Original article

Hsp70 enhances presentation of FMDV antigen to bovine CD4+ T cells in vitro

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Abstract – Foot-and-mouth disease virus (FMDV) is the causative agent of a highly contagious acute vesicular disease affecting cloven-hoofed animals, including cattle, sheep and pigs. The current vaccine induces a rapid humoral response, but the duration of the protective antibody response is variable, possibly associated with a variable specific CD4+ T cell response. We investigated the use of heat shock protein 70 (Hsp70) as a molecular chaperone to target viral antigen to the Major Histocompatibility Complex (MHC) class II pathway of antigen presenting cells and generate enhanced MHC II-restricted CD4+ T cell responses in cattle. Monocytes and CD4+ T cells from FMDV vaccinated cattle were stimulated in vitro with complexes of Hsp70 and FMDV peptide, or peptide alone. Hsp70 was found to consistently improve the presentation of a 25-mer FMDV peptide to CD4+ T cells, as measured by T cell proliferation. Complex formation was required for the enhanced effects and Hsp70 alone did not stimulate proliferation. This study provides further evidence that Hsp70:peptide complexes can enhance antigen-specific CD4+ T cell responses in vitro for an important pathogen of livestock.

1. INTRODUCTION

Foot-and-mouth disease virus (FMDV) causes a highly contagious, clinically acute vesicular disease, affecting cloven-hoofed animals, including the economically important cattle, sheep and pigs. Infection of susceptible animals with FMDV results in a rapid neutralising antibody response that becomes detectable as early as 3–4 days post-infection. Isotype switching typically occurs between 4 and 7 days, and results predominantly in production of IgG1, but with detectable levels of IgG2. The onset of the IgG1 response coincides with clinical resolution and viral clearance, with serum levels of IgG peaking at approximately 14–28 days [2, 30]. The early antibody response has recently been demonstrated to be T-independent. Selective depletion of CD4+ T cells from cattle during the acute phase of infection had no effect on the magnitude or duration of clinical signs or clearance of virus from the circulation [21]. The CD4+ T cell depleted animals generated a similar neutralising antibody response to the control animals and rapid class switching to IgG antibody still occurred.

Following infection with FMDV, protective immune responses in cattle can be maintained

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for several years. Memory CD4^{+} T cell responses are usually weak and not easily detectable and therefore are unlikely to significantly contribute to the observed memory response. Instead, viral persistence is thought to form a major contribution to the extended duration of immunity after natural infection [20]. Currently the most effective vaccine against FMDV is a chemically-inactivated whole virus preparation with adjuvant. This vaccine, like natural infection, induces a rapid neutralising antibody response, but it remains to be determined whether this is also T-independent [10]. The vaccine is usually sufficient to protect against clinical disease, but not infection. Although vaccinated cattle may only become sub-clinically infected, they are just as likely to become carriers following virus exposure as non-vaccinated animals, which is a major concern for international trade [1]. Vaccine-induced immunity is short-lived and repeat vaccinations maybe required every 6 months.

While the T cell response is not known to directly affect the outcome of infection, more effective CD4^{+} T cell responses might support the induction of sterile immunity and extend the duration of the protective immune response. In support of this hypothesis, high levels of interferon-gamma (IFN-γ) detected in vaccinated cattle in in vitro restimulation assays prior to challenge with FMDV correlated with the animals' ability to control viral replication, suggesting that cell-mediated immunity, as well as humoral immunity, is important for vaccine-induced immunity [27].

Targeting antigen to the MHC class II pathway of antigen presenting cells, particularly dendritic cells, may improve CD4^{+} T cell activation. Various methods are currently being studied to improve antigen delivery, including the use of heat shock proteins as antigen chaperones. The ability of heat shock protein 70 (Hsp70) to enhance cross-presentation of antigen to CD8^{+} T cells via the MHC class I pathway has been demonstrated for a number of viral antigens [6–8, 23, 25, 28, 31, 33, 34] and clinical trials in phases I–III are on-going [5, 22, 32].

However, the ability of Hsp70 to enhance presentation of antigen to CD4^{+} T cells via the MHC class II pathway is less well estab-

lished. The possible role of Hsp70 related chaperones in peptide transport and loading onto MHC II was first noted by DeNagel and Pierce [9] who hypothesised that chaperones may improve the efficiency of loading. The over expression of Hsc73, a constitutively expressed member of the Hsp70 family, was also shown to enhance the processing and presentation of exogenous antigen in macrophages by binding the antigen following its internalisation, and transporting it to MHC II molecules [26]. This is supported by in vitro quantitative binding assays, which demonstrated that Hsp70 can interact directly with peptides destined for MHC II loading, as well as whole proteins [17].

