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(54) Title: METHOD FOR ANTIGEN-SPECIFIC TOLERANCE INDUCTION IN HUMANS USING THE SMALL HEAT SHOCK PROTEIN ALPHA B-CRYSTALLIN.

(57) Abstract: The present invention relates to a CRYAB protein for use in a method of inducing tolerance to HSPB5 in humans, wherein the CRYAB protein is administered at a dosage range that results in a serum concentration of the CRYAB protein that induces a tolerogenic response in macrophages without inducing a pro-inflammatory response in T cells.

Title: Method for antigen-specific tolerance induction in humans using the small heat shock protein alpha B-crystallin.

FIELD OF THE INVENTION

The invention is in the field of medicine. In particular, the invention is in the field of medicaments used in the treatment of inflammatory diseases.

BACKGROUND OF THE INVENTION

It is known that the small heat shock protein alpha B-crystallin (CRYAB, or, according to more recent nomenclature, HSPB5) can exert powerful anti-inflammatory effects. In several experimental animal models that are commonly used to mimic human inflammatory disorders, systemic administration of HSPB5 prevents disease from developing, or suppresses disease symptoms once these have already developed. These prophylactic and therapeutic anti-inflammatory effects of HSPB5 have been documented in animal models of chemically induced neuroinflammation, autoimmune neuroinflammation, ischemic optic neuropathy, stroke, spinal cord injury, ischemia reperfusion and chronic obstructive pulmonary disorders. Exogenous HSPB5 also suppresses stress-induced cardiac arrhythmia in laboratory animals. Clearly, HSPB5 exerts powerful anti-inflammatory effects in diverse experimental inflammatory conditions *in vivo*, protecting a variety of different tissues and cell types from damage. Accordingly, HSPB5 has been described as a potential medicament for a number of human diseases and disorders.

In WO2008073466, methods are described for inhibiting an inflammatory disease by intravenously administering a soluble form of HSPB5. In WO9533997, therapy with HSPB5 in multiple sclerosis is described. In WO2012008834, the use of HSPB5 encapsulated in

biodegradable microparticles as an anti-inflammatory agent is described. In this latter document it is revealed that the anti-inflammatory therapeutic effects of HSPB5 are the result of its ability to activate a non-classical, immune-regulatory state of activation in macrophages. When activated by HSPB5, macrophages produce a range of immune-regulatory and anti-inflammatory factors, several of which are known to promote the restoration of immune tolerance. WO2012008834 also discloses that activation is triggered by HSPB5 binding to the macrophage pattern-recognition receptors CD14, Toll-like receptor (TLR) 1 and TLR2. The macrophage scavenger receptor-A (MSR-A) and the macrophage scavenger receptor with collagenous structure (MARCO) additionally contribute to HSPB5-mediated activation of macrophages by facilitating capture and binding of HSPB5.

To date, all data on the therapeutic potential of HSPB5 have been collected in experimental mouse models. However, a major hurdle exists when contemplating the use of HSPB5 as a medicament for humans based on these animal data. The reason is that, in contrast to laboratory animals, humans possess established immunity to HSPB5. This is reflected by the presence of memory T cells in the human circulation that specifically react against HSPB5, and of HSPB5-specific antibodies in human bodily fluids. By comparison, laboratory animals such as mice and rats are profoundly unresponsive to self-HSPB5. Consistent with generally accepted views on the way central immunological tolerance is established, the state of non-responsiveness to HSPB5 in laboratory animals is associated with clearly detectable thymic expression of HSPB5, whereas the state of non-tolerance to self-HSPB5 in humans is associated with the absence of HSPB5 from the thymus and other lymphoid elements. In the absence of central or peripheral tolerance for HSPB5 in humans, natural infection of humans by common viruses that induce HSPB5 expression in the infected host cell causes the establishment of anti-HSPB5 immunity, as a side-effect of infection.

This fundamental difference between humans and laboratory animals is of critical importance when considering HSPB5 as a medicament in humans. The main obstacle for application of free soluble HSPB5 as a therapeutic agent in humans (as opposed to encapsulated HSPB5 as proposed in for instance WO2012008834) is the fact that all humans possess memory T cells against HSPB5, which secrete considerable amounts of interferon-gamma (IFN-γ) when encountering their antigen at sufficient levels. IFN-γ is a powerful driver of classical activation of macrophages, a state in which they promote inflammation rather than suppress it. Therefore, the therapeutic use of HSPB5 in humans may well be associated with activation of IFN-γ-secreting human memory T cells, and will not result in the desired anti-inflammatory non-classical, immune-regulatory state of macrophage activation by HSPB5 as seen in laboratory animals. Instead of promoting the resolution of inflammation by producing anti-inflammatory factors such as IL-10, IFN-γ-exposed macrophages will start to stimulate tissue-damaging inflammatory processes by producing large amounts of TNF-α and IL-12. Mainly for this reason, it is not known if, and if so, how HSPB5 might be applied for therapeutic use in humans as this cannot be simply inferred from animal data.

SUMMARY OF THE INVENTION

It has now been found by the present inventors that restoring the immunological tolerance to the HSPB5 protein in humans is possible, and that this can be achieved by intravenous administration of HSPB5 at a very specific and surprisingly narrow dosage range. It is further found that only a single administration is effective in inducing tolerance, and that several consecutive daily administrations do not result in the required induction of tolerance, but instead promote T-cell responsiveness to HSPB5 rather that suppressing it. This is a clear-cut case where animal data cannot be directly transposed to humans. In fact, therapeutic administration of HSPB5 in mice

for suppressing experimental inflammation has consistently been found to occur only after repeated intravenous administrations.

Restoring immunological tolerance for HSPB5 at the T-cell level (which is defined as a state in which T cells no longer proliferate and secrete IFN-γ in response to this antigen) is of critical relevance to the application of HSPB5 as an anti-inflammatory therapeutic product in humans, since it eliminates the subversive effects of IFN-γ that is otherwise secreted by HSPB5-reactive T cells. Tolerance induction to HSPB5 is of particular relevance to the therapy of multiple sclerosis. This is because the cellular autoimmune response to myelin –the driver of inflammatory lesions in the central nervous system that cause multiple sclerosis - is dominated by the memory T-cell reaction to HSPB5. Thus, restoring self-tolerance to HSPB5 is an important therapeutic goal in itself in the treatment of multiple sclerosis.

