ResearchGate

See discussions, stats, and author profiles for this publication at: https://www.researchgate.net/publication/277130361

Role of Mycobacterial Heat Shock Protein 70 (mHSP70) as Genetic Vaccine Adjuvants

Article *in* World Applied Sciences Journal · May 2011

CITATIONS
7

READS
106

2 authors:

Seyyed Mahmoud Ebrahimi
Baqiyatallah University of Medical Sciences
34 PUBLICATIONS **308** CITATIONS
SEE PROFILE

Majid Tebianian
Razi Vaccine and Serum Research Institute
66 PUBLICATIONS **387** CITATIONS
SEE PROFILE

Some of the authors of this publication are also working on these related projects:

Metabolomic studies of Anti-cancer Probiotic bacteria View project

Monitoring immune responses following vaccination by Razi Vet and human vaccine View project

All content following this page was uploaded by Seyyed Mahmoud Ebrahimi on 24 May 2015.

The user has requested enhancement of the downloaded file.

World Applied Sciences Journal 14 (10): 1569-1575, 2011
ISSN 1818-4952
© IDOSI Publications, 2011

Role of Mycobacterial Heat Shock Protein 70 (mHSP70) as Genetic Vaccine Adjuvants

[1]Seyyed Mahmoud Ebrahimi and [2]Majid Tebianian

[1]Research Center of Virus and Vaccine,
Baqiyatallah University of Medical Sciences, P.O. Box 14155-3651,Tehran, Iran
[2]Department of Biotechnology, Razi Vaccine and Serum Research Institute (RVSRI),
P.O. Box 31975/148, Karaj, Tehran, Iran

Abstract: One major challenge in developing effective vaccines is to design a vaccine that can induce effective immune responses to the desired antigen with no or very limited side effects. The immunogenicity can be improved by using appropriate carriers and adjuvant molecules. Heat shock proteins (HSPs) are some of the most conserved proteins present in all prokaryotes and eukaryotes which posses highly immunogenic effect and function as adjuvants that may play a crucial role in integrating innate and adaptive immunity. *Mycobacterium tuberculosis* HSP70 (mHSP70) consists of three functionally distinct domains: an N-terminal 44 kDa ATPase portion (amino acids 1-358), followed by an 18 kDa peptide-binding domain (amino acids 359-494) and a 10 kDa fragment (amino acids 495-610). However, the C-terminal portion (amino acids 359-610) was proven to stimulate the production of CC chemokines, interleukin-12 (IL-12), tumor necrosis factor-alfa (TNF-á), nitric oxide (NO) and the maturation of dendritic cells (DCs). In addition, a cytotoxic cell-inducing function was demonstrated in the ATPase portion (amino acids 161-370) of mHSP70. In this review, we peruse about several HSP-based vaccines as background for more detailed discussions about *M. tuberculosis* HSP molecule structure, function and usage as a novel vaccine adjuvants.

Key words: Adjuvant % HSP70 % Vaccine % Immune Responses

INTRODUCTION

The term of adjuvant derived from the latin word *adjuvare*, meaning help or aid, was first used by Ramon in 1926 and defined as a group of structurally heterogeneous compounds that enhance or modulate the immunogenicity of the poorly immunogenic vaccine proteins or peptides [1,2]. In vaccine development the choice of the adjuvant is often as important as the selection of the vaccine antigens themselves, which is sufficient to mimic natural infection or traditional vaccine.

The most appropriate adjuvant for a given vaccine antigen will depend on a large extent on the type of immune response that is required for protective immunity. Moreover, some adjuvants are strikingly potent, but also very harmful to the host. Therefore, the potency of an adjuvant often conflicts with host safety and tolerability.

Adjuvants can be used for various purposes; a) to enhance the immunogenicity of recombinant antigens, b) to reduce the amount of antigens or the number of immunizations needed for protective immunity, c) to improve the efficacy of vaccine in newborns, the elderly or immunocompromised persons and, d) as antigen delivery systems for the uptake of antigens by the mucosa [3-5].