A series of studies conducted in mice or with human T cell clones showed that the presentation of MHC II-restricted epitopes could be improved by the exogenous delivery of peptides chaperoned by heat shock proteins Hsp70 and Gp96 [11, 31, 35, 37]. In addition, enhanced antigen-specific proliferation of CD4^{+} T cells from immunised human donors, to peptides representing MHC II-restricted epitopes from tetanus toxoid and influenza haemagglutinin, was also observed using exogenously added human Hsp70 complexes [16]. This effect was most pronounced at low doses of antigen and decreasing APC:T cell ratios, particularly relevant in the context of vaccination.

Here we describe the capacity of bovine Hsp70 to enhance recognition of FMDV peptide by bovine memory CD4^{+} T cells in vitro and show this enhanced recognition is dependent on internal processing of the antigen complex. This study provides further evidence of the potential use of Hsp70 as an antigen delivery vehicle for FMDV vaccines.

2. MATERIALS AND METHODS

2.1. Recombinant Hsp70 purification

The expression vector pQE9 carrying the bovine cardiac Hsp70 gene (GenBank accession number AY662497) with an N-terminal histidine tag was kindly provided by Dr Lakshmikuttyamma (University of Saskatchewan, Canada). Protein was expressed in Escherichia coli M15 (pREP4) cells

and purified using His-Select Nickel Affinity Gel (Sigma, Poole, UK) as described in Lakshmikuttyamma et al. [24]. Buffer was exchanged by dialysis against PBS in Slide-A-Lyser 3.5kDA MWCO dialysis cassettes and protein was concentrated using Centricon YM-50 spin columns (Millipore, Livingston, UK). Endotoxin was depleted using Detoxigel endotoxin removing gel (Pierce, Dorchester, UK). Quantitation of endotoxin content was performed using the *Limulus amoebocyte* lysate assay (QCL-1000, Cambrex Bioscience, St. Albans, UK). The resulting endotoxin content was below 21 EU/mg of purified Hsp70 protein.

Protein was quantified using a BCA reagent (Pierce) against a standard of bovine serum albumin. Purity was determined by SDS-PAGE and silver staining using the SilverSNAP Stain Kit II (Pierce). For Western blotting, protein was transferred to Hybond-C nitrocellulose membrane and probed with an anti-human Hsp70 monoclonal antibody stated by the manufacturer to cross-react with bovine Hsp70 (SPA-810, Stressgen, Cambridge Bioscience, Cambridge, UK).

2.2. Peptides

The FMDV 25-mer peptide BC2 LVGALLRTA-TYYFADLEVAVKHEGN was synthesised in-house using standard fluorenylmethoxycarbonyl chemistry and corresponds to amino acid residues 61–85 on the structural protein 1D (VP1) of FMDV serotype O/UKG/35/2001. BC2 is an extended peptide of the 15-mer p252 which was demonstrated, in a screen of over 400 pentadecapeptides, to contain an epitope recognised by CD4$^+$ T cells from cattle expressing the class II allele DRB3*0701 which is linked to the MHC I haplotype A31 [13]. The MHC class II restriction of p252 has been demonstrated [14], but the DRB3*0701 molecule has not been functionally demonstrated to present the epitope. In some control experiments, an irrelevant peptide from the Core protein of classical swine fever virus (CSFV) was used. This peptide corresponds to amino acid residues 81–95 (KLEKALLAWAVIAIV).

2.3. Hsp70 peptide complex formation

To demonstrate Hsp70:BC2 complex formation, a competitive binding assay was performed as previously described [28]. Briefly, BC2 was first biotinylated using the EZ-Link Biotinylation kit (Pierce). Hsp70 (2 μM) was incubated with the biotinylated peptide (60 μM), with or without an excess of unlabelled peptide, in PBS in a volume of 55 μL for 1 h at 37 °C. Samples containing approximately 1 μg of Hsp70 were resolved on a non-reducing 10% SDS-PAGE gel. To minimise complex dissociation during preparation, the loading buffer contained 0.1% SDS and no reducing agent, and the samples were not boiled prior to loading. The proteins were then transferred to Hybond-C nitrocellulose membrane and probed with HRP-conjugated streptavidin (Southern Biotechnology, Cambridge Bioscience). The control CSFV peptide was also biotinylated and incubated with Hsp70 to form a complex; Control CSFV peptide/Hsp70 complex formation was verified by Western blot analysis as above.

For the proliferation experiments, Hsp70 (3 μg) and peptide (15 ng) were incubated for at least 1 h at 37 °C in 30 μL PBS. Before adding to cells, the complex was diluted in medium and 100 μL was added per well to give the indicated final concentration, giving a peptide-to-Hsp70 ratio of approximately 1:10. Hsp70 or peptide only controls were treated identically.

2.4. Cattle

Calves (*Bos taurus*) were British Holstein Friesians conventionally reared at the Institute for Animal Health (IAH, Compton, UK). The animals used in this study were from a partially inbred herd in which MHC class I haplotypes had been characterised at the level of expressed genes [12]. The class II genes of these cattle have not been fully characterised, so for the purposes of this study, animals which were previously found to respond to p252 by proliferation assay were considered responders to the extended BC2 peptide. Some of the cattle had previously been vaccinated twice with a single bovine dose of commercially available FMDV serotype O inactivated vaccine (Merial, Pirbright, UK). All experiments were approved by the Institute's ethical review process and were in accord with national guidelines on animal use.

