–antigen complexes, or recombinant hsp combined with selected antigens *in vitro*, offer an innovative approach against challenging diseases where broad antigen coverage is critical.

Keywords: Cancer; dendritic cells; heat-shock proteins; infectious disease; vaccines.

Introduction

Seminal studies published by the groups of Steinman and others showed that dendritic cells (DC) function as the key professional antigen-presenting cells (APC), providing a link between the innate and adaptive arms of the immune system.[1] Dendritic cells are central to the generation of adaptive immunity, continuously sampling their vicinity for antigens against which the body might need to react, such as from invading pathogenic microbes. Antigens are taken up by DC in soluble or particulate forms, often facilitated by opsonization by antibody or complement, processed by a series of enzymes and then loaded onto MHC molecules for presentation to T-cells during priming of an immune response.[2] MHC class II usually presents antigenic peptides derived from extracellular organisms to $CD4^+$ T-cells, whereas MHC class I presents peptides derived from intracellular organisms (or cytoplasmic proteins) to $CD8^+$ T-cells. This ensures that the optimum T-cell response is generated: $CD4^+$ T helper cells for antibodies and cell-mediated immunity against extracellular organisms, and $CD8^+$ cytotoxic T-cells against intracellular organisms and cancers. The DC also receive inflammatory signals during infections and cancers; pathogen-associated molecular patterns or danger signals, which are recognized via receptors such as Toll-like receptors and stimulate cytokine secretion and co-stimulatory molecule expression, which further facilitates T-cell responses. Hence, various vaccination strategies aim to target DC because of their pivotal role in adaptive immunity.

Delivering antigens to DC, using strategies that target uptake via surface receptors, including DEC-205, mannose receptor and FcγR1, is an innovative area for developing vaccines and therapeutics. Heat-shock proteins (hsp) carry an antigenic profile or fingerprint of the cells from which they are derived, possess adjuvant activity and bind to receptors on DC to promote uptake. This review highlights the role of hsp in antigen delivery to DC, which forms the basis of a strategy for developing vaccines against cancer and infectious diseases.

Heat-shock protein biology

Within cells, hsp undertake critical and conserved physiological roles. They function as chaperones and co-chaperones binding intracellular polypeptide chains and misfolded proteins, preventing aggregation and supporting folding and transport.[3] Most hsp have at least two functional domains: a polypeptide-binding domain, and an ATPase domain controlling binding and release of polypeptide substrate. Heat-shock proteins are present in organisms as diverse as bacteria and man, protecting proteins from damage during normal physiological activity as well as stressful conditions.[4] As a consequence of their physiological functions, hsp transport multiple proteins as 'cargo'. Cellular levels of hsp are high, for example in bacteria, hsp70 alone accounts for 1–2% of cellular proteins after heat induction.[5] In eukaryotic cells hsp levels are increased by stressful stimuli including heat, oxidative stress, starvation, hypoxia, irradiation, viral infection and cancerous transformation.[6,7] The hsp are grouped into several distinct families named according to the molecular weight of family members (Table 1). In man, hsp90, hsp70, hsp60/Chaperonin and hsp40 families have been characterized.[8] In prokaryotes, GroEL (hsp60) and DnaK (hsp70) are the main hsp families.

Stress proteins are ubiquitous and can be detected readily in normal human plasma samples.[9] Absolute levels of extracellular hsp vary markedly between individuals. For example, reported levels for human plasma hsp60 range between < 1 ng/ml and 1 mg/ml[9] and between 100 pg/ml and 160 ng/ml for serum hsp72.[10] Levels of hsp are dynamic during normal physiological activities; exercise increases hsp72 levels in serum by fourfold to eightfold.[11] Therefore, extracellular hsp are continuously present in the circulation of normal individuals and can be increased transiently by several fold without apparent pathology.

In addition to functioning as intracellular protein chaperones, hsp modulate the immune system by stimulating both innate and adaptive responses. The term 'chaperokine' has been used to describe the dual activity of hsp functioning as both chaperone and cytokine.[12] Once released from a host or pathogen cell, hsp bind to cellular receptors to trigger an innate immune response, including maturation of DC and secretion of pro-inflammatory cytokines and chemokines, for example RANTES (Regulated on Activation Normal T-cell Expressed and Secreted), through Toll-like receptor activation.[13] Processing of cargo proteins carried by hsp occurs, leading to antigen presentation on MHC. Hence hsp link the innate and acquired immune responses to pathogens and have the potential to function as vaccine adjuvants in infections and cancer.[14] For example, hsp70 is an effective and safe adjuvant in neonatal mice and functions effectively via mucosae to generate protective cell-mediated immune responses against herpes simplex virus type-1.[15] Moreover, modified hsp are also capable of inducing cytokine responses. For example, a fusion protein containing Bacillus Calmette–Guérin (BCG)-derived hsp70 and *Mycobacterium leprae*-derived major membrane protein binds to human DC stimulating production of interleukin-12 p70 through Toll-like receptor 2.[16]

Table 1. Heat-shock protein (hsp) families

Family	Family members	Intracellular location
Small hsp	**hsp10**, GroES, **hsp16**, **α-crystallin**, **hsp20**, **hsp25**, hsp26, **hsp27**	Cytosol
hsp40	**hsp40**, **DnaJ**, SIS1	Cytosol
hsp47	**hsp47**	Endoplasmic reticulum
Calreticulin	**Calreticulin, calnexin**	Endoplasmic reticulum
hsp60	**hsp60**, hsp65, GroEL	Cytosol and mitochondria
hsp70	**hsp72**, **Hsc70 (hsp73), hsp110/SSE**, DnaK	Cytosol
	SSC1, SSQ1, ECM10	Mitochondria
	Grp78 (BiP), Grp170	Endoplasmic reticulum
hsp90	**hsp86**, HTPG	Cytosol
	gp96 (Grp94, hsp108, endoplasmin)	Endoplasmic reticulum
hsp100	hsp104, **hsp110**	Cytosol
	CLP proteins	Cytosol
	hsp78	Mitochondria

Heat-shock proteins expressed in mammals are shown in bold.

Cellular receptors for hsp

Dendritic cells and other cell types possess multiple receptors that bind hsp but the identities and functions of those proposed to modulate the immune system *in vivo* are not fully understood.[17] The expression profile of these receptors is broad, including, but not limited to, multiple immune, epithelial, endothelial and fibroblast cells and multiple cell types of the central nervous system. Receptors for which evidence supports a role in hsp binding and their distribution on immune cells are shown (Table 2).