To allow exploitation of the therapeutic anti-inflammatory activity of HSPB5 as an activator of immune-regulatory macrophages in humans, and to exert direct therapeutic effects in multiple sclerosis, the pro-inflammatory response to HSPB5 by IFN-γ-secreting memory T cells must be suppressed. The present inventors found that this is possible, and that it will occur in a surprisingly narrow dosage range within which low dosages of intravenously administered HSPB5 actively induce restoration of T-cell tolerance to HSPB5, and do so in an antigen-specific manner. Since macrophages and T cells use different receptors for mounting a response to HSPB5, a limited but marked difference appears to exist in the concentration of HSPB5 that is required to activate either cell type.

Without wishing to be bound by theory, it is believed that this finding can be explained by the fact that macrophages respond to lower concentrations of HSPB5 than do T cells. While it is believed that macrophages use pattern-recognition receptors, T cells appear to use their T-cell receptor to register processed forms of HSPB5 presented by MHC

molecules. It is contemplated by the present inventors that this difference in responsiveness to HSPB5 concentrations may be responsible for the presence of a narrow sub-immunogenic window of HSPB5 concentrations wherein HSPB5 is allowed to activate the beneficial tolerogenic macrophage response, while avoiding the activation of the pro-inflammatory T-cell response. This narrow sub-immunogenic concentration window now appears to be responsible for the observed narrow therapeutic dosage range wherein intravenous HSPB5 induces antigen-specific tolerance of human T cells for HSPB5 itself.

In a first aspect, the present invention provides a CRYAB protein for use in a method of inducing tolerance to HSPB5 in humans, wherein the CRYAB protein is administered at a dosage range that results in a serum concentration of the CRYAB protein that induces a tolerogenic response in macrophages without inducing a pro-inflammatory response in T cells.

In an alternative aspect, the present invention provides a CRYAB protein for use in a method of inducing tolerance to HSPB5 in humans, wherein the CRYAB protein is prepared for administration at a dosage range that results in a serum concentration of the CRYAB protein that induces a tolerogenic response in macrophages without inducing a pro-inflammatory response in T cells.

In preferred embodiments of these aspects, the tolerogenic response in macrophages is characterized by secretion of IL-10 by macrophages, and the pro-inflammatory response in T cells is characterized by secretion of IFN-γ by human memory T cells.

In yet other preferred embodiments of the above aspects, the CRYAB protein is administered or prepared for administration in a single dosage regime.

In yet another preferred embodiment, the CRYAB protein is administered intravenously or prepared for intravenous administration.

In still a further preferred embodiment, the CRYAB protein has a sequence of any of SEQ ID NO 2-11, preferably SEQ ID NO:2.

In still a further preferred embodiment, the dosage range results in a peak serum concentration of CRYAB protein of between 1 and 15 μg/ml, more preferably between 1 and 10 μg/ml, more preferably around 3-5 μg/ml in the subject.

In still a further preferred embodiment, the dosage range of a single unit dose is 5-20 mg, preferably 7-18 mg, more preferably 7.5-17.5 mg, still more preferably 8-17 mg, still more preferably 9-16 mg, still more preferably 10-15 mg, still more preferably 11-14 mg, still more preferably 12-13 mg, still more preferably 12.5 mg of CRYAB protein once per day.

In still a further preferred embodiment, the dosage range for a single unit dose is 5-20 mg, preferably 7-18 mg, more preferably 7.5-17.5 mg, still more preferably 8-17 mg, still more preferably 9-16 mg, still more preferably 10-15 mg, still more preferably 11-14 mg, still more preferably 12-13 mg, still more preferably 12.5 mg of CRYAB protein once per day.

The single unit dose is preferably provided in a unit dosage form being referred to herein as is a composition containing an amount of drug, i.e. CRYAB protein or a pharmaceutically-acceptable salt thereof, that is suitable for administration to a human, in a single dose, according to good medical practice. These compositions preferably contain from about 5 mg (milligrams) to about 20 mg, with preferred embodiments as indicated above of CRYAB protein or a pharmaceutically-acceptable salt thereof.

The above single unit doses, which are applicable to all aspects of this invention, are indicated for human adults of average weight of 65-80 kg, preferably 65 kg. It is contemplated that the blood volume of a child is smaller than that of an adult, therefore, in preferred embodiments of this invention a proportionally smaller dose may be administered to children. Similar, considerations apply to adults with higher or lower blood volumes. The skilled person will appreciate that it is not always necessary to correct

the single unit dose of a medicament for body weight. It is therefore also envisaged that the single unit-doses as indicated herein comprise the same amount of active agent, regardless of the body weight of the subject. Preferably, the serum concentration is such that it induces a tolerogenic response in macrophages without inducing a pro-inflammatory response in T cells.

In still further preferred embodiments, the induction of tolerance to HSPB5 provides anti-inflammatory effects in the prophylactic and therapeutic treatment of inflammatory diseases, including celiac disease, vasculitis, dermatitis, lupus erythematosus, sarcoidosis, chronic obstructive pulmonary disease (COPD), irritable bowel disease, Crohn's disease, ulcerative colitis, lichen planus, lichen sclerosus, atherosclerosis, arthritis, psoriasis, eczema, emphysema, hay fever, periodontitis, atherosclerosis, rheumatoid arthritis, cancer, neuroinflammation, Alzheimer's disease (AD), Parkinson's disease, dementia, multiple sclerosis, ischemic optic neuropathy, optic neuritis, encephalitis, diabetes, inflammatory neuropathy, and inflammatory processes resulting from acute injury including stroke, spinal cord injury, ischemia-reperfusion, and inflammation-induced cardiac arrhythmia.

In yet another preferred embodiment, the induction of tolerance to HSPB5 provides anti-inflammatory effects in the prophylactic and therapeutic treatment of multiple sclerosis.

In another aspect, the present invention provides the use of a CRYAB protein for the preparation of a medicament for inducing tolerance to HSPB5 in humans, wherein the medicament is prepared for intravenous administration in a single dosage regime and at a dosage range that results in a serum concentration of HSPB5 that induces a tolerogenic response in macrophages but that does not induce a pro-inflammatory response in T cells.