2.5. Culture media

Culture medium comprised RPMI 1640 supplemented with 10% heat-inactivated foetal calf serum (Autogen, Calne, UK), 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 10 μg/mL gentamycin and 50 mM 2-mercaptoethanol, subsequently termed complete medium.

2.6. Cells

Monocytes were isolated from bovine peripheral blood mononuclear cells (PBMC) using anti-human

CD14 paramagnetic microbeads (Miltenyi-Biotec, Woking, UK). Labelled cells were isolated from a Midimacs LS column (Miltenyi-Biotec) according to the manufacturer's instructions.

CD4$^+$ T cells were isolated from PBMC using mouse anti-bovine CD4$^+$ antibody (clone CC30, IAH Compton) [18] followed by rat anti-mouse IgG1 microbeads (Miltenyi-Biotec) as above. Typically, purity following selection of CD4$^+$ was over 95% as determined by flow cytometry.

2.7. CD4$^+$ T cell proliferation assay

Proliferation experiments were performed as previously described [15]. Briefly, triplicate wells of monocytes (5×10^3/well) were incubated with antigens as indicated and CD4$^+$ T cells (2×10^5/well) in a total volume of 200 μL of complete medium in u-bottomed 96 well microtitre plates. Medium alone and Pokeweed mitogen (PWM) stimulated cells (2 μg/mL) were used as negative and positive controls, respectively. A sample of the antigen used for vaccination (diluted 1/1000 in complete medium) was used for assays with cells from vaccinated animals. After 5 days, cells were pulsed with 37 kBq [3H] thymidine, per well, diluted in complete medium and incubated for a further 16 h before harvesting onto filter mats. The radioactive thymidine incorporated into the DNA of proliferating cells was determined by liquid scintillation counting using a Trilux Microbeta counter (Wallac, Beaconsfield, UK) and expressed as counts per minute (cpm). For monocyte fixation experiments, monocytes were fixed in 0.5% paraformaldehyde for 30 min at 4 °C and then washed, added to antigens and CD4$^+$ T cells and assayed as above.

2.8. Interferon-gamma ELISA

Cultures were set up as for proliferation assays, but after 72 h the supernatants were harvested and assayed for IFN-γ by capture ELISA. Nunc Maxisorp ELISA plates were coated with anti-IFN-γ antibody (clone cc330, Serotec) diluted in 0.1 M sodium bicarbonate buffer (2 μg/mL) overnight at room temperature. The wells were washed with PBS/0.05% Tween 20 and blocked for 1 h with blocking buffer (1 mg/mL sodium casein in PBS) and washed again. Samples, including an IFN-γ standard titration and a negative control of blocking buffer only (50 μL per well), were added to the wells and incubated for 1 h at room temperature. The wells were washed, incubated with biotinylated anti-IFN-γ antibody (clone cc302, Serotec (diluted in blocking buffer (2 μg/mL))) for

1 h at room temperature and then washed again. An HRP-conjugated streptavidin antibody diluted 1/500 in blocking buffer was added for 45 min at room temperature before development with o-Phenylenediamine dihydrochloride (OPD; Sigma) according to manufacturer's instructions. The reaction was stopped with 25 μL/well of 2 M sulphuric acid and the absorbance read at 450 nm. IFN-γ concentration was determined against a standard of recombinant bovine IFN-γ in the range of 10–0.0137 ng/mL.

2.9. Data analysis

Statistical analyses were performed using the statistical software GraphPad Prism version 4. For proliferation assays, the responses from medium, BC2, Hsp70:BC2, Hsp70 + BC2 and Hsp70 stimulated cells were analysed by one-way ANOVA followed by Tukey's post-test comparison to detect differences between Hsp70:peptide complexes versus peptide alone. p values < 0.05 were considered statistically significant.

3. RESULTS

3.1. Purification and characterisation of recombinant bovine Hsp70

The identity and purity of the preparation was confirmed by silver staining and Western blot. Apart from a single clear band at approximately 70 kDa, no additional bands were observed on a silver stained gel after 1 μg of Hsp70 was resolved. The gel was exposed until a band of the expected size stained with high intensity (Fig. 1A). A single protein of the predicted size was also recognised by Western blot analysis using a monoclonal antibody raised against human Hsp70 (Fig. 1B).