The relative contribution made by each receptor type to the binding and internalization of hsp by DC is poorly understood. For example, with the exception of Scavenger Receptor type A and CD91, the hsp binding functionality of each receptor has not been assessed *in vivo*. Furthermore, investigations show that for gp96, non-specific endocytosis/pinocytosis mechanisms account for a fraction of internalization.[39]

Heat-shock proteins deliver antigen peptides to DC for T-cell priming

Heat-shock proteins deliver peptides as cargo to DC (Fig. 1) leading to MHC presentation for priming of adaptive immunity.[40] Increased levels of pathogen-derived hsp caused by inflammatory stimuli such as fever, result in a concomitant increase in pathogen-specific antigens carried as hsp complexes.[41] The uptake of hsp complexes by DC enables efficient capture and presentation of pathogen-specific antigens and the mounting of a specific immune response against the infectious agent through the generation of $CD4^+$ T-cell responses.[42] The capture of pathogen-specific antigens 'chaperoned' in hsp complexes also results in their uptake and MHC class I restricted presentation to specific T-cells, so eliciting $CD8^+$ cytotoxic T-cell responses.[43] It has been shown through the use of inhibitors, that hsp90 plays a significant natural role in chaperoning antigenic peptides in presentation.[44]

Human DC pulsed with peptide-loaded mycobacterial hsp70 generate potent antigen-specific cytotoxic T-cell responses, dependent on an hsp70-stimulated calcium signalling cascade.[45] Delivery of peptides is achieved significantly through extracellular hsp binding to cellular receptors, followed by internalization.[46] Antigens need to be bound or linked to hsp to facilitate uptake, simple mixing is not adequate. The hsp70–peptide complexes reach endosomal compartments that fuse with vesicles containing recycling MHC class I–peptide complexes. Protein fragments chaperoned by hsp and not intact proteins are sufficient for priming $CD8^+$ T-cell responses.[47]

Highly purified human recombinant hsp70 enhances cross-presentation of exogenous antigens on MHC class I resulting in better antigen-specific T-cell stimulation.[48] Here T-cell stimulation was a function of the degree of complex formation between hsp70 and peptides and correlated with improved antigen delivery to endosomal compartments. hsp70 enhanced cross-presentation by different APC including DC and B cells and antigen-specific T-cell activation occurred in the absence of innate signals transmitted by hsp70.[48] Heat shock protein 90-mediated cross-presentation of ovalbumin-derived antigens involves binding of hsp90–ovalbumin complexes to Scavenger Receptor expressed by Endothelial Cells-I on the surface of APC.[49] Internalization is driven through a regulated, endocytic pathway.[49] Peptides are loaded either directly onto MHC class I in endosomes, or undergo cytosomal processing by aminopeptidases and proteases. Extracellular hsp90 can therefore convey antigenic peptides through an efficient endocytosis pathway in APC and facilitate presentation in a regulated manner.[49] Heat-shock proteins can also mediate by the same mechanism cross-presentation of exogenous HIV antigens.[50]

Distinct hsp possess differing abilities to induce cross-presentation of antigens. For example, an extract prepared from human melanoma lines contained the four major chaperone proteins hsp/HSC 70, hsp90, Grp94/gp96 and calreticulin. These hsp were functional, enhancing presentation of exogenous peptides, but superior activity was observed for the hsp70-rich preparation.[51] Small hsp fragments are sufficient to link peptides and to be taken up by receptors on APC including CD91 and Scavenger Receptor type A, and can be used in immunotherapy of tumours and vaccine development.[52]

Broad antigen coverage requires vaccines containing multiple hsp

To replicate a physiological response to natural infections, so as to maximize immune protection, it is necessary for a vaccine to contain multiple hsp families and associated antigens, hence delivering a broad range of antigens thereby maximizing antigen coverage and protection. The identity and range of cargo carried are dependent upon the types and diversity of hsp present within a vaccine.

Table 2. Heat shock protein (hsp) receptors on cells of the immune system

Ligand(s)	Receptor	Receptor cellular distribution	Reference
gp96, hsp90, hsp70, Calreticulin	CD91	Dendritic cell (DC), macrophage, monocyte,	18–20
hsp70	LOX-1	DC, macrophage, monocyte	21,22
gp96, hsp60, hsp70, hspB8, α-Crystallin	Toll-like receptor 2/4	DC, macrophage, mast cell, monocyte, T-cell, microglia, neutrophil	23–25
hsp70	CD14	DC, macrophage, monocyte, Kupffer	26,27
hsp70	CD40	DC, macrophage, monocyte, B cell, microglial,	28–30
gp96, Calreticulin	Scavenger Receptor type A	DC, macrophage, microglial	31–33
mycobacterial hsp70	CCR5	DC, macrophage, T-cell, microglia,	34–36
Calreticulin, gp96, hsp110, Grp170, hsp70	Scavenger Receptor expressed by Endothelial Cells (SREC)-I	DC, macrophage	37,38