Role of Adjuvants in the Immune Responses: Adjuvants as nonspecific enhancers of immune response appear to exert different mechanisms to improve the immune response to vaccine antigens, such as: a) Improve antigen delivery to antigen presenting cells (APCs), increase cellular infiltration, inflammation and trafficking to the injection site, b) Promote the activation state of APCs by upregulating costimulatory signals or MHC expression, inducing cytokine release, c) Enhance antigen processing and presentation by APCs and enhance the speed, magnitude and duration of the immune response, d) Modulate antibody avidity, affinity as well as the magnitude, isotype or subclass induction, e) Stimulate cell-mediated immunity and lymphocyte proliferation nonspecifically [2].

Corresponding Author: Seyyed Mahmoud Ebrahimi, Research Center of Virus and Vaccine, Baqiyatallah University of Medical Sciences, P.O. Box 14155-3651,Tehran, Iran. Tel: +98 21 66949644, Fax: +98 21 66949643.
E-mails: smebrahimi@shirazu.ac.ir & sm.ebrahimi@rvsri.ir.

Classification of Adjuvants: Adjuvants can be classified according to their source, mechanism of action or physicochemical properties [6]. They were classified into three groups based on their principal mechanisms of action ; a) Immunostimulatory adjuvants, being substances that increase the immune response to the selective antigen by directly activating antigen presenting cells (APCs) through specific receptors, such as toll-like receptors (TLRs), known as adjuvant receptors, b) carriers, being immunogenic proteins that provide T-cell help and c) particulate or vehicle adjuvants (Vaccine delivery systems), serve as a matrix for antigens, mainly function to localize vaccine components and to target vaccines to APCs. So, delivery systems are used to promote the interaction of both antigens and immunostimulators with the key cells of the innate immune system. Immunostimulatory adjuvants provide the inflammatory context necessary for optimal antigen-specific immune activation by activating APCs and amplifying the innate immune response [7].

Most Commonly Used Adjuvants: Adjuvants, currently licensed for human use include alum, squalane oil/water emulsion (MF59), influenza virosomes and some cytokines as IFN-(_and IL-2. A number of adjuvants are currently under investigation as DNA motifs, monophosphoryl lipid A, cholera toxin (CT), *E. coli* heat labile toxin (LT), Flt3 ligand (a pleotropic glycoprotein), immunostimulating complexes (ISCOMs), liposomes, saponins and non-ionic block copolymers [1, 3].

The Limitation of Currently Used Adjuvants: It is obvious from different studies that the currently licensed vaccine adjuvants are not sufficiently effective for the induction of efficient and appropriate immune responses. Several adjuvants, including microbial components have been evaluated for their ability to induce efficient immune responses in animal models as well as in preclinical/clinical studies. HSPs are one of the widely studied vaccine candidates [8-11].

Heat Shock Proteins (HSPs): Heat shock proteins (HSPs) are some of the most conserved proteins present in all prokaryotes and eukaryotes. These proteins undertake crucial functions in maintaining cell homeostasis and are essential for life since they behave as chaperons [12].

HSPs are expressed both constitutively (cognate proteins) and under stressful conditions (inducible forms). Constitutively expressed HSPs appear to serve as molecular chaperons (Figure 1), recognizing and binding

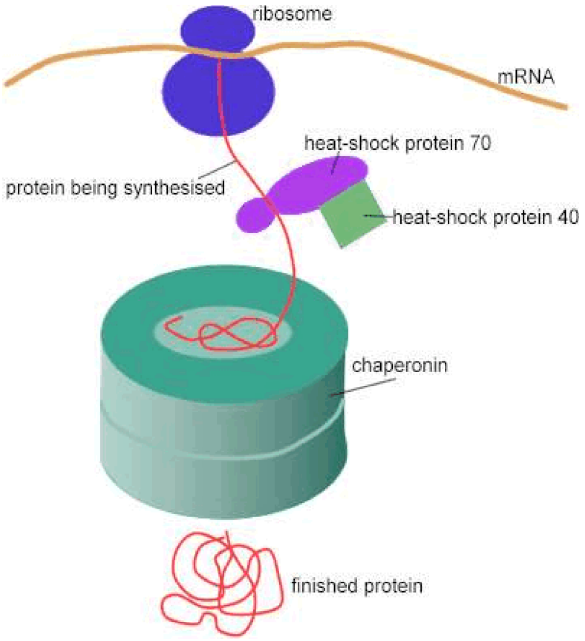

Fig. 1: A diagram showing interaction between heat-shock proteins and a chaperonin in protein folding. The figure was derived from the web site (http://www.palaeos.com/Eukarya/Lists/EuGlossary/Images/Hsp70.gif).