In another aspect, the present invention provides a CRYAB protein for use in a method of inducing a anti-inflammatory response by inducing tolerance to HSPB5 in humans suffering from an inflammatory disease, wherein the CRYAB protein is administered intravenously or prepared for administration by a single dosage regime at a dosage range that results in a serum concentration of the CRYAB protein that induces a tolerogenic response in macrophages without inducing a pro-inflammatory response in T cells, wherein said inflammatory disease is selected from celiac disease, vasculitis, dermatitis, lupus erythematosus, sarcoidosis, chronic obstructive pulmonary disease (COPD), irritable bowel disease, Crohn's disease, ulcerative colitis, lichen planus, lichen sclerosus, atherosclerosis, arthritis, psoriasis, eczema, emphysema, hay fever, periodontitis, atherosclerosis, rheumatoid arthritis, cancer, neuroinflammation, Alzheimer's disease (AD), Parkinson's disease, dementia, multiple sclerosis, ischemic optic neuropathy, optic neuritis, encephalitis, diabetes, inflammatory neuropathy, and inflammatory conditions resulting from acute injury including stroke, spinal cord injury, ischemia-reperfusion, and inflammation-induced cardiac arrhythmia.

In preferred embodiments of this aspect, the tolerogenic response in macrophages is characterized by secretion of IL-10 by macrophages, and wherein said pro-inflammatory response in T cells is characterized by secretion of IFN-γ by human memory T cells.

In further preferred embodiments of this aspect, the CRYAB protein has a sequence of any of SEQ ID NO 2-11, preferably SEQ ID NO:2.

In still further preferred embodiments of this aspect, the dosage range results in a peak serum concentration of CRYAB protein of between 1 and 15 μg/ml, more preferably between 1 and 10 μg/ml, suitably around 3-5 μg/ml. Very suitably, a single dosage range may be between 5-20 mg, preferably 12.5 mg of CRYAB protein.

In yet another aspect, the present invention provides a method of treating or preventing multiple sclerosis in a subject, comprising the step of administering HSPB5 to a subject suffering from or having the risk of suffering from multiple sclerosis at a dosage range that results in a serum concentration of HSPB5 in said subject that induces a tolerogenic response in macrophages without inducing a pro-inflammatory response in T cells.

In a preferred embodiment of this aspect, the tolerogenic response in macrophages is characterized by secretion of IL-10 by macrophages, and wherein said pro-inflammatory response in T cells is characterized by secretion of IFN-γ by human memory T cells.

In a further preferred embodiment of this aspect, the medicament is administered or prepared for administration in a single dosage regime.

In yet another preferred embodiment of this aspect, the medicament is administered intravenously or prepared for intravenous administration.

In still another preferred embodiment of this aspect, said HSPB5 has the sequence of SEQ ID NO:2

In a further preferred embodiment of this aspect, the dosage range results in a peak serum concentration of HSPB5 of 1-10 μg/ml.

In still a further preferred embodiment of this aspect, the dosage range is 5-20 mg, preferably 12.5 mg of HSPB5.

The present invention discloses which dosage window allows intravenously administered of CRYAB protein to induce T-cell tolerance in humans. It also discloses that - within the time frame of more than 8, more preferably more than 9, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36 or 38 hours - a single intravenous dose of HSPB5 is effective in suppressing peripheral HSPB5-specific T cells responses, while three consecutive daily administrations result in the opposite effect.

DESCRIPTION OF THE DRAWINGS

Figure 1 shows the induction by CRYAB protein HSPB5 of a series of macrophage transcripts encoding known tolerogenic factors. Twelve different isolates of human monocyte-derived macrophages were exposed to 50 μg/mL HSPB5 and prior to stimulation as well as after 1 and 4 h after stimulation, RNA was isolated and used for genome-wide microarray transcript profiling. For each gene, transcript levels at 1 or 4 h were calculated and expressed relative to levels found in control macrophages, examined prior to stimulation. Fig. 1 shows the average result for the 12 different cultures examined. In the text, the tolerogenic role of the various mediators is further discussed. Abbreviations: MCPIP: monocyte chemoattractant protein-induced protein; TRAF1: TNF receptor-associated factor 1; CLIC4: chloride intracellular channel; ADORA2A: adenosine receptor A2A; IDO1: indoleamine 2, 3- dioxygenase; KYNU: kynureninase; TSG-6: TNF-alpha-stimulated gene 6 protein; ADM: adrenomedullin.

Figure 2 compares the HSPB5-induced accumulation of IL-10 in macrophages with the accumulation of IFN-γ in HSPB5-specific memory T cells. This comparison reveals the concentration window in which HSPB5 induces a tolerogenic response in macrophages while not yet inducing a pro-inflammatory response in T cells. Human monocyte-derived macrophages were cultured and 4 h after activation by HSPB5 at different concentrations up to 80 μg/mL, IL-10 levels were quantified in the culture medium using ELISA. In Fig. 2, a representative example is given in which IL-10 levels are expressed relative to the maximum levels found in the experiment. A similar series of experiments evaluated the dose-response relationship in the production of IFN-γ by human memory T cells. Again, a representative set of data is visualized in Fig. 2, expressed as relative secretion levels. For both IL-10 and IFN-γ, maximum levels found in the culture medium varied between about 1,000 and 2,000 pg/mL, with the highest levels consistently found in response to the highest concentration of HSPB5.. While in such cell culture experiments, concentrations between 1 and 20 μg/mL HSPB5

induced production of IL-10, and concentrations between 10 and 20 μg/mL HSPB5 induced even marked production of IL-10, activating production of IFN-γ required concentrations for more than 20 μg/mL.

Figure 3 shows how exposure to IFN-γ abrogates the tolerogenic IL-10 response of human macrophages to HSPB5. Human monocyte-derived macrophages were stimulated with 50 μg/mL HSPB5, or only medium as a control, and after 4 h, IL-10-encoding transcripts were quantified by real-time PCR. Secretion of IL-10 into the culture medium at that time point was additionally quantified by ELISA. Exposure of macrophage to IFN-γ prior to HSPB5-mediated activation consistently led to a time-dependent blockade of IL-10 transcription as well as secretion, IL-10 being a marker for the tolerogenic macrophage response to HSPB5. Exposure of macrophage to IFN-γ on its own did not trigger any detectable IL-10 response, consistent with the well-documented pro-inflammatory effects of IFN-γ.