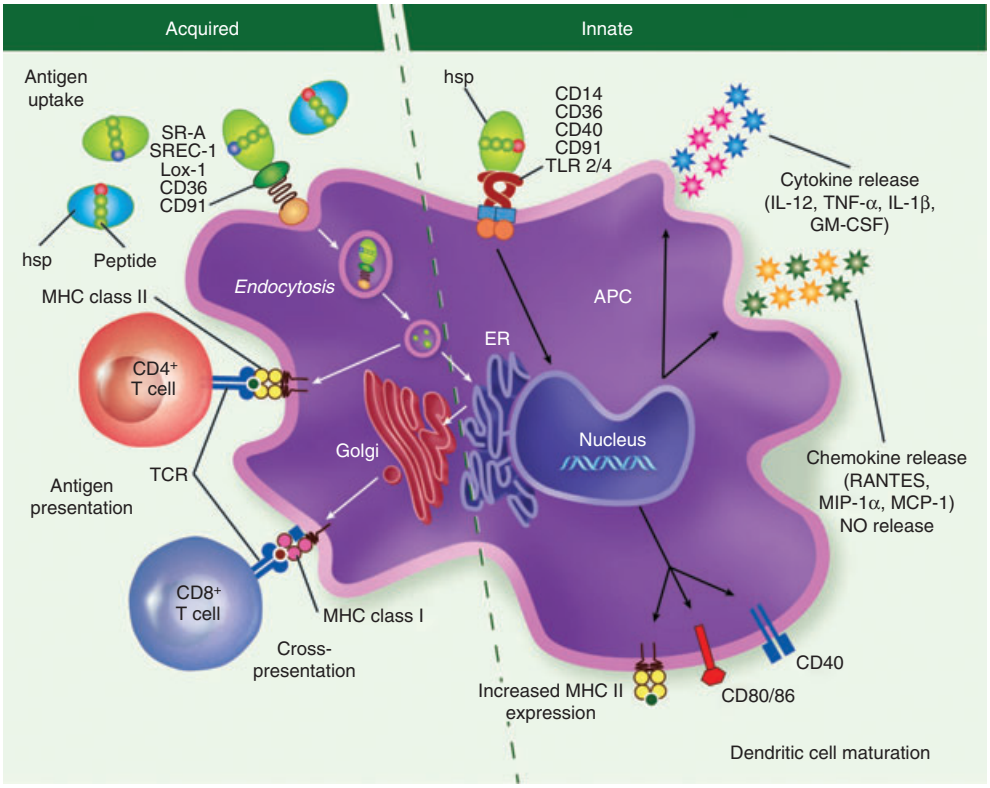

Figure 1. The binding of heat-shock proteins (hsp) to target receptors on the surface of antigen-presenting cells (APC), including dendritic cells (DC), can lead to 1. receptor internalization followed by processing of cargo and presentation on MHC; 2. the induction of cell signalling leading to increased levels of MHC and co-stimulatory molecules; 3. increased production/release of modulatory molecules including cytokines. ER, endoplasmic reticulum; GM-CSF, granulocyte–macrophage colony-stimulating factor; hsp, heat-shock protein; IL-12, interleukin-12; MCP-1, monocyte chemotactic protein 1; MIP-1α, macrophage inflammatory protein-1α; NO, nitric oxide; RANTES, Regulated on Activation Normal T-cell Expressed and Secreted; TCR, T-cell receptor; TLR, Toll-like receptor; TNF-α, tumour necrosis factor-α

Gp96, hsp70 and hsp90 each bind distinct antigen peptide precursors.[53] For *Escherichia coli*, GroEL binds to approximately 250 of the 2400 cytosolic proteins and a recent study demonstrated that for folding *in vivo*, 57 proteins are *bona fide* obligate GroEL substrates.[41]

Deletion of GroEL is lethal in *E. coli*, as is the deletion of the two chaperones Trigger Factor and DnaK (hsp70)[54] that chaperone a significant subset of GroEL target proteins. For cancer, a chaperone-rich cell lysate is more effective than purified hsp alone in generating tumour-specific responses in multiple murine models.[55,56] The chaperone-rich cell lysate vaccine has a more pronounced immunological effect per unit material of protein than any one of the individual chaperone proteins that it contains used independently as vaccines.[57]

Heat-shock proteins as cancer therapeutics

Immune responses can be generated by hsp against tumour antigens, despite immune evasion processes mediated for example by regulatory T-cells. The potential role for hsp in the immune response to cancer was recognized by Srivastava and colleagues, who proposed that hsp complexed with antigenic peptides, released from tumour cells (or virus-infected cells) *in vivo* during lysis, are taken up by APC,[58] and the potential use of hsp in cancer immunotherapy has been demonstrated extensively. Of interest, immunization of mice with gp96 can induce a regulated immune response resulting either in tumour immunity or down-regulation, depending on the immunization dose used.[59] Heat-shock protein-based vaccines have been shown to activate tumour-specific immunity, triggering the proliferation and cytotoxic capabilities of cancer-specific $CD8^+$ T-cells, inhibiting tumour growth.[60] The hsp also activate natural killer cells to impart anti-tumour responses.[61] Exogenous antigens chaperoned by hsp are presented by MHC class I molecules and recognized by $CD8^+$ T lymphocytes offering one mechanism for the classical phenomenon of cross-presentation as well as offering a role within the immune danger theory.[62,63] Lysates from heat-shocked tumour cells provide an optimal source of tumour antigens, generating DC with improved cross-presentation capacity.[64]

Examples of hsp-based therapeutics in cancer trials are detailed in Table 3. To date, one hsp vaccine, Vitespen, is licensed and marketed. The hsp gp96, the master chaperone for Toll-like receptors[65], is the major component of Vitespen. Chaperoning by gp96 increases uptake over unchaperoned peptides *in vitro* by two orders of magnitude and immunization of mice with 5 ng gp96–peptide complexes, results in generation of a peptide-specific $CD4^+$ T-cell response.[66] In April 2008, Vitespen was approved in Russia as a patient-specific adjuvant treatment of kidney cancer for individuals at intermediate-risk for disease recurrence. Outside Russia, Vitespen is an investigational vaccine designed to treat cancer with the intent of minimizing side-effects. It has been studied extensively in clinical trials in Phase I and II settings, demonstrating efficacy in some but not all trials. Phase III studies have been completed in which over 1300 patients with renal cell carcinoma or malignant melanoma have been treated. Essentially neither toxicity nor autoimmunity induced by Vitespen was observed.[67]

Although pre-clinical studies with Vitespen were promising, clinical studies show limited efficacy.[68] This outcome may be a consequence of differences in the hsp content of Vitespen used for initial *in vivo* models compared with the vaccine used for clinical trials.[69] Pre-clinical studies reported utilised vaccines containing gp96 or hsp70, while clinical studies utilised vaccine containing only gp96. Critically, gp96 and hsp70 have distinct functions as endoplasmic reticulum (ER) luminal and cytoplasmic chaperones, respectively, and thus bind distinct client proteins. Heat-shock protein 70 binds a variety of cytoplasmic proteins and isolation of this hsp from tumour cells will result in the purification of intact hsp–client protein complexes. In contrast, gp96 binds membrane proteins such as integrins and Toll-like receptors and is essential for chaperoning peptides in the ER.[70] As the clinical production processes used do not contain detergents,[71] peptides bound to gp96 in Vitespen are unlikely to result from tumour client proteins. Hence differences between the bound peptides in gp96 isolated from the homogeneous tumour tissue in the animal models and the heterogeneous tumour tissue from patients in the clinical trials may also account for the limited efficacy reported for Vitespen.[68] Other key issues concerning the future development of such a vaccine are the correct and effective dose of hsp, and which patients to target.