3.2. Hsp70 binds FMDV peptide BC2

The capacity of recombinant bovine Hsp70 to bind the FMDV 25-mer peptide BC2 was tested. The peptide was first biotinylated and then complexes were formed by co-incubating with Hsp70 for 1 h at 37 °C. Complex formation was then confirmed by non-reducing SDS-PAGE followed by Western blot analysis using streptavidin-HRP to detect biotin. No signal was detected at ~ 70 kDa in the
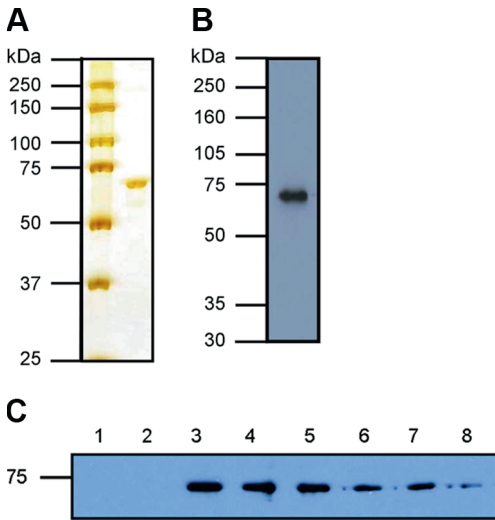
Figure 1. Purification of recombinant bovine Hsp70 and formation of Hsp70:peptide complexes. (A) Purified Hsp70 (1 μg) was resolved by SDS-PAGE on a 10% gel and detected by silver staining. Left hand lane indicates molecular weight markers. (B) Purified Hsp70 (250 ng) was analysed by Western blot using an anti-Hsp70 monoclonal antibody (SPA-810, Stressgen). (C) 2 μM Hsp70 was incubated with 60 μM biotinylated peptide and increasing amounts of unlabelled peptide in 55 μL PBS at 37 °C for 1 h to form complexes. Fractions containing an equivalent of 1 μg Hsp70 were resolved by non-reducing SDS-PAGE on a 10% gel, followed by Western blot analysis using streptavidin-HRP to detect biotinylated peptide. Controls of Hsp70 and biotinylated peptide only were run in lanes 1 and 2 respectively. Lanes 4–8 additionally contain unlabelled peptide at 6 μM, 60 μM, 150 μM, 300 μM and 600 μM (0.1×, 1×, 2.5×, 5× and 10× molar concentration of labelled peptide). (A color version of this figure is available at www.vetres.org.)

control lanes containing either Hsp70 or biotinylated-BC2 alone; however a clear signal was observed when both components were pre-incubated prior to gel loading. As increasing amounts of unlabelled peptide were added to the mix to compete out the labelled peptide, the anti-biotin signal decreased accordingly. Equal quantities of Hsp70 were loaded in each well (Fig. 1C). For subsequent experiments,

complexes were formed with 3 μg Hsp70 and 15 ng peptide. A clear signal was also detected at ∼ 70 kDa when Hsp70 was incubated with biotinylated CSFV peptide, but not in the control lanes containing Hsp70 or biotinylated peptide alone (data not shown).

3.3. Enhanced proliferation of memory CD4$^+$ T cells to Hsp70 complexed BC2

Three cattle vaccinated against FMDV (FMD10, FMD17 and FMD18) were available with a MHC haplotype recognising the epitope contained within p252. When monocytes and autologous CD4$^+$ T cells isolated from the three cattle were stimulated with a sample of the antigen used for FMDV vaccination, similar proliferation responses were observed. To determine an appropriate concentration of BC2 peptide to use in the complexes, a titration of the peptide was performed (Fig. 2A). 5 ng/mL was found to be a suboptimal concentration and was therefore used in subsequent experiments with Hsp70 complexes formed at a peptide-to-Hsp70 ratio of approximately 1:10 as previously determined to be optimal [16]. Preliminary experiments indicated that an excess molar concentration of Hsp70 in the complex did not increase the proliferative response.