Figure 4 shows how exposure to IFN-γ makes macrophages produce IL-12 instead of IL-10 in response to HSPB5. Human monocyte-derived macrophages were stimulated with 50 μg/mL HSPB5, or only medium as a control, and after 24 h, secretion of IL-12p70 into the culture medium was quantified by ELISA. Exposure of macrophage to IFN-γ for a period of 24 h prior to HSPB5 exposure consistently led to the HSPB5-induced accumulation of IL-12, whereas neither HSPB5 nor IFN-γ on their own trigger any detectable IL-12 secretion.

Figure 5 shows the carboxyfluorescein succinimidyl ester (CFSE)-based proliferation assay by which the response of human memory ($CD45RO^+$) T cells to HSPB5 is measured. Human peripheral blood mononuclear cells (PBMC) were labelled with the fluorescent tracer CFSE, and subsequently placed in culture along with 50 μg/mL HSPB5, or only with medium supplied with PBS as a control. CFSE accumulates and persists intracellularly in all cells during labelling. When cells proliferate in response to HSPB5, the CFSE label is divided over the resulting daughter

cells, which consequently loose half the amount of label relative to their parent cell. During the 9-day assay, responsive T cells divide an average of 8 times, reducing the intensity of the CFSE label in the resulting population of daughter cells about 256 times. Upon flowcytometry, as visualized in Fig. 5, this reduction in CFSE fluorescence intensity (plotted on the horizontal axis) is readily detectable by a shift of cellular signals to the left. While analyzing cells, additional fluorescent labels attached to specific antibodies that bind to cellular surface markers can be used to identify the different cell types contained in PBMC. In this experiment, antibodies to the CD45RO marker were used to identify memory T cells. The intensity of this marker (plotted on the vertical axis) does not change upon cell division, thus allowing a distinction between $CD45RO^+$ parent as well as daughter T cells (in the upper part of the plot) and other cell types (in the lower part of the plot). The result as depicted in Fig. 5 therefore shows selective proliferation of human memory T cells in response to HSPB5, resulting in a population of daughter cells that is about 4% larger than in control cultures without HSPB5. Given that this sub-population arises from an average of 8 cycles of division, this implies that the parent population of PBMC must have originally contained about 1 HSPB5-responsive memory T cell in every 3,000 (the reciprocal value of 4%/256 divided by the fraction of all $CD45RO^+$ cells in the sample, which is 0.469).

Figure 6 shows the pharmacokinetic profile of intravenous HSPB5 in healthy humans, illustrating that peak serum levels measured at 10 min after administration of different doses are consistently about 0.26 -0.32 µg/mL per mg of administered protein. Healthy human subjects were given single intravenous doses of 4, 12.5, 25 or 37.5 mg HSPB5 and at various time points after administration, serum was collected and HSPB5 levels contained therein were evaluated using a mass-spectrometry-based quantitation method. The highest serum levels were found in the first samples, taken after 10 min. Levels in subsequent samples indicate rapid

clearance of HSPB5 from the circulation of all subjects, with a half-life of around 1 h. The data illustrated in Fig. 6 reflect the average outcome for dose groups of eight subjects each.

Figure 7 shows the effects of single intravenous doses of either 4 mg HSPB5 (group 1), 12.5 mg (group 2), 25 mg (group 3) or 37.5 mg (group 4) on the response of either $CD4^+$ or $CD45RO^+$ human T cells to HSPB5, illustrating the selective ability only of the single 12.5-mg dose to suppress these responses. Healthy human subjects (eight subjects per dose group) were given the different single intravenous doses indicated, and blood samples were taken either immediately before administration, as well as after 8, 28 and approximately 220 days thereafter. PBMC isolated from these blood samples were analysed for T-cell responsiveness to 200 μg/mL HSPB5 using the CFSE proliferation assay as explained and illustrated in Fig. 5. The test concentration of 200 μg/mL used for comparison of responses was based on the observation that the dose-dependence of human proliferative responses were consistently very similar to those for IFN-γ induction by HSPB5 (see Fig. 1), requiring at least 50-100 μg/mL to produce a maximum response. The data illustrate the statistically significant reduction in both CD4+ and $CD45RO^+$ T-cell responses to HSPB5 after 8 and 28 days resulting from a single 12.5-mg dose of HSPB5, but not from any other dose.

Figure 8 confirms the antigen specificity of the T-cell suppressive effects of 12.5 mg intravenous HSPB5. Healthy human subjects (eight subjects per dose group) were given the different single intravenous doses indicated, and blood samples were taken either immediately before administration, as well as after 8, 28 and approximately 220 days thereafter. PBMC isolated from these blood samples were analyzed for T-cell responsiveness to 0.2 μg/mL tetanus toxoid using the CFSE proliferation assay as explained and illustrated in Fig. 5. As the result of routine national vaccination programmes, almost all adult humans are responsive to tetanus

toxoid, rendering it a suitable control antigen. The test concentration of 0.2 μg/mL tetanus toxoid used for comparison of responses was based on the observation that human proliferative T-cell responses were consistently maximal in response to this concentration. The data show the effects of single intravenous doses of either 4 mg HSPB5 (group 1), 12.5 mg (group 2), 25 mg (group 3) or 37.5 mg (group 4) on the response of $CD45RO^+$ human T cells to the control antigen tetanus toxoid. The result confirms the antigen specificity of the tolerance-inducing effect. While HSPB5 treatment suppresses the T-cell response to HSPB5 itself (Fig. 7), the response to tetanus toxoid remains largely unaffected.

Figure 9 confirms that the T-cell suppressive effects of 12.5 mg intravenous HSPB5 are not observed upon administration of phosphate-buffered saline as a placebo instead. Healthy human subjects (eight subjects per dose group) were given the same volume of phosphate-buffered saline that was used to administer HSPB5 in a single intravenous injection, and blood samples were taken either immediately before administration, as well as after 8, 28 and approximately 220 days thereafter. PBMC isolated from these blood samples were analyzed for T-cell responsiveness to 200 μg/mL HSPB5 using the CFSE proliferation assay as explained and illustrated in Fig. 5. The data show no significant change in either CD4+ or $CD45RO^+$ human T cell responses to HSPB5 as the result of such a placebo treatment.