to nascent polypeptide chains and partially folded intermediates of proteins, preventing their aggregation and misfolding HSPs also participate in protein synthesis, suitable protein folding, assembly, trafficking and degradation [13-16]. Under stress situations, including environmental (heat shock, exposure to heavy metals or UV radiation), pathological (infections or fever, malignancies, inflammation or autoimmunity) or physiological stress (growth factor deprivation, cell differentiation, hormonal stimulation or tissue development) [17-19], synthesis of HSP is markedly increased to protect cells from damage [14, 20-21]. HSPs are classified based on their homology, related function and molecular mass. The most studied HSP families are HSP60, HSP70 and HSP90 [15].Among different studied HSP families, the HSP70 family is well characterized and has attracted much attention because of its versatile functions in the immune system. It is considered as the 'workhouse' of the chaperons, because of its promiscuity to assist in folding new polypeptide chains [22-24].

***M. tuberculosis* HSP70 Structure:** Microbial HSP70 especially that derived from *Mycobacterium tuberculosis* (mHSP70) is a 70 kD molecular chaperone possessing three functionally distinct domains: an N-terminal 44 kDa

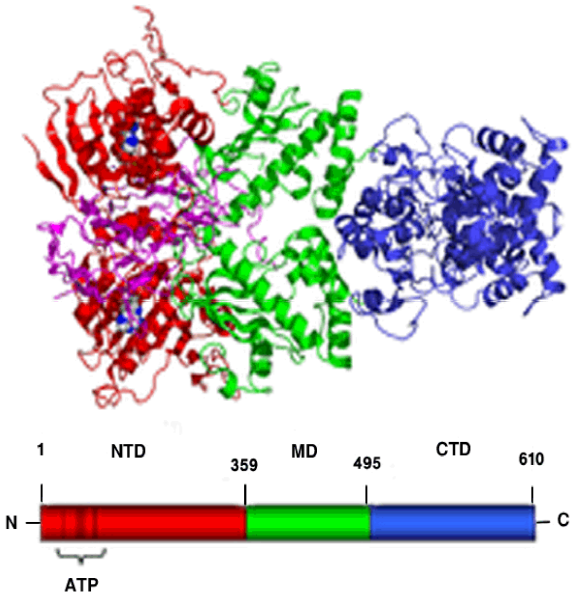

Fig. 2: Crystallogrphic structure of HSP70. NTD= N-terminal domain (red), MD=middle domain (green), CTD=C-terminal domain (blue). The figure was derived from the web site (http://www-personal.umich.edu/~zuiderwe).

ATPase portion (amino acids 1-358), followed by an 18 kDa peptide-binding domain (amino acids 359-494) and a 10 kDa fragment (amino acids 495-610) (Figure 2). The amino-terminus of HSP70, with mixed alpha-helices and betapleated sheets, binds and hydrolyzes ATP molecules which were driven from folding action of HSP70 [25]. The carboxy-terminal domain of mHSP70 (28 kD) is a loose beta barrel which binds 6-amino acid hydrophobic protein motifs, the object of folding activity [26].

HSP 70 as Adjuvant and Carrier: Besides the chaperone activity, HSP70 molecules can function as adjuvants [27-28]. HSP70s prepared from tumor cells or virus-infected cells are capable of eliciting $CD8^+$ CTL responses *in vivo* and *in vitro* against a variety of antigens expressed in the cells from which these immunogenic proteins have been purified [29]. Extremely small quantities of HSP70 bound peptide, around 120 pM, can generate a CTL response *in vivo*, whereas 2000-fold higher concentrations of free peptide was unable to do so [30-31]. However, a mutated mHSP70 with markedly decreased peptide binding affinity due to a point mutation in the peptide binding domain, could still induce the production of pro-inflammatory cytokines by Dendritic cells (DCs), but did not lead to CTL generation. Thus, the delivery of antigen can be independent of DC stimulation [32]. Extracellular mHSP70 can complex with antigenic peptides and simultaneously activate professional APCs. This interaction triggers a cascade of events, including re-presentation of chaperoned peptides to MHC I restricted $CD8^+$ and MHC II restricted $CD4^+$ T cells, secretion of proinflammatory cytokines and phenotypic and functional maturation of DCs [28, 33-37]. These properties combine to make mHSP70 a potent adjuvant that integrates innate and adaptive immune responses.