Other hsp provide alternatives to gp96 for cancer vaccine development. Vaccination with hsp70 derived from the Meth A sarcoma, established dose-dependent immunity to challenge with Meth A sarcoma in mice.[72] A suggestion to use cell lines or allogeneic tumours as a more generic source of hsp complex carrying common cancer antigens,[73] has not been taken further. High molecular weight chaperone complexes, hsp110- or grp170-tyrosinase-related protein 2 peptide ($TRP2_{175–192}$), were superior to conventional chaperones as a vaccine platform to deliver tumour-derived antigens.[74] In addition, the immunization with chaperones combined to two different melanoma antigens (gp100, TRP2) significantly improved anti-tumour efficacy compared with either of the single antigen vaccines,[74] demonstrating that hsp combination vaccines can offer increased efficacy. In a Phase II clinical trial, vaccination with autologous tumour-derived gp96–peptide complex vaccine (hsp complex-96) together with granulocyte–macrophage colony-stimulating factor and interferon-α was associated with mild local and systemic toxicity.[75] Vaccination was proven to instigate both tumour-specific T-cell-mediated and natural killer cell responses in some patients. However, neither immunological nor clinical responses were improved compared with those recorded in a previous study investigating hsp complex-96 monotherapy. A recent study has provided the first evidence in man of patient-specific immune responses against autologous tumour-derived peptides bound to gp96.[76]

Over-expression of hsp70 increases significantly the immunogenicity of cancer cell extracts; with the mechanism of cell death influencing both hsp70 expression

Table 3. Examples of heat-shock protein (hsp) -based cancer therapeutics in clinical trials

Therapeutic	Disease targeted	Trial phase	References
Vitespen Gp96 based vaccine derived from patient tumour	Various cancers including, but not limited to, kidney, liver, ovarian, colorectal, glioma and melanoma	Marketed (Russia) Disease-dependent Phase II and III	76
hspE7 fusion protein consisting of BCG hsp65 linked to HPV 16 E7	Cervical diseases caused by human papillomavirus (HPV)	Phase II	82
hspPC-96 Gp96 based vaccine, administered with GM-CSF and interferon-α, derived from patient tumour	Melanoma	Phase I	75
hsp70.PC-F hsp70 based vaccine, derived from patient tumour	Breast cancer	Phase I	80
Chaperone-rich cell lysates from patient tumour	Breast cancer	Phase I	83

levels and the immunogenicity of cell extracts.[77] In addition to hsp complex from hsp70 (hsp70C), synthetic peptide-mimics of hsp70C can modulate positively the immune response against tumours[78] and therefore provide an additional approach for therapeutic intervention. Heat shock protein 70 derived from tumours of characterized antigenic makeup could be used as a generic subunit tumour vaccine.[73] Vaccines derived from tumours or cell lines that have undergone heating to increase the abundance of hsp may provide an innovative approach. For example, vaccination with heated autologous prostate cancer cells elicits protection against tumour challenge in 60% of vaccinated rats, compared with 0% protection in control rats receiving vaccines from non-shocked cells, together with an increase in the T helper type 1 (interferon-γ) response.[79]

Heat shock protein 70 extracted from DC fused to patient-derived ovarian cancer or breast cancer cells (hsp70.PC-F) were tested as tumour vaccines.[80] The hsp70.PC-F induced T-cells expressing higher levels of interferon-γ and with increased killing capacity for tumour cells, compared with those induced by hsp derived from tumour cells, although these were characterized by a higher content of tumour antigens and the detection of hsp such as hsp90 and hsp110.[80] In a mouse tumour model, vaccination with hsp60, hsp70, gp96 and hsp110/peptides, combined with treatment with a low-dose of cyclophosphamide plus interleukin-12, induced a marked immunological response directed against autologous tumours.[81]

Heat-shock protein vaccines: an innovative solution for infectious diseases

Heat-shock proteins possess broad utility as vaccine components. For example, marketed adjuvants often possess side-effects (e.g. ulceration); hsp adjuvants avoid such effects. The abilities of hsp to drive innate stimulation and deliver antigens are now being exploited in prophylactic vaccines against infectious diseases. In one approach, hsp-based vaccines have been produced by over-expressing the influenza virus nucleoprotein in cultured cells before purification of gp96.[84] The gp96 preparation was well tolerated in mice; with preliminary results suggesting that a cellular immune response was induced, providing a novel strategy to develop vaccines against virus targets.[84]

There are several published approaches to prepare hsp complexes, including ion exchange and hydroxyapatite column chromatography and immunoprecipitation with antibodies coupled to magnetic beads.[85] In an innovative approach, hsp70C have been extracted from plant cells expressing viral antigens[86,87] using the same ADP-chromatography purification protocol described for animal hsp70,[88] a method able to prevent the release of the naturally chaperoned peptides. Plant-derived hsp70C were shown to activate the immune system inducing both activation of MHC class I-restricted polyclonal T-cell responses and antibody production in mice of different haplotypes without the need of adjuvant co-delivery.[87] These results indicate that hsp70C derived from plants producing recombinant antigens may be used to formulate multi-epitope vaccines.

Several investigational prophylactic vaccines containing hsp and hsp complex are in development. For example, a tuberculosis vaccine based on hsp complex from BCG (T-BioVax) has demonstrated good efficacy in the mouse *Mycobacterium tuberculosis* aerosol challenge model.[89,90] ImmunoBiology Ltd is also developing a vaccine against meningitis (MenBioVax) derived from heat-shocked *Neisseria meningitidis*. Both T-BioVax and MenBioVax contain multiple hsp families derived from the stressed bacterium of interest to maximize efficacy. MenBioVax provides protection against lethal challenge in a mouse model of meningococcal septicaemia. Sera obtained from mice immunized with this vaccine show promising bactericidal and opsonophagocytic responses against a panel of *N. meningitidis* strains.[91]

HerpV, a vaccine consisting of 32 synthetic 35mer HSV-2 peptides representative of all phases of viral replication, non-covalently complexed with recombinant human hsp70 protein, is well tolerated and safe.[92] This was the first hsp-based vaccine to show immune responses against viral antigens in humans.[92] Vaccinated subjects demonstrated a statistically significant $CD4^+$ T-cell response to HSV-2 antigens, with the majority of subjects also having a significant $CD8^+$ T-cell response.