Figure 10 shows the effects of three consecutive daily intravenous doses of either 10 mg HSPB5 (group 5), or 25 mg (group 6) on the response of either $CD4^+$ or $CD45RO^+$ human T cells to HSPB5. Healthy human subjects (nine subjects per dose group) were given three consecutive daily doses as indicated, and blood samples were taken either immediately before the first administration, as well as after 8, 28 and approximately 220 days thereafter. PBMC isolated from these blood samples were analyzed for T-cell responsiveness to 200 μg/mL HSPB5 using the CFSE proliferation assay as explained and illustrated in Fig. 5. The data illustrate the statistically

significant increase in both CD4+ and $CD45RO^+$ T-cell responses to HSPB5 resulting from both dose regimens. The marked increase in T-cell reactivity to HSPB5 induced under these conditions confirms that at three consecutive daily intravenous doses, even 10 mg HSPB5 is no longer a sub-immunogenic dose.

Figure 11 provides the alignment of CRYAB proteins as defined herein indicating the alpha-crystallin domain in the boxed area.

Figure 12 provides the homologies (sequence identity levels) between alpha-crystallin domains in the CRYAB proteins as defined herein.

Figure 13 shows the relationship between the dose-normalized peak serum concentration of HSPB5 and the body weight of the recipient human subject. In the framework of a Phase I clinical study, 51 healthy adult human subjects were given varying doses of HSPB5 of 4 mg, 10 mg, 12.5 mg, 25 mg and 37.5 mg in a single intravenous bolus injection. Peak serum concentrations, normalized for the dose given, and expressed as µg/mL per mg administered HSPB5, were defined as the concentration measured in a blood sample taken 10 min after injection. This definition was based on extended pharmacokinetic profiling over 8 hours, showing peak serum concentrations consistently occurring in blood samples taken 10 min after administration (not shown). The data in Fig. 13 show that body weight is significantly associated with peak serum concentrations of HSPB5, but they also show that this association is quantitatively very weak (r^2=0.2774). The solid line reflects the best linear fit as established by linear regression analysis. The dotted line indicates the 95% confidence interval. Fig. 13 illustrates that generally, peak serum levels of HSPB5 in humans are between 0.2 and 0.5 µg/mL per mg protein administered, clarifying that an optimum peak serum concentration between 1 and 20 µg/mL, preferably between 3 and 15 µg/mL, more preferably 5-10 µg/mL for the purposes of the invention, is achieved by administrating doses of between 5 mg (minimum of 5 mg x 0.2 µg/mL/mg = 1 µg/mL) and 40 mg (maximum of 40 mg x 0.5

μg/mL/mg = 20 μg/mL). Taken together, the data indicate that it is not useful to correct the dose of HSPB5 for body weight.

Figure 14 shows the relationship between the absolute dose of intravenous HSPB5 and the peak serum concentration of HSPB5 in RR-MS patients. In the framework of a randomized, placebo-controlled, double blind Phase IIa clinical study, three groups of 8 relapsing-remitting (RR) MS patients were given either 7.5, 12.5 or 17.5 mg intravenous HSPB5 and peak serum concentrations of HSPB5 were evaluated by mass spectroscopy in blood samples taken 10 min after the injection. The highly significant linear relationship confirms the data in Fig. 13 in showing that controlling the absolute intravenous dose alone, without any correction for body weight, provides adequate control over peak serum concentrations of HSPB5. Data in Fig. 13 represent group mean averages ± standard deviation. In accordance with the extrapolation of the data obtained after administering each of the HSPB5 doses (dotted line in Fig. 13), the serum concentrations of HSPB5 in the RR-MS patients that received placebo were all below detection levels of the assay of 12 ng/mL.

Figure 15 provides an evaluation of the number of active MS lesions on MRI at 36 weeks. In the framework of a randomized, placebo-controlled, double blind Phase IIa clinical study, groups of 8 relapsing-remitting MS patients were given three injections of either placebo (phosphate-buffered saline), or varying doses of human HSPB5 in saline. Injections were given at the start of the study, and after 8 as well as 16 weeks (see bottom panel). Patients were monitored by MRI at the indicated time points up to 36 weeks after the first injection, and scored for the number of active, gadolinium-enhancing (Gd+) T1 weighted multiple sclerosis lesions. Data points represent mean values ± standard deviation. The solid line represents the result of linear regression analysis, while the p value expresses the probability of the linear relationship thus calculated to be a chance event.

Figure 16 provides an evaluation of the volume of active MS lesions on MRI at 36 weeks. In the framework of a randomized, placebo-controlled double blind Phase IIa clinical study, groups of 8 relapsing-remitting MS patients were given three injections of either placebo (phosphate-buffered saline), or varying doses of human HSPB5 in saline. Injections were given at the start of the study, and after 8 as well as 16 weeks (see bottom panel). Patients were monitored by MRI at the indicated time points up to 36 weeks after the first injection, and scored for the total volume of active, gadolinium-enhancing (Gd+) T1 weighted multiple sclerosis lesions. Data points represent mean values ± standard deviation. The solid line represents the result of linear regression analysis, while the p value expresses the probability of the linear relationship thus calculated to be a chance event.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

The term "CRYAB protein" is used herein to refer to a proteinaceous compound comprising an alpha-crystallin-like domain having an amino acid sequence similarity of at least about 56%, preferably 60%, preferably at least 70%, more preferably at least 80%, more preferably at least 85%, still more preferably at least 90%, even more preferably at least 95%, and particularly preferably at least 97%, 98% and most preferably at least 99%, with the alpha-crystallin domain comprising residues 68-148 of CRYAB (SEQ ID NO: 1). Preferably the amino acid sequence similarity relates to a region of at least 40 contiguous amino acids, more preferably at least 50, more preferably at least 60, more preferably at least 70, more preferably at least 73, more preferably at least 74, more preferably at least 75, more preferably at least 77, most preferably at least 80 contiguous amino acids. The term

"CRYAB protein" includes reference to anyone of the proteins as listed in SEQ ID NO 2-11.

In the context of a therapeutic or prophylactic medicament, the term CRYAB protein refers to an exogenous compound administered to a subject.

The terms "HSPB5", "alpha B-crystallin" and CRYAB" are used interchangeably herein. The term "HSPB5", as used herein, includes reference to the protein having the amino acid sequence of SEQ ID NO:2 and homologous protein sequences having a sequence identity of at least 90%, preferably at least 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% with SEQ ID NO:2, over the full length of the protein. Preferably, said homologous protein sequences comprise the alpha-crystallin domain comprising residues 68-148 of CRYAB (SEQ ID NO: 1) or alpha-crystallin-like domains having at least 90%, preferably at least 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% with SEQ ID NO:1, over the length of the alpha-crystallin domain. Preferably, said homologous protein sequences comprise proteins capable of inducing tolerance to HSPB5 in humans as described herein.