mHSP70 contains strong T-cell epitopes and serves as a carrier of antigens, effectively inducing antigen specific B cells as well as $CD4^+$ and $CD8^+$ T-cell responses without requiring an adjuvant [38- 45].

HSP70 Receptors and Mechanism of Adjuvanticity: The immunomodulatory functions of HSPs are based on the specific interaction of them with the receptors present on professional APCs (such as dendritic cells and macrophages) having three distinct consequences: (i) stimulation of chemokines production which attract immunological cells; (ii) activation of dendritic cells (DCs), thus initiating innate immune responses; and (iii) capability of delivering peptides to major histocompatibility complex molecules for the priming of adaptive immunity, therefore integrating innate and adaptive immune events [8,46-47].

The use of novel vaccine adjuvants or carrier proteins, which are known to enhance the immunogenicity of the subunit antigens and provide T-cell help, can improve the potency of vaccines based on small and low immunogen antigens. The potential of mHSP70 to function as adjuvants when fused to or co-delivered with protein antigens, make them attractive vaccine candidates [48-49].

Fusing mycobacterial HSP70 to HIV-1 gag p24 [41,50], Influenza M2e [9-11] and synthetic malarial antigen $(NANP)_{40,}$ a synthetic peptides consisting of 40 (Asn-Ala-Asn-Pro) repeats, [38], enhanced the immunogenicity of the antigens and obviated the need for adjuvant.

Mice immunized with a kineto plamid membrane protein-11 (KMP11) covalently fused to HSP70 from *Trypanosoma cruzi* elicited a CTL response against the Jurkat-A2/Kb cells expressing the KMP11 protein [51]. Moreover, HSP70 has been used as a carrier for group C meningococcal oligosaccharide, inducing antibodies against oligosaccharide in mice [52]. Furthermore, chimeric proteins formed by antigens coupled to the C-terminal fragment of HSP70 from *M. tuberculosis* [53-54] and N-terminal fragment from *Leishmania infantum* [44], induced humoral and cell mediated immune responses to the coupled antigens.

It was shown that the C-terminal fragment of mHSP70 acted as a carrier in mice when fused to the malarial antigen EB200 (HSP70-EB200) and considerably induced MHC responses [55]. The effects of two truncated HSP70 molecules, N-terminal domain $HSP70_{1-360}$, amino acids 1-360 and C-terminal domain $HSP70_{359-610}$, amino acids 359-610, of mycobacterial HSP70 was evaluated on the potency of antigen-specific immunity generated by a hepatitis B virus (HBV) DNA vaccination. It was shown that only the $HSP70_{359-610}$-fused HBV DNA vaccination resulted in a significant increase in hepatitis B surface antigen (HBsAg)-specific humoral response, while the $HSP70_{1-360}$ -fused vaccine did not. Moreover, $HSP70_{359-610}$-fused DNA vaccine did not induce anti-HSP70 antibody [48]. In other studies, the effects of $HSP70_{359-610}$ on foot and mouth virus (FMDV) and Japanese encephalitis virus (JEV) in mice were evaluated separately and the HSP70 markedly enhanced both the humoral and cell-mediated immune responses [56-57].

Hsp70 in Association with Autoimmunity: One of the concerns for the use of HSPs as adjuvants in human vaccines is the association of HSPs with the induction of autoimmune conditions.

One peculiar aspect of HSPs is their sequence conservation, leading to homologies between bacterial and mammalian members of the same HSP family. Therefore, immunization with mHSP70 might lead to the induction of immune responses against self-HSP, which may end up in autoimmune reactions.