Equal concentrations of BC2 peptide or BC2:Hsp70 complex, pre-formed in a 30 μL volume, were tested for their ability to stimulate the same cells. In comparison to treatment with BC2 in the absence of Hsp70, BC2:Hsp70 complex resulted in significantly higher proliferation ($p < 0.001$; one-way ANOVA followed by post-hoc Tukey) for all three animals (Figs. 2B–D). However, variation in the magnitude of the enhancement was observed between animals. Using cells from FMD17 the enhancement was approximately 60-fold (Fig. 2C), compared to approximately 30-fold for FMD10 (Fig. 2B) and threefold for FMD18 (Fig. 2D). These results were consistent across three separate experiments for each animal. When monocytes were fixed before the addition of antigens, in order to prevent internal processing, the T cell response to the Hsp70:BC2 complex was significantly diminished ($p < 0.001$; two-way ANOVA followed by post-hoc
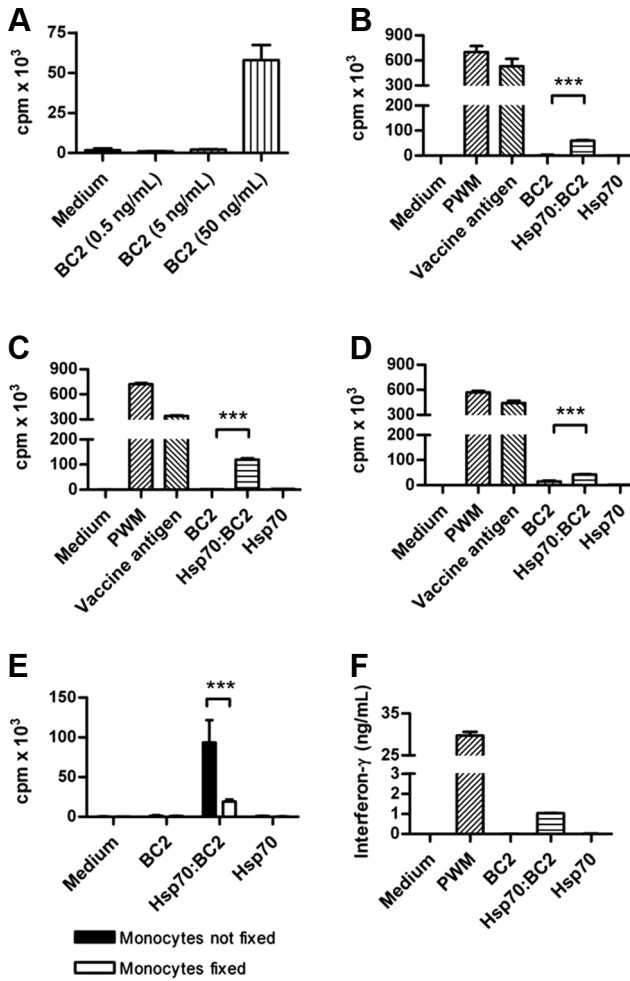
Figure 2. CD4^{+} T cell proliferation induced by monocytes to BC2 and Hsp70:BC2 complexes. (A) Monocytes isolated from cattle vaccinated against FMDV were co-incubated with autologous CD4^{+} T cells and FMDV 25-mer peptide BC2 at 0.5, 5 and 50 ng/mL. After 5 days, wells were pulsed with 37 kBq [3H] thymidine and incubated for a further 16 h before harvesting. Incorporated radioactivity was determined by liquid scintillation counting and expressed as counts per minute (cpm). Data are presented as the cpm $\times 10^3$/min mean ± S.D. of triplicate cultures. (B–D) Monocytes isolated from cattle vaccinated against FMDV were co-incubated with autologous CD4^{+} T cells and either FMDV 25-mer peptide BC2 (5 ng/mL), Hsp70 (1 μg/mL) or BC2 and Hsp70, at the same final concentrations, but pre-incubated to form a complex. Responses to Pokeweed mitogen (PWM) and vaccine antigen are also indicated. Proliferation was assessed as above. One representative data set of three is shown for each of the three animals. Significant differences in proliferation between BC2 and Hsp70:BC2 stimulated cells are indicated (*** $p < 0.001$). (E) Monocytes were fixed with 0.5% paraformaldehyde before incubation with antigens and T cells. Significant differences in proliferation between Hsp70:BC2 stimulated cells using fixed or unfixed monocytes are indicated (*** $p < 0.001$). (F) 72 h after stimulation, supernatants from proliferation assays were assayed for IFN-γ production by capture ELISA. Data are presented as ng/mL mean ± S.D. of triplicate wells.

Bonferroni). Across five separate experiments, the T cell response had a mean reduction of 83%. Results from one experiment are shown in Figure 2E. Cultures were set up alongside the proliferation assays to assess the production of IFN-γ from CD4$^+$ T cells induced by monocytes which had been stimulated with Hsp70 complexes. After 72 h, the concentration of IFN-γ in 200 μL of the culture supernatant was measured by capture ELISA and was found to correlate closely with CD4$^+$ T cell proliferation. Results from one of three replicate experiments are shown in Figure 2F.

With only three cattle of an appropriate haplotype vaccinated against FMDV, the data set was expanded by using monocytes from four unvaccinated A31 homozygous cattle with CD4$^+$ T cells from FMD17 (A31 heterozygous). Again, significant enhancement of proliferation ($p < 0.001$; two sample t-test) was induced by the Hsp70:BC2 complex in all experiments, varying between 10- and 25-fold above that observed for BC2 in the absence of Hsp70 (Fig. 3).

To determine whether the enhancement of proliferation was antigen-specific or due to non-specific activation of the T cells, a series of controls were performed. The first set used a CSFV peptide, not recognised by the cattle, complexed to Hsp70. Counts of 1 000 or less were observed with both the Hsp70:CSFV peptide complex and Hsp70 in the absence of any peptide (Fig. 4A). This result was consistent across four separate experiments using cells from different animals. The second control utilised an animal with a different MHC haplotype (A18/A19) that did not recognise the epitope contained within BC2. The BC2 non-responding animal showed a very similar response to whole vaccine antigen, but no proliferation (cpm < 1 500) was induced by the Hsp70:BC2 complex (Fig. 4B).