The terms "dose" or "dosage" are used interchangeably herein and are refer to any amount of active therapeutic agent.

The terms "single unit dose" and "single dosage" are used interchangeably herein and refer to a single administration of any amount of an active therapeutic agent.

The term "single dosage regimen" is defined herein as a systematic plan for therapy relating to the quantity of an active agent that is taken in or administered at any one time in a single dosage form. The term "single dosage regimen", as used herein, also encompasses repeated administration of a single dosage in a certain time interval. The skilled person will understand that, in the context of the present invention, repeated administration of a single dosage is also encompassed in the term

"single dosage regimen". The reason for this is that the influx of new T cells into the repertoire, and renewed priming of these T cells for a CRYAB protein, for example for the CRYAB protein having the amino acid sequence of SEQ ID NO:2, will gradually restore responsiveness in humans to HSPB5. In the context of the invention, repeated administration of a single dosage takes into consideration the dynamics of the adaptive immune system, as it takes up to several weeks for an adaptive response to fully develop. For this reason, consecutive daily administrations, for example three consecutive daily administrations of CRYAB (or similar high-frequency administrations) are not envisaged here.

The terms "single administration" and "single dosage regimen" are used interchangeably herein.

As used herein, the term "single dosage form" refers to not more than one unitary dosage form being administered to a subject. "Unitary" refers to a dosage form that consists of one part or unit. Thus, a "single dosage form" is administered to a subject in its entirety.

The term "dosage form" as used herein refers to the physical form in which a drug is produced and dispensed, such as a tablet, a capsule, or an injectable.

The term "dosage range", as used herein, refers to a specific range (outer limits included) of active therapeutic agent that is to administered The term "dosage range" is also used herein to indicate a specific amount of active therapeutic agent.

The term "serum concentration" is defined herein as the measure of a compound found in the blood's liquid, and in particular refers to the concentration of the exogenous compound in the blood liquid as occurring shortly after administration. As shown in Fig 6 the serum concentration of intravenously administered exogenous active shows a peak concentration several minutes after the administration. This peak concentration is contemplated as the intended serum concentration herein.

The term "immunological tolerance" is defined herein as the development of a state of unresponsiveness of T-cells to a particular antigen (*in casu*, HSPB5) capable under other conditions (e.g. under non-tolerogenic conditions) of inducing immunity, resulting from previous contact with the antigen and having no effect on the response to non–cross-reacting antigens.

The term "inducing a tolerogenic response in macrophages" is defined herein as leading to or causing a response in macrophages that produces immunological tolerance. In particular, when reference is made to tolerance, tolerance to HSPB5 (in particular human self-HSPB5, i.e. endogenous HSPB5 that is naturally present in the subject or is naturally produced *in vivo* by the subject) is contemplated herein.

The term "inducing a pro-inflammatory response in T cells" is defined herein as an antigen-induced level of proliferation and IFN-γ secretion by T cells in cell culture assays that is at least 2 times above the level of production measured under the same condition in the absence of antigen.

The term "secretion of IL-10 by macrophages" is defined herein as a level of IL-10 production that is at least 2 times above the level of production measured under the same condition in the absence of the stimulus.

The term "intravenous" is defined herein as administration into a vein.

The term "cancer" as used herein refers in particular to those types of cancer where inflammatory processes play an important role in the disease development, such as cervical carcinoma due to papilloma virus infection, hepatocellular carcinoma due to hepatitis B and C virus infection, gastric cancer due to *Helicobacter pylori* colonization, lung cancer due to cigarette smoke, and colon cancer due to long-standing inflammatory bowel disease.

The term "multiple sclerosis" is defined herein as the disease also known as disseminated sclerosis or encephalomyelitis disseminata, which is a inflammatory disease in which myelin sheaths around axons of the brain and spinal cord are damaged, leading to loss of myelin and scarring.

The term "macrophage" comprises white blood cells within tissues, resulting from expansion and differentiation of monocytes. Typically, macrophages are about 21 micrometers in diameter. Macrophages are stationed at strategic points where microbial invasion or accumulation of dust is likely to occur. Macrophages in aspects of the invention include different types of macrophages, determined by their location in the body. These macrophages have specific names. Preferred macrophages include dust cells or alveolar macrophages which are located in the pulmonary alveolus of lungs, histiocytes located in connective tissue, Kupffer cells located in the liver, microglia located in neural tissue, epithelioid cells located in granulomas, osteoclasts located in bones, sinusoidal lining cells located in spleen and mesangial cells located in the kidney. Methods of identifying macrophages *in vitro* are well known to a skilled person and include the use of preferably monoclonal antibodies against membrane bound markers present on macrophages for identification. Preferred membrane markers comprise CD13, CD14 and CD68.

The term "activation of a macrophage" is used to designate a regulatory process wherein a macrophage undergoes physiological changes resulting in an activated form.

The term "activated macrophage" refers to a functional state of a macrophage that develops in response to a given stimulus, characterized amongst others by the expression levels of specific cytokines and/or chemokines that are at least 2 times higher than are measured under the same condition in the absence of the stimulus. The term "activated macrophage" as used herein refers to macrophages characterized for

example by an increased production of mediators such as IL-10, TNF-alpha, IL-6, CCL1, and CCL5.

Preferred methods of determining the levels of the specific cytokines and/or chemokines associated with macrophage activation involve the use of commercially available enzyme-linked immunosorbent assays (ELISA) or PCR-amplification of transcripts of specific cytokines and/or chemokines. Preferably, the secretion of at least one of these of specific cytokines and/or chemokines is significantly higher in activated macrophages compared to secretion levels of unstimulated macrophages. Preferably, the secretion levels of said at least one cytokine is at least 2, 5, 10, 15, 25, 50 or 100 higher when compared to unstimulated or non-activated macrophages, being macrophages from the same source, and cultured under identical conditions, but in the absence of the stimulus.