Qazi *et al.* [58] analyzed sera from mHSP70 immunized and untreated mice for their capacity to bind to a number of antigens to detect auto-antibodies and pointed out that the presence of cross-reactive antibodies does not necessarily have to be correlated with autoimmunity. Cross-reactive antibodies are frequently detected in sera from healthy individuals and commonly induced in primary immune responses shortly after challenge with the antigens. Moreover, the concept of HSPs as a cause for autoimmunity is not clearly established. For instance, it has been shown that the prevention of certain autoimmune conditions could be achieved by pretreatment of animals with mycobacterial HSP70 or with some defined epitopes in the HSP sequences [58,59].

The use of HSPs as carriers is particularly interesting for the development of vaccine strategies, since most humans have been in contact with microbial HSPs through natural contacts with bacterial agents or tuberculosis vaccines. Therefore, the C-terminal domain of HSP70, $HSP70_{359-610}$, seems to be safe and enough immunogenic and its function in the form of linkage with small and low immunogen antigens as described above have persuaded researchers to consider them as attractive vaccine adjuvants.

CONCLUSIONS

One major challenge in developing effective vaccines is to design a vaccine that can induce effective immune responses to the desired antigen with no or very limited side effects. Poor immunogenicity and MHC restriction hamper the potential of many candidate antigens. The immunogenicity can be improved by using appropriate carriers and adjuvant molecules.

So, it seems by linking some antigen with limited potency to an appropriate carrier such as c-terminal 28-kDa domain of mHSP70 ($HSP70_{359-610}$), containing an 18-kDa peptide binding region (aa359-540), we can render it very immunogenic and increase the potency of these vaccines, without any side effects.

We expect several vaccines HSP-based adjuvant will be considered as a novel vaccine strategy toward effective controlling of infections of global importance.

REFERENCES

1. Marx, P.A., R.W. Compans, A. Gettie, J.K. Staas, R.M. Gilley, M.J. Mulligan, G.V. Yamshchikov, D. Chen and J.H. Eldridge, 1993. Protection against vaginal SIV transmission with microencapsulated vaccine. Sci., 260: 1323-7.
2. Gupta, R.K., E.H. Relyveld, E.B. Lindblad, B.Bizzini, S. Ben-Efraim and C.K. Gupta, 1993. Adjuvants-a balance between toxicity and adjuvanticity. Vaccine. 11: 293-306.
3. Vogel, F.R., 1995. Immunologic adjuvants for modern vaccine formulations. Ann. N.Y. Acad. Sci., 754: 153-60.
4. Douce, G., C. Turcotte, I. Cropley, M. Roberts, M. Pizza, M. Domenghini, R. Rappuoli and G. Dougan, 1995. Mutants of *Escherichia coli* heat-labile toxin lacking ADP ribosyltransferase activity act as nontoxic, mucosal adjuvants. Proc. Natl. Acad. Sci. USA., 92: 1644-8.
5. McElrath, M.J., 1995. Selection of potent immunological adjuvants for vaccine construction. Semin. Cancer. Biol., 6: 375-85.
6. Vogel, F.R., 1998. Adjuvants in perspective. Dev. Biol. Stand. 92: 241-8.
7. Allison, A.C. and N.E. Byars, 1991. Immunological adjuvants: desirable properties and side effects. Mol. Immunol., 28: 279-84.