The requirement for complex formation between Hsp70 and BC2 was examined by performing proliferation assays as before, but without pre-incubation of the Hsp70 and BC2. Significantly more proliferation ($p < 0.001$; two sample t-test) was induced by the Hsp70:BC2 complex than by both components added together without pre-incubation (Fig. 4C).

4. DISCUSSION

Presentation of antigen by the MHC II pathway to CD4$^+$ T cells is one of the central requirements of an adaptive immune response. Improving delivery of antigen to antigen presenting cells is therefore an aim of many vaccine strategies. Enhanced presentation of peptides chaperoned by heat shock proteins has been demonstrated in the murine and human system [11, 16, 31, 35, 37]. Our study confirms these findings in cattle cells using FMDV peptide antigen.

An essential aspect of heat shock protein function is to form complexes with peptides. Bovine recombinant Hsp70 was found to form complexes with biotinylated FMDV peptide BC2. The binding was determined to be specific and saturable by competition assay. The actual molar ratio of Hsp70 binding to peptide in the complexes was not determined in this study, but could be tested by quantitative mass spectrometry. For proliferation experiments, Hsp70:peptide complexes were formed with a molar ratio of approximately 1:10, previously demonstrated to be optimal [16].

Proliferation assays were used to evaluate the ability of Hsp70 to enhance the amount of antigen presented via the MHC II pathway. Peptide BC2 had previously been determined to contain an epitope that is recognised by cattle of the DRB3*0701 haplotype and stimulates a CD4$^+$ T cell recall response [13]. Bovine MoDC were originally considered for use as antigen presenting cells, however preliminary experiments indicated that differentiated MoDC compared to directly isolated monocytes did not endocytose Hsp70 efficiently. Macropinocytosis has been demonstrated to be a major mechanism for Hsp70 uptake by human MoDC [3]. However bovine MoDC, unlike human or mouse, do not constitutively macropinocytose[1] [38] accounting for the inefficient uptake of Hsp70 by bovine cells. This defect in bovine MoDC model system is not thought to be representative of bovine dendritic cells in vivo, since DC isolated directly from afferent lymph take up Lucifer Yellow, a marker for macropinocytosis [19]. By using directly isolated

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[1] Unpublished data.
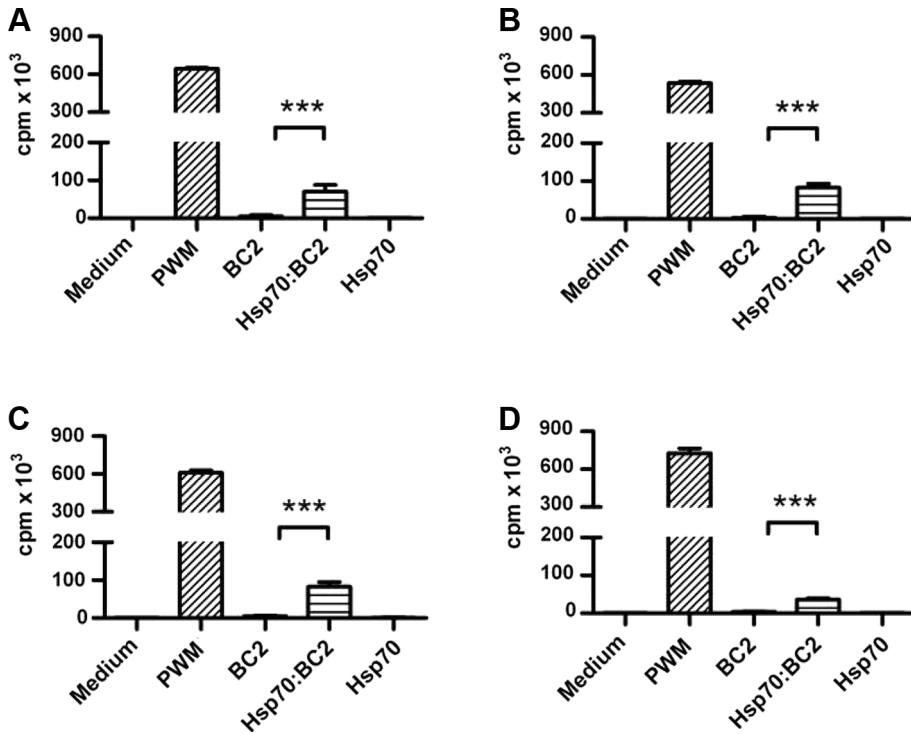
Figure 3. CD4^{+} T cell proliferation induced by MHC-matched monocytes to Hsp70 complexes. (A–D) Monocytes isolated from naïve A31 homozygous cattle were co-incubated with CD4^{+} T cells from FMD17 (A31/A14) and either FMDV 25-mer peptide "BC2" (5 ng/mL), Hsp70 (1 µg/mL) or BC2 and Hsp70, at the same final concentrations, pre-incubated to form a complex. Responses to Pokeweed mitogen (PWM) are also indicated. After 5 days, wells were pulsed with 37 kBq [3H] thymidine and incubated for a further 16 h before harvesting. Incorporated radioactivity was determined by liquid scintillation counting and expressed as counts per minute (cpm). Data are presented as the cpm $\times 10^3$/min mean ± S.D. of triplicate cultures. One representative data set of three is shown for each of the four animals. Significant differences in proliferation between BC2 and Hsp70:BC2 stimulated cells are indicated (*** $p < 0.001$).