Development of hsp vaccines is based on the need to emulate safely, the mechanism by which protection is established during a normal infection. Extracellular, pathogen-derived hsp are routinely present during infection and transport pathogen proteins as cargo.[93] During infection, because of bacterial lysis, multiple pathogen hsp will be visible to the host in parallel. The identity of cargo proteins will depend upon the family and type of hsp chaperone.[40] The meningococcal stress protein MSP63, a member of the hsp60 family, has been shown in man to be immunogenic during natural meningococcal infection.[94] Genes encoding hsp, including DnaK, GroEL, GroES, DnaJ, GrpE and ClpB, were shown by transcriptional profiling to be up-regulated several fold in *N. meningitidis* in human blood during bacteraemia.[95]

The similarity of pathogen-derived hsp to human hsp raises the hypothetical possibility of enhanced self recognition induced by vaccines enriched for pathogen hsp. Theoretically, this could occur as a consequence of the presentation of host proteins to DC by vaccine-derived hsp and the induction of autoimmune responses induced by the vaccine hsp. The potential for antibodies produced in mice against plant hsp70 to cross-react, either with murine hsp70 or human hsp70, has been investigated and found to be absent despite the significant structural similarities between the three isoforms.[86]

Significantly, as a consequence of the manufacturing process, hsp are present in many marketed vaccines against infectious diseases, notably in whole cell vaccines and vaccines derived from cell extracts. The extensive, safe use of vaccines containing hsp therefore provides compelling evidence against safety concerns. For example, whole cell vaccines are used widely and possess acceptable safety profiles.[96] Antibodies to hsp65 were found in sera from children vaccinated with DTP (diphtheria, tetanus, pertussis) vaccine administered extensively in Europe and the USA.[97] Antibodies against BCG hsp develop naturally in infants in 6–12 months, even without BCG vaccination.[98] The safety of human exposure to *N. meningitidis* hsp was obtained from administration of marketed vaccines that contain hsp.[99] Such vaccines have been used since the 1980s and the safety records are excellent.

Perspective: new vaccine approaches are needed urgently

From the pioneering work of Benjamin Jesty and subsequent developments from Edward Jenner to the present day, vaccines have delivered and continue to deliver significant improvements to global health. Smallpox is eradicated, polio has been controlled and the frequency of childhood diseases such as measles has reduced. However, the most successful vaccines have been against diseases where the causal pathogen does not have major anti-immune defence mechanisms. Many pathogens, including hepatitis C and human immunodeficiency viruses, *M. tuberculosis*, *Helicobacter pylori* and *Plasmodium falciparum* have evolved complex immune evasion strategies and probably require high level effector T-cell activation for their eradication. So far, these pathogens have proved intractable to existing vaccination strategies. Bioterrorism, emerging and re-emerging infectious diseases and changes in population demographics and target groups will shape the need for new vaccines.[100] These challenges drive the requirement for new efficacious vaccines produced at low cost and therefore innovative technologies are urgently required. Several such approaches involve the targeting of vaccine antigens to DC, the key controllers of the immune response.

Heat-shock proteins possess significant properties that support their inclusion in the next generation of vaccines to target DC: first, hsp are natural adjuvants; second, hsp deliver multiple antigens that can induce adaptive immune responses to provide broad coverage against pathogens and effective cancer therapy; and third, data show that they are safe constituents of existing vaccines. Most marketed vaccines generate antibody responses but hsp vaccines can also generate cellular immunity, a tightly regulated process varying between individuals in part because of MHC differences. Heat-shock protein complexes derived from cells carry a broad antigenic peptide fingerprint, which helps to avoid both pathogen and immune escape mechanisms. Critically, manufacturing approaches for hsp-containing vaccines against infectious diseases provide low cost production. Although hsp vaccines provide an exciting and innovative strategy for the development of much needed new vaccines, data from clinical trials are now needed to confirm that they provide an effective new approach in man.

Acknowledgements

We wish to acknowledge TSB grant number 1204_BCF_CDS_R1 21601-155139 awarded from the UK innovation agency, the Technology Strategy Board, as part of the UK government-backed Biomedical Catalyst. Dr McNulty is a project manager for ImmunoBiology Ltd, a vaccine development company based at the Babraham Research Campus, Cambridge.

Disclosures

ImmunoBiology Ltd develops innovative anti-infective vaccines based on hsp and has a number of patents in this field.

References

1 Steinman RM. Decisions about dendritic cells: past, present, and future. *Annu Rev Immunol* 2012; **30**:1–22.
2 Neefjes J, Jongsma ML, Paul P, Bakke O. Towards a systems understanding of MHC class I and MHC class II antigen presentation. *Nat Rev Immunol* 2011; **11**:823–36.
3 Hartl FU, Hayer-Hartl M. Converging concepts of protein folding *in vitro* and *in vivo*. *Nat Struct Mol Biol* 2009; **16**:574–81.
4 Sarge KD. Male germ cell-specific alteration in temperature set point of the cellular stress response. *J Biol Chem* 1995; **270**:18745–8.
5 Herendeen SL, VanBogelen RA, Neidhardt FC. Levels of major proteins of *Escherichia coli* during growth at different temperatures. *J Bacteriol* 1979; **139**:185–94.
6 Morimoto R, Tissieres A, Georgopoulos C. Stress Proteins in Biology and Medicine. New York: Cold Spring Harbor Laboratory Press, 1990.
7 Kalmar B, Greensmith L. Induction of heat shock proteins for protection against oxidative stress. *Adv Drug Deliv Rev* 2009; **61**:310–8.
8 Kampinga HH, Hageman J, Vos MJ *et al.* Guidelines for the nomenclature of the human heat shock proteins. *Cell Stress Chaperones* 2009; **14**:105–11.
9 Shamaei-Tousi A, Steptoe A, O'Donnell K *et al.* Plasma heat shock protein 60 and cardiovascular disease risk: the role of psychosocial, genetic, and biological factors. *Cell Stress Chaperones* 2007; **12**:384–92.
10 Sandström ME, Siegler JC, Lovell RJ, Madden LA, McNaughton L. The effect of 15 consecutive days of heat–exercise acclimation on heat shock protein 70. *Cell Stress Chaperones* 2008; **13**:169–75.
11 Fischer CP, Hiscock NJ, Basu S, Vessby B, Kallner A, Sjoberg LB, Febbraio MA, Pedersen BK. Vitamin E isoform-specific inhibition of the exercise-induced heat shock protein 72 expression in humans. *J Appl Physiol* 2006; **100**:1679–87.
12 Asea A, Kraeft SK, Kurt-Jones EA, Stevenson MA, Chen LB, Finberg RW, Koo GC, Calderwood SK. HSP70 stimulates cytokine production through a CD14-dependent pathway, demonstrating its dual role as a chaperone and cytokine. *Nat Med* 2000; **6**:435–42.
13 Lehner T, Wang Y, Whittall T, McGowan E, Kelly CG, Singh M. Functional domains of HSP70 stimulate generation of cytokines and chemokines, maturation of dendritic cells and adjuvanticity. *Biochem Soc Trans* 2004; **32** (Pt 4):629–32.
14 Segal BH, Wang XY, Dennis CG, Youn R, Repasky EA, Manjili MH, Subjeck JR. Heat shock proteins as vaccine adjuvants in infections and cancer. *Drug Discov Today* 2006; **11**:534–40.
15 Pack CD, Gierynska M, Rouse BT. An intranasal heat shock protein based vaccination strategy confers protection against mucosal challenge with herpes simplex virus. *Hum Vaccin* 2008; **4**:360–4.