8. Ebrahimi, S.M., M. Tebianian and H. Paykari, 2010a. Cloning, expression and purification of the truncated C-terminal fragment of *Mycobacterium tuberculosis* HSP70 gene in prokaryotic system as a tool for vaccine research. Middle- East J. Scientific Sci., 3: 128-133.
9. Ebrahimi, S.M., M. Tebianian, H. Toghyani, A. Memarnejadian and H. R. Attaran, 2010b. Cloning, expression and purification of the influenza A (H9N2) virus M2e antigen and truncated Mycobacterium tuberculosis HSP70 as a fusion protein in Pichia pastoris. Protein Expression and Purification, 70: 7-12.
10. Ebrahimi, S.M., M. Tebianian, A. Mirjalili, H. Paykari, H. R. Varshovi, H.Toghyani, S. Moradi Bidhendi and H.R. Attaran, 2009. Fusion and sequence analysis of the influenza A (H9N2) virus M2e and C-terminal fragment of Mycobacterium tuberculosis HSP70 (H37Rv). Archives of Razi Institute, 64: 71-76.
11. Ebrahimi, S.M. and M. Tebianian, 2010. Heterologous expression, purification and characterization of the influenza A virus M2e gene fused to Mycobacterium tuberculosis HSP70359-610 in prokaryotic system as a fusion protein. Mol. Biol. Rep., 37(6): 2877-2883.
12. Smith, D.F., L. Whitesell and E. Katsanis, 1998. Molecular chaperones: biology and prospects for pharmacological intervention. Pharmacol. Rev., 50: 493-514.
13. Lindquist, S. and E.A. Craig, 1988. The heat-shock proteins. Annu. Rev. Genet., 22: 631-677.
14. Jaattela, M., 1999. Heat shock proteins as cellular lifeguards. Ann. Med., 31: 261-71.
15. Fink, A.L., 1999. Chaperone-mediated protein folding. Physiol. Rev., 79: 425-449.
16. Hartl, F.U. and M. Hayer-Hartl, 2002. Molecular chaperones in the cytosol: from nascent chain to folded protein. Sci., 295: 1852-58.
17. Jacquier-Sarlin, M.R., K. Fuller, A.T Dinh-Xuan, M.J. Richard and B.S. Polla, 1994. Protective effects of hsp70 in inflammation. Experientia, 50: 1031-8.
18. Moseley, P.L., 1998. Heat shock proteins and the inflammatory response. Ann. NY. Acad. Sci., 856: 206-13.
19. Feder, M.E. and G.E. Hofmann, 1999. Heat-shock proteins, molecular chaperones and the stress response: evolutionary and ecological physiology. Annu. Rev. Physiol., 61: 243-82.
20. Gething, M.J., S. Blond-Elguindi, J. Buchner, A. Fourie, G. Knarr, S. Modrow, L. Nanu, M. Segal and J. Sambrook, 1995. Binding sites for Hsp70 molecular chaperones in natural proteins. Cold. Spring Harb. Symp. Quant. Biol., 60: 417-28.
21. Laroia, G., R. Cuesta, G. Brewer and R.J. Schneider, 1999. Control of mRNA decay by heat shock-ubiquitinproteasome pathway. Sci., 284: 499-502.
22. Beckmann, R.P., L.E. Mizzen and W.J Welch, 1990. Interaction of Hsp 70 with newly synthesized proteins: implications for protein folding and assembly. Sci., 248: 850-4.
23. Liberek, K., D. Skowyra, M. Zylicz, C. Johnson and C. Georgopoulos, 1991. The *Escherichia coli* DnaK chaperone, the 70-kDa heat shock protein eukaryotic equivalent, changes conformation upon ATP hydrolysis, thus triggering its dissociation from a bound target protein. J. Biol. Chem., 266: 14491-6.
24. Hartl, F.U., 1996. Molecular chaperones in cellular protein folding. Nature, 381: 571-9.
25. Flaherty, K.M., C. De Luca-Flaherty and D.B. McKay, 1990. Three-dimensional structure of the ATPase fragment of a 70K heat-shock cognate protein. Nature, 346: 623-628.
26. Zhu, X., Z. Zhao, W.F. Burkholder, A. Gragerov, C.M. Ogata, M.E. Gottesman and W.A. Hendrickson, 1996. Structural analysis of substrate binding by the molecular chaperone DnaK. Sci., 272: 1606-1614.
27. Vabulas, R.M., P. Ahmad-Nejad, S. Ghose, C.J. Kirschning, R.D. Issels and H.Wagner, 2002. HSP70 as endogenous stimulus of the Toll/interleukin-1 receptor signal pathway. J. Biol. Chem., 277: 15107-12.
28. Asea, A., S.K. Kraeft, E.A. Kurt-Jones, M.A. Stevenson, L.B. Chen, R.W. Finberg, G.C. Koo and S.K. Calderwood, 2000. HSP70 stimulates cytokine production through a CD14-dependant pathway, demonstrating its dual role as a chaperone and cytokine. Nat. Med., 6: 435-42.
29. Srivastava, P., 2002. Roles of heat-shock proteins in innate and adaptive immunity. Nat. Rev. Immunol., 2: 185-94.
30. Minton, K., 2004. Antigen presentation: Shocking stimulation. Nature Reviews Immunol., 4: 162.
31. Javid, B., P.A. MacAry, W. Oehlmann, M.Singh and P.J. Lehner, 2004. Peptides complexed with the protein HSP70 generate efficient human cytolytic T-lymphocyte responses. Biochem. Soc. Trans., 32: 622-5.
32. MacAry, P.A., B. Javid, R.A. Floto, K.G. Smith, W. Oehlmann, M. Singh and P.J. Lehner, 2004. HSP70 peptide binding mutants separate antigen delivery from dendritic cell stimulation. Immunity. 20: 95-106.