monocytes, the problems of variability and loss of function associated with cultured cells can be eliminated. Recent evidence suggests that monocytes are relevant antigen presenting cells in vivo with the ability to endocytose antigen in the periphery and ferry it to the lymph nodes where presentation to T cells can take place [29]. Consequently, bovine monocytes were used as APC in subsequent experiments.

To determine whether Hsp70 facilitated antigen presentation via MHC II molecules, CD4^{+} T cells from immunised cattle, of the appropriate haplotype to recognise the epitope contained

within BC2, were stimulated in vitro with either BC2 chaperoned by Hsp70 or BC2 alone and Hsp70 alone. Using monocytes as APC with autologous CD4^{+} T cells, increased proliferation to Hsp70:BC2 complexes compared to BC2 in the absence of Hsp70 was consistently observed for all of the three animals available, using a suboptimal concentration of peptide. The same result was observed using monocytes from an additional four naïve animals and MHC half-matched CD4^{+} T cells from a vaccinated animal. Variation in the enhancement of proliferation was observed between animals. Although
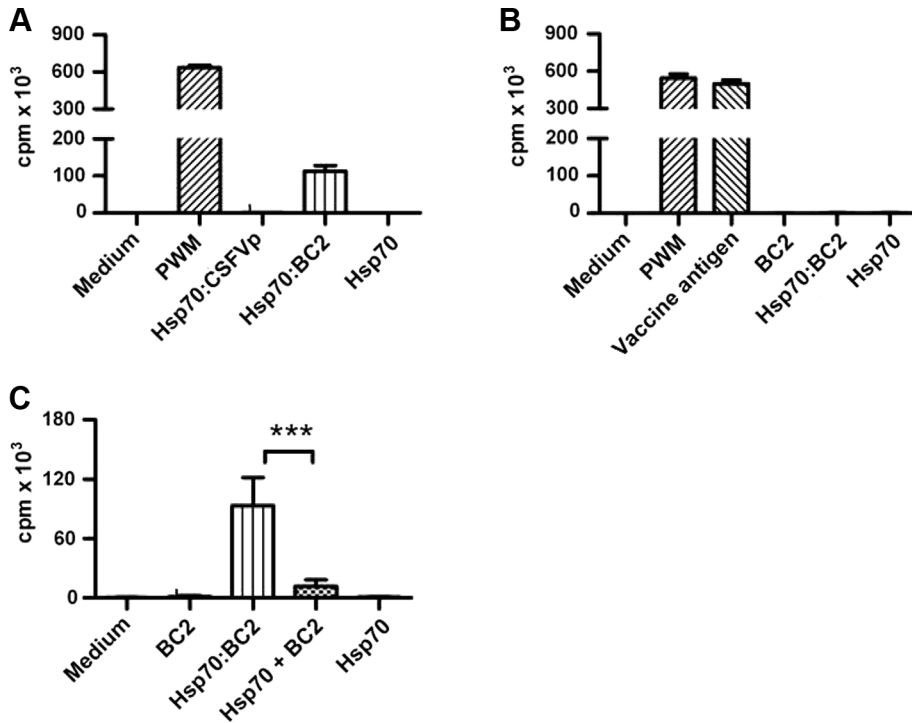
Figure 4. Enhancement of CD4^{+} T cell proliferation by Hsp70 complexes is antigen-specific and requires complex formation. (A) Monocytes isolated from FMD17 were co-incubated with autologous CD4^{+} T cells and either Hsp70 (1 μg/mL) alone, or Hsp70 complexed to FMDV 25-mer peptide BC2 (5 ng/mL) or a classical swine fever virus (CSFV) peptide (5 ng/mL). After 5 days, wells were pulsed with 37 kBq [3H] thymidine and incubated for a further 16 h before harvesting. Incorporated radioactivity was determined by liquid scintillation counting and expressed as counts per minute (cpm). Data are presented as the cpm $\times 10^3/$ min mean ± S.D. of triplicate cultures. (B) Monocytes isolated from FMD7 (A18/A19) which had previously been vaccinated against FMDV, but did not recognise the BC2 peptide, were co-incubated with autologous CD4^{+} T cells and antigens as indicated. Proliferation was assessed as above. (C) Monocytes isolated from FMD17 were co-incubated with autologous CD4^{+} T cells and either FMDV 25-mer peptide BC2 (5 ng/mL), Hsp70 (1 μg/mL) or BC2 and Hsp70, at the same final concentrations, but either pre-incubated to form a complex or added together without pre-incubation. Significant differences in proliferation between Hsp70:BC2 complex and Hsp70 + BC2 stimulated cells are indicated (*** $p < 0.001$). Proliferation was assessed as above.