16 Mukai T, Maeda Y, Tamura T, Matsuoka M, Tsukamoto Y, Makino M. Induction of cross-priming of naive $CD8^+$ T lymphocytes by recombinant bacillus Calmette–Guérin that secretes heat shock protein 70-major membrane protein-II fusion protein. *J Immunol* 2009; **183**:6561–8.

17 Binder RJ. HSP receptors: the cases of identity and mistaken identity. *Curr Opin Mol Ther* 2009; **11**:62–71.

18 Hart JP, Gunn MD, Pizzo SV. A CD91-positive subset of $CD11c^+$ blood dendritic cells: characterization of the APC that functions to enhance adaptive immune responses against CD91-targeted antigens. *J Immunol* 2004; **172**:70–8.

19 Nilsson A, Vesterlund L, Oldenborg PA. Macrophage expression of LRP1, a receptor for apoptotic cells and unopsonized erythrocytes, can be regulated by glucocorticoids. *Biochem Biophys Res Commun* 2012; **417**:1304–9.

20 Marzolo MP, von Bernhardi R, Bu G, Inestrosa NC. Expression of α_2-macroglobulin receptor/low density lipoprotein receptor-related protein (LRP) in rat microglial cells. *J Neurosci Res* 2000; **60**:401–11.

21 Yoshimoto R, Fujita Y, Kakino A, Iwamoto S, Takaya T, Sawamura T. The discovery of LOX-1, its ligands and clinical significance. *Cardiovasc Drugs Ther* 2011; **25**:379–91.

22 Delneste Y, Magistrelli G, Gauchat J *et al.* Involvement of LOX-1 in dendritic cell-mediated antigen cross-presentation. *Immunity* 2002; **17**:353–62.

23 Kaisho T, Akira S. Regulation of dendritic cell function through Toll-like receptors. *Curr Mol Med* 2003; **3**:373–85.

24 Hayashi F, Means TK, Luster AD. Toll-like receptors stimulate human neutrophil function. *Blood* 2003; **102**:2660–9.

25 Juarez E, Nuñez C, Sada E, Ellner JJ, Schwander SK, Torres M. Differential expression of Toll-like receptors on human alveolar macrophages and autologous peripheral monocytes. *Respir Res* 2010; **11**:2.

26 Mahnke K, Becher E, Ricciardi-Castagnoli P, Luger TA, Schwarz T, Grabbe S. CD14 is expressed by subsets of murine dendritic cells and upregulated by lipopolysaccharide. *Adv Exp Med Biol* 1997; **417**:145–59.

27 Marchant A, Duchow J, Delville JP, Goldman M. Lipopolysaccharide induces up-regulation of CD14 molecule on monocytes in human whole blood. *Eur J Immunol* 1992; **22**:1663–5.

28 McLellan AD, Sorg RV, Williams LA, Hart DN. Human dendritic cells activate T lymphocytes via a CD40: CD40 ligand-dependent pathway. *Eur J Immunol* 1996; **26**:1204–10.

29 Suttles J, Stout RD. Macrophage CD40 signaling: a pivotal regulator of disease protection and pathogenesis. *Semin Immunol* 2009; **21**:257–64.

30 Tan J, Town T, Paris D *et al.* . Activation of microglial cells by the CD40 pathway: relevance to multiple sclerosis. *J Neuroimmunol* 1999; **97**:77–85.

31 Husemann J, Loike JD, Anankov R, Febbraio M, Silverstein SC. Scavenger receptors in neurobiology and neuropathology: their role on microglia and other cells of the nervous system. *Glia* 2002; **40**:195–205.

32 Harshyne LA, Zimmer MI, Watkins SC, Barratt-Boyes SM. A role for class A scavenger receptor in dendritic cell nibbling from live cells. *J Immunol* 2003; **170**:2302–9.

33 Gordon S. Macrophage-restricted molecules: role in differentiation and activation. *Immunol Lett* 1999; **65**:5–8.

34 Sorce S, Myburgh R, Krause KH. The chemokine receptor CCR5 in the central nervous system. *Prog Neurobiol* 2011; **93**:297–311.

35 Granelli-Piperno A, Moser B, Pope M *et al.* Efficient interaction of HIV-1 with purified dendritic cells via multiple chemokine coreceptors. *J Exp Med* 1996; **184**:2433–8.

36 Oppermann M. Chemokine receptor CCR5: insights into structure, function, and regulation. *Cell Signal* 2004; **16**:1201–10.

37 Tamura Y, Osuga J, Adachi H *et al.* Scavenger receptor expressed by endothelial cells I (SREC-I) mediates the uptake of acetylated low density lipoproteins by macrophages stimulated with lipopolysaccharide. *J Biol Chem* 2004; **279**:30938–44.

38 Beauvillain C, Meloni F, Sirard JC *et al.* The scavenger receptors SRA-1 and SREC-I cooperate with TLR2 in the recognition of the hepatitis C virus non-structural protein 3 by dendritic cells. *J Hepatol* 2010; **52**:644–51.

39 Pockley AG, Fairburn B, Mirza S, Slack LK, Hopkinson K, Muthana M. A non-receptor-mediated mechanism for internalization of molecular chaperones. *Methods* 2007; **43**:238–44.

40 Colaco CA. Towards a Unified Theory of Immunity; DCs, stress proteins and antigen capture. *Cell Mol Biol* 1998; **6**:883–90.

41 Fujiwara K, Ishihama Y, Nakahigashi K, Soga T, Taguchi H. A systematic survey of *in vivo* obligate chaperonin-dependent substrates. *EMBO J* 2010; **29**:1552–64.

42 Tobian AA, Canaday DH, Boom WH, Harding CV. Bacterial heat shock proteins promote CD91-dependent class I MHC cross-presentation of chaperoned peptide to $CD8^+$ T-cells by cytosolic mechanisms in dendritic cells versus vacuolar mechanisms in macrophages. *J Immunol* 2004; **172**:5277–86.

43 Tobian AA, Canaday DH, Harding CV. Bacterial heat shock proteins enhance class II MHC antigen processing and presentation of chaperoned peptides to $CD4^+$ T-cells. *J Immunol* 2004; **173**:5130–7.