The term “inflammatory disease” refers to a pathological state of the body in which the activity of the immune system is pathologically stimulated or suppressed. In a preferred embodiment, said activity is the primary cause of the inflammatory disease. Preferably, said inflammatory disease is an acute or chronic inflammatory disorder of the skin, mucosa, the lungs, the nervous system the vascular system, the pancreas or of a joint, preferably dermatitis, psoriasis, eczema, Crohn’s disease, ulcerative colitis, paradontitis, lichen planus, lichen sclerosis, chronic obstructive pulmonary disorder, emphysema, Alzheimer disease, Parkinson disease, dementia, optic neuritis, encephalitis, inflammatory peripheral neuropathies, atherosclerosis, vasculitis, rheumatoid arthritis or diabetes.

The term "pharmaceutically acceptable carrier" as used herein refers to a carrier for administration of said microparticle. The pharmaceutically acceptable carrier can comprise any substance or vehicle suitable for delivering said microparticle to a therapeutic target. The term refers to any pharmaceutical carrier that does not itself induce the production of antibodies harmful to the individual receiving the composition, and which

may be administered without undue toxicity. Suitable carriers may be one or more optional stabilizers, diluents, or excipients.

As used herein, "a pharmaceutically effective amount" as used herein refers to an amount of the CRYAB protein as described herein effective to elicit a detectable IL-10 level secreted by a macrophage. Preferably, the protein will induce accumulation of IL-10 to concentrations of at least 200 pg/mL over a 24-h period in a culture well containing between 100,000 and 150,000 human macrophages and a total medium volume of 200 µL.

The term "therapeutically effective amount" as used herein refers to the quantity of the CRYAB protein according to the invention necessary to prevent, cure or at least partially arrest the symptoms of the disorder and its complications. Amounts effective to achieve this goal will, of course, depend on the severity of the disease and the weight and general state of the patient.

Embodiments

The present invention provides a CRYAB protein for use in a method of inducing tolerance to HSPB5 in humans, wherein the CRYAB protein, preferably a recombinant CRYAB protein, preferably human, is administered at a dosage range that results in a serum concentration of the CRYAB protein that induces a tolerogenic response in macrophages without inducing a pro-inflammatory response in T cells. In the description below, reference is made to the use of exogenous HSPB5 as an example of a CRYAB protein having the alpha-crystallin domain as an active compound in therapeutic and prophylactic formulations. It is stressed that where reference is made to HSPB5 in that context, a generalization to a CRYAB protein as defined herein is contemplated as being a part of this invention.

Preferably, the amino acid sequence of a CRYAB protein in aspects of this invention is the protein having the amino acid sequence of SEQ ID

NO:2. It is stressed herein that the insertion, deletion and/or replacement of 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10, or more, amino acid residues in a CRYAB protein, for example the CRYAB protein having the amino acid sequence of SEQ ID NO:2, results in a CRYAB protein that can be used in the present invention, preferably said protein is capable of inducing tolerance to HSPB5 in humans as described herein.

A method of treating multiple sclerosis in a subject comprises the step of administering HSPB5 to a subject at a dosage range that results in a serum concentration of HSPB5 protein that induces a tolerogenic response in macrophages without inducing a pro-inflammatory response in T cells. HSPB5 is preferably human and is preferably recombinant. The CRYAB protein may be produced in a form wherein it comprises a tag, for example a FLAG tag that aids in its purification when the protein is produced recombinantly. Preferably, the administered CRYAB protein is not post-translationally modified. A DNA construct coding for the CRYAB protein is preferably comprised in a suitable expression vector. CRYAB protein is preferably produced in a host cell suited for the production of CRYAB protein, for example in *E. coli* cells or in insect cells. Further methods steps may comprise (i) administer intravenously, or prepare for intravenous administration, a contrast agent, preferably gadolinium, to said subject and (ii) perform a magnetic resonance imaging (MRI) scan to assess the number and/or volume of active inflammatory lesions in the brain of said subject using standard methodologies for monitoring or diagnosing inflammatory disease, such as MS in a subject, for instance, wherein a computer-implemented algorithm is used to provide the number and volume of active inflammatory lesions in the brain of an MS subject, and (iii) administer a single dosage of the CRYAB protein to a subject in need thereof.

The establishment and maintenance of immunological tolerance for proteins is a complicated process, key aspects of which have been gradually uncovered over the past decades. As explained above, expression

of self-proteins in the thymus is well known to induce elimination of potentially reactive T cells, or induction of natural regulatory T cells (nTregs), securing a state of profound unresponsiveness to the protein in question. This state of unresponsiveness is generally referred to as "central tolerance". Yet, some self-proteins are not expressed in the thymus, and also to certain non-self proteins (such as for example food-derived proteins, or proteins from beneficial commensal gut bacteria), a state of unresponsiveness is generally required. To achieve a state of unresponsiveness to non-thymic antigens, the immune system relies on what is generally referred to as "peripheral tolerance". As early as in the 1920s, it was observed that loss of antigen-specific T-cell reactivity in mice, or so-called "cumulative immunological paralysis", could be induced by systemic administration of soluble protein antigens. Work in the 1960s subsequently revealed that such "immunological paralysis" could be achieved by systemic administration of protein at either rather low or rather high doses, with intermediate doses being ineffective. This phenomenon has subsequently been referred to as "low-dose" and "high-dose" tolerance, respectively. It was also noted in these studies that either low or high systemic doses that effectively induced tolerance were not necessarily the same for every protein, but differed for different proteins.

An important next step in the understanding especially of the phenomenon of "low-dose tolerance" was the finding in 1971 that the underlying process did not simply rely on the elimination of antigen-specific T cells, but involves an active process. Upon adoptive transfer of T cells from mice that had been subjected to low-dose tolerance induction, an otherwise responsive recipient mouse became equally tolerant. Accordingly, low-dose tolerance was renamed "infectious tolerance". In contrast, high-dose tolerance reflects activation-induced cell death in T cells, or a state of unresponsiveness, also referred to as "anergy". Following high-dose tolerance induction, the state of tolerance is non-transferable.

The current invention is directed to intravenous delivery of the protein, preferably in the form of a solubilized liquid formulation such as in the form of an injectable, preferably wherein the protein in solubilised in physiological salt solution suitable for injection. When applied to humans, intravenous delivery has the advantage of allowing maximum dose control, circumventing the proteolytic environment of the stomach, and circumventing any variations or complications that may be caused by the heterogeneous composition of the gut microflora amongst humans. Intravenous tolerance induction results in systemic activation of inducible regulatory T cells (iTreg), and does not favour a iTreg response to only develop at mucosal sites. For clinical applications of HSPB5 (as an example of a CRYAB protein as defined herein) as an anti-inflammatory medicament, systemic tolerization is therefore the preferred strategy. For application of HSPB5 (or another CRYAB protein) as a specific tolerogen in multiple sclerosis, systemic tolerization is even essential, since pathogenic myelin-reactive T cells enter the central nervous system of multiple sclerosis patients from the circulation.