33. Castellino, F., P.E. Boucher, K. Eichelberg, M. Mayhew, J.E. Rothman, A.N. Houghton and R.N. Germain, 2001. Receptor-mediated uptake of antigen/heat shock protein complexes results in major histocompatibility complex class I antigen presentation via two distinct processing pathways. J. Exp. Med., 191: 1957-64.
34. Basu, S., R.J. Binder, T. Ramalingam and P.K. Srivastava, 2001. CD91 is a common receptor for heat shock proteins gp96, hsp90, hsp70 and calreticulin. Immunity. 14: 303-13.
35. Harmala, L.A. and E.G. Ingulli, J.M. Curtsinger, M.M. Lucido, C.S. Schmidt, B.J.Weigel, B.R. Blazar, M.F. Mescher and C.A. Pennell, 2002. The adjuvant effects of *Mycobacterium tuberculosis* heat shock protein 70 result from the rapid and prolonged activation of antigen-specific CD8+ T cells *in vivo*. J. Immunol., 169: 5622-9.
36. Tobian, A.A., D.H. Canaday and C.V. Harding, 2004a. Bacterial heat shock proteins enhance class II MHC antigen processing and presentation of chaperoned peptides to CD4+ T cells. J. Immunol., 173: 5130-7.
37. Tobian, A.A., D.H. Canaday, W.H. Boom and C.V. Harding, 2004b. 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., 172: 5277-86.
38. Barrios, C., A.R. Lussow, J. Van Embden, R. Van der Zee, R. Rappuoli, P. Costantino, J.A. Louis, P.H. Lambert and G. Del Giudice, 1992. Mycobacterial heat-shock proteins as carrier molecules. II: The use of the 70-kDa mycobacterial heat-shock protein as carrier for conjugated vaccines can circumvent the need for adjuvants and Bacillus Calmette Guérin priming. Eur. J. Immunol., 22: 1365-72.
39. Barrios, C., C. Georgopoulos, P.H. Lambert and G. Del Giudice, 1994. Heat shock proteins as carrier molecules: *in vivo* helper effect mediated by *Escherichia coli* GroEL and DnaK proteins requires cross-linking with antigen. Clin. Exp. Immunol., 98: 229-33.
40. Del Giudice, G., 1994. Hsp70: a carrier molecule with built-in adjuvanticity. Experientia, 50: 1061-6.
41. Suzue, K. and R.A. Young, 1996a. Adjuvant-free hsp70 fusion protein system elicits humoral and cellular immune responses to HIV-1 p24. J. Immunol., 156: 873-9.
42. Roman, E. and C. Moreno, 1996. Synthetic peptides non-covalently bound to bacterial hsp 70 elicit peptidespecific T-cell responses *in vivo*. Immunol., 88: 487-92.
43. Rico, A.I., G. Del Real, M. Soto, L. Quijada, A.C. Martinez, C. Alonso and J.M. Requena, 1998. Characterization of the immunostimulatory properties of *Leishmania infantum* HSP70 by fusion to the *Escherichia coli* maltose-binding protein in normal and nu/nu BALB/c mice. Infect. Immun., 66: 347-52.
44. Rico, A.I., S.O. Angel, C. Alonso and J.M. Requena, 1999. Immunostimulatory properties of the *Leishmania infantum* heat shock proteins HSP70 and HSP83. Mol. Immunol., 36: 1131-9.
45. Udono, H., T. Yamano, Y. Kawabata, M. Ueda and K. Yui, 2001. Generation of cytotoxic T lymphocytes by MHC class I ligands fused to heat shock cognate protein 70. Int. Immunol., 13: 1233-42.
46. Cusi, M.G., C. Terrosi, G.G. Savellini, G. Di Genova, R. Zurbriggen and P.Correale, 2004. Efficient delivery of DNA to dendritic cells mediated by influenza virosomes. Vaccine, 22: 735-739.
47. Calarota, S.A. and D.B Weiner, 2004. Enhancement of human immunodeficiency virus type 1-DNA vaccine potency through incorporation of T-helper 1 molecular adjutants. Immunol. Rev., 199: 84-99.
48. Li, X., X. Yang, L. Li, H. Liu and J. Liu, 2006. A truncated C-terminal fragment of *Mycobacterium tuberculosis* HSP70 gene enhanced potency of HBV DNA vaccine. Vaccine, 24: 3321-3331.
49. Qazi, K.R., W. Oehlmann, M. Singh, M.C López and C. Fernández, 2007. Microbial heat shock protein 70 stimulatory properties have different TLR requirements. Vaccine, 25: 1096-1103.
50. Suzue, K. and R.A. Young, 1996b. Heat shock proteins as immunological carriers and vaccines. EXS 77: 451- 65.
51. Maranon, C., M.C. Thomas, L. Planelles and M.C. Lopez, 2001. The immunization of A2/K(b) transgenic mice with the KMP11-HSP70 fusion protein induces CTL response against human cells expressing the *T. cruzi* KMP11 antigen: identification of A2-restricted epitopes. Mol. Immunol., 38: 279-87.
52. Perraut, R., A.R. Lussow, S. Gavoille, O. Garraud, H. Matile, C. Tougne, J. Van Embden, R.Van Der Zee, P.H. Lambert and J. Gysin, 1993. Successful primate immunization with peptides conjugated to purified protein derivative or mycobacterial heat shock proteins in the absence of adjuvants. Clin. Exp. Immunol., 93: 382-6.