differences in the frequency or responsiveness of antigen-specific CD4^{+} T cells may have contributed to this variation, further variation was observed between experiments using monocytes from four separate animals with CD4^{+} T cells from a single MHC half-matched animal indicating that there was a difference at the level of the antigen presenting capacity as well. It is unclear whether this is related to the relative ability of the monocytes from different animals to interact

with Hsp70 or variation in the capacity to present the epitope on their MHC II molecules to CD4^{+} T cells. The requirement for antigen processing was demonstrated by comparing T cell responses using either lightly fixed monocytes or non-fixed monocytes as antigen presenting cells. The fixed monocytes which were unable to endocytose or process antigen did not effectively stimulate proliferation of T cells. As a measure of the effector status of the activated T cells, IFN-γ production

was measured in the supernatants of stimulated cells by capture ELISA and was found to closely correlate with proliferation.

The proportion of reactive T cells that were specific for the epitope in BC2 was not defined in this assay. The induction of self-HSP T cell reactivity in the form of $CD4^+$ $CD25^+$ regulatory T cells and regulatory Th2-type cytokine responses has been reported to control autoimmune responses [36], therefore a proportion of the proliferating cells could be against the HSP element rather than the chaperoned peptide. However both Hsp70 alone and Hsp70 complexed with an irrelevant CSFV peptide did not induce measurable T cell proliferation, suggesting that anti-Hsp70 regulatory T cells did not make a major contribution to the proliferation observed. Similarly, if a microbial contaminant was responsible for non-specific T cell proliferation, then responses would be expected in the Hsp70 and Hsp70:CSFV controls. The MHC restriction of the epitope allowed a further control. An animal vaccinated against FMDV, but of a haplotype that did not recognise the epitope within BC2 did not show any enhancement of proliferation to Hsp70: BC2, providing further evidence that the proliferation observed was not against the HSP element or a contaminant of the Hsp70:BC2 preparation. However, it is possible that that not all the proliferating T cells were specific to the chaperoned antigen. Non-specific proliferation of bystander T cells may have occurred as a result of cytokines released from proliferating antigen-specific T cells [16]. Peptide tetramers were not available for more detailed analysis of antigen specificity.

Regardless of the proportion of memory T cells specific to the antigen, the consistent enhancement of proliferation by chaperoned peptide suggest that Hsp70 was influencing the presentation of peptide. The mechanism by which this may occur is currently unknown, however the results of the monocyte fixation experiments indicate that active processing by antigen presenting cells is required for enhancing the T cell response. The amount of presented antigen may be increased by improved uptake, loading or protection from degradation by Hsp70 [3]. A direct interaction between purified HLA-DR and mammalian Hsp70 has been demonstrated which might be of particular importance in the enhanced activation of T cells with Hsp70-chaperoned peptides by Hsp70 transferring bound peptide directly to MHC II molecules [17]. Alternatively, Hsp70 could alter the turnover of MHC class II:antigen complexes, increasing the length of time the complex is displayed on the cell surface and allowing activation of T cells at a lower antigen concentration, but this has not been proven [16].

Evidence has been provided that enhanced cross-presentation of HSP chaperoned antigen to $CD8^+$ T cells rests solely on the ability of heat shock proteins to form complexes and not on their capacity to non-specifically stimulate the immune system [3, 4]. The requirement for complex formation of Hsp70 and BC2 was tested in $CD4^+$ T cell proliferation assays with BC2 alone, Hsp70 complexed to BC2 and Hsp70 plus uncomplexed BC2. The enhancement of proliferation was only observed when Hsp70 and BC2 were pre-incubated to form complexes, providing further evidence that the enhancement observed was not due to non-specific stimulation of either the APC or T cells.

The relevance of these studies in vivo rests on the ability of antigen presenting cells in situ to take up the complexes. The data presented suggests that the formation of these complexes can increase presentation of antigen to $CD4^+$ T cells under limiting conditions. The fold enhancement required in vitro for a demonstrable difference in immunogenicity in vivo remains to be determined, but in the case of FMDV where cellular responses to vaccine are variable and may relate to antigen payload [27], the amplification seen here could potentially make a significant difference to an individual's anti-FMDV T cell response. However for this to be a useful vaccine concept, greater antigenic coverage would be required to overcome the MHC restriction of a single peptide. Future work should consider forming heat shock protein complexes with larger viral proteins and extending the findings to an in vivo cattle study.

In conclusion, we have demonstrated that bovine Hsp70 can improve the delivery of FMDV antigen to the MHC II pathway of bovine antigen presenting cells resulting in

enhanced T cell stimulation. This study further demonstrates the potential of heat shock proteins as antigen delivery vehicles for vaccine purposes. Further work is required to understand how the HSP complexes are handled by APC in vivo.

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