44 Callahan MK, Garg M, Srivastava PK. Heat-shock protein 90 associates with N-terminal extended peptides and is required for direct and indirect antigen presentation. *Proc Natl Acad Sci USA* 2008; **105**:1662–7.

45 Floto RA, MacAry PA, Boname JM *et al.* Dendritic cell stimulation by mycobacterial Hsp70 is mediated through CCR5. *Science* 2006; **314**:454–8.

46 Thériault JR, Adachi H, Calderwood SK. Role of scavenger receptors in the binding and internalization of heat shock protein 70. *J Immunol* 2006; **177**:8604–11.

47 Binder RJ, Srivastava PK. Peptides chaperoned by heat-shock proteins are a necessary and sufficient source of antigen in the cross-priming of $CD8^+$ T-cells. *Nat Immunol* 2005; **6**:593–9.

48 Bendz H, Ruhland SC, Pandya MJ *et al.* Human heat shock protein 70 enhances tumor antigen presentation through complex formation and intracellular antigen delivery without innate immune signaling. *J Biol Chem* 2007; **282**:31688–702.

49 Murshid A, Gong J, Calderwood SK. Heat shock protein 90 mediates efficient antigen cross presentation through the scavenger receptor expressed by endothelial cells-I. *J Immunol* 2010; **185**:2903–17.

50 SenGupta D, Norris PJ, Suscovich TJ *et al.* Heat shock protein-mediated cross-presentation of exogenous HIV antigen on HLA class I and class II. *J Immunol* 2004; **173**:1987–93.

51 Bleifuss E, Bendz H, Sirch B *et al.* Differential capacity of chaperone-rich lysates in cross-presenting human endogenous and exogenous melanoma differentiation antigens. *Int J Hyperthermia* 2008; **24**:623–37.

52 Bolhassani A, Rafati S. Mini-chaperones Potential immuno-stimulators in vaccine design. *Hum Vaccin Immunother* 2013; **9**:153–61.

53 Ishii T, Udono H, Yamano T *et al.* Isolation of MHC class I-restricted tumor antigen peptide and its precursors associated with heat shock proteins hsp70, hsp90, and gp96. *J Immunol* 1999; **162**:1303–9.

54 Teter SA, Houry WA, Ang D *et al.* Polypeptide flux through bacterial HSP70: DnaK cooperates with trigger factor in chaperoning nascent chains. *Cell* 1999; **97**:755–65.

55 Feng H, Zeng Y, Graner MW, Likhacheva A, Katsanis E. Exogenous stress proteins enhance the immunogenicity of apoptotic tumor cells and stimulate antitumor immunity. *Blood* 2003; **101**:245–52.

56 Zeng Y, Graner MW, Katsanis E. Chaperone-rich cell lysates, immune activation and tumor vaccination. *Cancer Immunol Immunother* 2006; **55**:329–38.

57 Kislin KL, Marron MT, Li G, Graner MW, Katsanis E. Chaperone-rich cell lysate embedded with BCR-ABL peptide demonstrates enhanced anti-tumor activity against a murine BCR-ABL positive leukemia. *FASEB J* 2007; **21**:2173–84.

58 Srivastava PK, Udono H, Blachere NE, Li Z. Heat shock proteins transfer peptides during antigen processing and CTL priming. *Immunogenetics* 1994; **39**:93–8.

59 Chandawarkar RY, Wagh MS, Kovalchin JT, Srivastava PK. Immune modulation with high dose of heat shock protein gp96: therapy of murine autoimmune diabetes and encephalomyelitis. *Int Immunol* 2004; **16**:615–24.

60 Calderwood SK, Stevenson MA, Murshid A. Heat shock proteins, autoimmunity, and cancer treatment. *Autoimmune Dis* 2012; **2012**:486069.

61 Lv LH, Wan YL, Lin Y *et al.* Anticancer drugs cause release of exosomes with heat shock proteins from human hepatocellular carcinoma cells that elicit effective natural killer cell antitumor responses *in vitro*. *J Biol Chem* 2012; **287**:15874–85.

62 Suto R, Srivastava PK. A mechanism for the specific immunogenicity of heat shock protein-chaperoned peptides. *Science* 1995; **269**:1585–8.

63 Todryk SM, Melcher AA, Dalgleish AG, Vile RG. Heat shock proteins refine the danger theory. *Immunology* 2000; **99**:334–7.

64 Aguilera R, Saffie C, Tittarelli A *et al.* Heat-shock induction of tumor-derived danger signals mediates rapid monocyte differentiation into clinically effective dendritic cells. *Clin Cancer Res* 2011; **17**:2474–83.

65 Yang Y, Liu B, Dai J, Srivastava PK, Zammit DJ, Lefrançois L, Li Z. Heat shock protein gp96 is a master chaperone for toll-like receptors and is important in the innate function of macrophages. *Immunity* 2007; **26**:215–26.

66 Matsutake T, Sawamura T, Srivastava PK. High efficiency CD91- and LOX-1-mediated re-presentation of gp96-chaperoned peptides by MHC II molecules. *Cancer Immun* 2010; **2**:7.

67 Testori A, Richards J, Whitman E *et al.* C-100-21 Study Group. Phase III comparison of vitespen, an autologous tumor-derived heat shock protein gp96 peptide complex vaccine, with physician's choice of treatment for stage IV melanoma: the C-100-21 Study Group. *J Clin Oncol* 2008; **26**:955–62.

68 Reitsma DJ, Combest AJ. Challenges in the development of an autologous heat shock protein based anti-tumor vaccine. *Hum Vaccin Immunother* 2012; **8**:1152–5.

69 Colaco C. Autologous heat-shock protein vaccines. *Hum Vaccin Immunother* 2013; **9**:275–6.

70 Kropp LE, Garg M, Binder RJ. Ovalbumin-derived precursor peptides are transferred sequentially from gp96 and calreticulin to MHC class I in the endoplasmic reticulum. *J Immunol* 2010; **184**:5619–27.

71 Janetzki S, Palla D, Rosenhauer V, Lochs H, Lewis JJ, Srivastava PK. Immunization of cancer patients with autologous cancer-derived heat shock protein gp96 preparations: a pilot study. *Int J Cancer* 2000; **88**:232–8.

72 Udono H, Srivastava PK. Heat shock protein 70-associated peptides elicit specific cancer immunity. *J Exp Med* 1993; **178**:1391–6.

73 Casey DG, Lysaght J, James T, Bateman A, Melcher AA, Todryk SM. Heat shock protein derived from a non-autologous tumour can be used as an anti-tumour vaccine. *Immunology* 2003; **110**:105–11.