53. Wang, Y., C.G. Kelly, M. Singh, E.G. McGowan, A.S. Carrara, L.A. Bergmeier and T. Lehner, 2002. Stimulation of Th1-polarizing cytokines, C-C chemokines, maturation of dendritic cells and adjuvant function by the peptide binding fragment of heat shock protein 70. J. Immunol., 169: 2422-9.
54. Lehner, T., Y. Wang, T. Whittall, E. McGowan, C.G. Kelly and M. Singh, 2004. Functional domains of HSP70 stimulate generation of cytokines and chemokines, maturation of dendritic cells and adjuvanticity. Biochem. Soc. Trans., 32: 629-32.
55. Qazi, K.R., M. Wikman, N.M. Vasconcelos, K. Berzins, S. Ståhl and C. Fernandez, 2005. Enhancement of DNA vaccine potency by linkage of *Plasmodiumfalciparum* malarial antigen gene fused with a fragment of HSP70 gene. Vaccine, 23: 1114-1125.
56. Chunxia, S., X. Duan, X. Wang, C. Wang, R. Cao, B. Zhou and P. Chen, 2006. Heterologous expression of FMDV immunodominant epitopes and HSP70 in *P. pastoris* and the subsequent immune response in mice. Veterinary Microbiol., 124: 256-263.
57. Fei-fei, G. and Y. Qiu, X. Gao, Y. Yang and P. Chen, 2006. Fusion expression of major antigenic segment of JEV E protein-hsp70 and the identification of domain acting as adjuvant in hsp70. Veterinary Immunology and Immunopathol., 113: 288-296.
58. Qazi, K.R., M.R. Qazi, E. Julian, M. Singh, M. Abedi-Valugerdi and C. Fernandez, 2005. Exposure to Mycobacteria Primes the Immune System for Evolutionarily Diverse Heat Shock Proteins. Infection and Immunity 73: 7687-7696.
59. Van Eden, W., J.E. Thole, R. Van Der Zee, A. Noordzij, J. D. Van Embden, E. J. Hensen and I.R. Cohen, 1988. Cloning of the mycobacterial epitope recognized by T lymphocytes in adjuvant arthritis. Nature, 331: 171-173.

View publication stats