74 Wang XY, Sun X, Chen X, Facciponte J, Repasky EA, Kane J, Subjeck JR. Superior antitumor response induced by large stress protein chaperoned protein antigen compared with peptide antigen. *J Immunol* 2010; **184**:6309–19.

75 Pilla L, Patuzzo R, Rivoltini L *et al.* A phase II trial of vaccination with autologous, tumor-derived heat-shock protein peptide complexes Gp96, in combination with GM-CSF and interferon-α in metastatic melanoma patients. *Cancer Immunol Immunother* 2006; **8**:958–68.

76 Crane CA, Han SJ, Ahn BJ *et al.* Individual patient-specific immunity against high-grade glioma after vaccination with autologous tumor derived peptides bound to the 96 KD chaperone protein. *Clin Cancer Res* 2013; **19**:205–14.

77 Melcher A, Todryk S, Hardwick N, Ford M, Jacobson M, Vile RG. Tumor immunogenicity is determined by the mechanism of cell death via induction of heat shock protein expression. *Nat Med* 1998; **4**:581–7.

78 Arnaiz B, Madrigal-Estebas L, Todryk S, James TC, Doherty DG, Bond U. A novel method to identify and characterise peptide mimotopes of heat shock protein 70-associated antigens. *J Immune Based Ther Vaccines* 2006; **4**:2.

79 Todryk SM, Eaton J, Birchall L, Greenhalgh R, Soars D, Dalgleish AG, Melcher AA, Pandha HS. Heated tumour cells of autologous and allogeneic origin elicit anti-tumour immunity. *Cancer Immunol Immunother* 2004; **53**:323–30.

80 Gong J, Zhang Y, Durfee J *et al.* A heat shock protein 70-based vaccine with enhanced immunogenicity for clinical use. *J Immunol* 2010; **184**:488–96.

81 Guo QY, Yuan M, Peng J, Cui XM, Song G, Sui X, Lu SB. Antitumor activity of mixed heat shock protein/peptide vaccine and cyclophosphamide plus interleukin-12 in mice sarcoma. *J Exp Clin Cancer Res* 2011; **30**:24.

82 Einstein MH, Kadish AS, Burk RD, Kim MY, Wadler S, Streicher H, Goldberg GL, Runowicz CD. Heat shock fusion protein-based immunotherapy for treatment of cervical intraepithelial neoplasia III. *Gynecol Oncol* 2007; **106**:453–60.

83 Murshid A, Gong J, Stevenson MA, Calderwood SK. Heat shock proteins and cancer vaccines: developments in the past decade and chaperoning in the decade to come. *Expert Rev Vaccines* 2011; **10**:1553–68.

84 Heikema A, Agsteribbe E, Wilschut J, Huckriede A. Generation of heat shock protein-based vaccines by intracellular loading of gp96 with antigenic peptides. *Immunol Lett* 1997; **57**:69–74.

85 Cuello-Carrión FD, Fanelli MA, Cayado-Gutiérrez N, Castro G, Ciocca DR. Isolation of heat shock protein complexes. *Methods Mol Biol* 2011; **787**:267–75.

86 Buriani G, Mancini C, Benvenuto E, Baschieri S. Plant heat shock protein 70 as carrier for immunization against a plant-expressed reporter antigen. *Transgenic Res* 2011; **20**:331–44.

87 Buriani G, Mancini C, Benvenuto E, Baschieri S. Heat-shock protein 70 from plant biofactories of recombinant antigens activate multiepitope-targeted immune responses. *Plant Biotechnol J* 2012; **10**:363–71.

88 Menoret A. Purification of recombinant and endogenous HSP70s. *Methods* 2004; **32**:7–12.

89 Colaco CA, Bailey CR, Keeble J, Walker KB. BCG (Bacille Calmette–Guérin) HSPCs (heat-shock protein–peptide complexes) induce T-helper 1 responses and protect against live challenge in a murine aerosol challenge model of pulmonary tuberculosis. *Biochem Soc Trans* 2004; **32**:626–8.

90 Walker KB, Keeble J, Colaco C. Mycobacterial heat shock proteins as vaccines – a model of facilitated antigen presentation. *Curr Mol Med* 2007; **7**:339–50.

91 Bailey C, Bignell C, Clarke S *et al.* A novel vaccine approach for *Neisseria meningitidis*: heat shock protein-antigen complexes (HspC) demonstrate cross-serotype immunogenicity. Meningitis and Septicaemia in Children and Adults: November 2009. London : Organised by Meningitis Research Foundation.

92 Wald A, Koelle DM, Fife K *et al.* Safety and immunogenicity of long HSV-2 peptides complexed with rhHsc70 in HSV-2 seropositive persons. *Vaccine* 2011; **29**:8520–9.

93 Cappello F, Conway de Macario E, Di Felice V, Zummo G, Macario AJ. *Chlamydia trachomatis* infection and anti-HSP60 immunity: the two sides of the coin. *PLoS Pathog* 2009; **5**:e1000552.

94 Pannekoek Y, Schuurman IG, Dankert J, van Putten JP. Immunogenicity of the meningococcal stress protein MSP63 during natural infection. *Clin Exp Immunol* 1993; **93**:377–81.

95 Hedman AK, Li MS, Langford PR, Kroll JS. Transcriptional profiling of serogroup B *Neisseria meningitidis* growing in human blood: an approach to vaccine antigen discovery. *PLoS ONE* 2012; **7**:e39718.

96 Pace JL, Rossi HA, Esposito VM, Frey SM, Tucker KD, Walker RI. Inactivated whole-cell bacterial vaccines: current status and novel strategies. *Vaccine* 1998; **16**:1563–74.

97 Arakere G, Kessel M, Nguyen N, Frasch CE. Characterization of a stress protein from group B *Neisseria meningitidis*. *J Bacteriol* 1993; **175**:3664–8.

98 Pilkington C, Costello AM, Rook GA, Stanford JL. Development of IgG responses to mycobacterial antigens. *Arch Dis Child* 1993; **69**:644–9.

99 Holst J, Martin D, Arnold R, Huergo CC, Oster P, O'Hallahan J, Rosenqvist E. Properties and clinical performance of vaccines containing outer membrane vesicles from *Neisseria meningitidis*. *Vaccine* 2009; **27** (Suppl. 2):B3–12.

100 Rappuoli R, Mandl CW, Black S, De Gregorio E. Vaccines for the twenty-first century society. *Nat Rev Immunol* 2011; **11**:865–72.