Doses of HSPB5 that fail to induce a tolerogenic macrophage response would likely be ineffective, but high HSPB5 doses that activate IFN-γ-secreting pro-inflammatory T-cells would be too. The present invention discloses the surprisingly narrow dose range within which intravenous HSPB5 is effective as a tolerogen in humans.

The intravenous doses of HSPB5 that are effective in inducing antigen-specific Tregs in humans cannot be inferred from currently available information, nor predicted on the basis of such information. Animal model data obtained with test antigens other than HSPB5, such as serum albumin or ovalbumin, do not provide sufficient guidance to establish an effective intravenous dose of HSPB5 to induce iTregs in humans. This is because HSPB5 induces unique tolerogenic innate immune responses in macrophages, while the other routine used model proteins do not. Given the

state of natural tolerance for HSPB5 in laboratory animals and, consequently, given the absence of an established memory T cell repertoire against HSPB5, there is no possibility to establish which doses of HSPB5 would be sub-immunogenic *in vivo* using such animals. For this reason, no animal model studies can be performed to examine low-dose tolerance induction with HSPB5. One study has documented high-dose tolerance induction in mice using intravenous HSPB5 [Verbeek et al. Immunology. 2007; 121: 416-426]. In this study, a highly artificial model was used consisting of HSPB5-deficient mice. Since these are the only laboratory animals that lack thymic expression of HSPB5, and are therefore non-tolerant, they will develop an immune response to HSPB5. In the above study by Verbeek et al., HSPB5-deficient mice were immunized with HSPB5 three weeks prior to the tolerizing experiment, to trigger the establishment of a HSPB5-reactive T cell repertoire. It was next shown that three consecutive daily intravenous doses of at least 2.5 μg of HSPB5 per gram body weight were required for effective suppression of the HSPB5-specific T-cell response. The effectiveness in tolerance induction increased with increased doses up to a dose of around 5 μg/g, pointing to activation-induced cell-death rather that activation of iTregs as the likely mechanism of action. When translating tolerogenic doses of HSPB5 found in this study to an equivalent human dose, considering that intravenous HSPB5 is limited to the circulation, and that both humans and mice have about 75-80 mL of blood per kg body weight, effective tolerogenic doses in mice would equal three consecutive daily doses, each of at least 150 mg HSPB5 for a 65-kg adult human. The present invention demonstrates that low-dose tolerance induction by activating iTregs instead is achieved with HSPB5 at intravenous doses much lower than that. As further explained below, the present invention also demonstrates that doses higher than the presently established effective dose window for tolerance induction likely result in the

amplification rather than suppression of the pre-existing T-cell reaction to HSPB5 in humans.

In line with the notion that low-dose tolerance induction with systemic HSPB5 cannot be studied in laboratory animals, none of the prior art studies that have previously documented the anti-inflammatory effects of HSPB5 in animal models of inflammation were aimed at inducing tolerance, nor documented such tolerance to be the result of the intervention. Instead, the prior art studies invariably aimed at inducing an immune-regulatory state of macrophage activation to achieve inhibition of the inflammatory process, and amelioration of clinical symptoms. This was consistently achieved in the various mouse models by using repeated daily dosages of 10 μg of HSPB5 given intravenously or, in some cases, intraperitoneally to mice. Given that HSPB5 exerts its anti-inflammatory effects in the circulation, and given the above blood volume: body weight ratio of mice and humans, the equivalent human dose of HSPB5 would be approximately 30 mg. Again, the present invention discloses such doses to be ineffective in inducing iTregs in humans and, instead, much more likely to promote T cell responses to HSPB5 rather than suppress them.

It was found by the present inventors that the preferred mode of administration is via a single dosage regime. Hence, it is preferred to prepare the CRYAB protein for administration in a single dosage regime. A single dosage means that a human subject receives one dose in a certain time interval, preferably a time period, which period for instance starts at the time of administration of a single dose and ends for instance at the time at which the suppression of HSPB5-reactive responses in T cells diminishes as explained in more detail below. Thus, a single dosage regime preferably comprises administering a CRYAB protein, for example the CRYAB protein having the amino acid sequence of SEQ ID NO:2, in a certain time interval, preferably a time period, which period, for instance, starts at the time of administration of a single dose and ends for instance at the time at which

the suppression of HSPB5-reactive responses in T cells diminishes. This period may be 2, 3, 4, 5, 6, or 7 days or more, or 1, 2, 3, or 4 weeks or more, or 1 month, 2 months, 3 months, 4 months, 5 months, 6 months,7 months, 8 months, 9 months, 10 months, 11 months or 12 months, or more, or a specific period anywhere between 2 days and 12 months. Thus, a single dosage regime preferably comprises a repeatable or repeated administration of a single dosage of a CRYAB protein, for example the CRYAB protein having the amino acid sequence of SEQ ID NO:2, in a single dosage form. wherein the administration is periodically repeated. Preferably, the period between single dosages of a CRYAB protein is about 8 weeks. The single dosage of a CRYAB protein, administered after the administration a previous single dosage of a CRYAB protein, may contain the same amount of a CRYAB protein as the previous single dosage of a CRYAB protein, or may contain a different amount. Preferably, the single unit dose of a subsequent, or repeated, dosage is between 5-20 mg of a CRYAB protein.

It was further found by the present inventors that the preferred mode of administration is via intravenous administration. Hence, it is preferred to prepare the CRYAB protein for intravenous administration.

It was further found by the present inventors that the preferred dosage range results in a peak serum concentration of CRYAB protein of between 1 and 10 μg/ml.

Finally, it was found by the present inventors that the preferred dosage range is 5-20 mg, preferably 12.5 mg of CRYAB protein in a single unit dosage form.

The following Examples are intended to further illustrate the invention.

EXAMPLES

Methods

Protein preparation

For all experiments, we used sterile clinical-grade recombinant human HSPB5 (Delta Crystallon BV, Leiden, The Netherlands), containing < 5 ng/mg (0.0005%) *E. coli* proteins, < 0.7 EU/mg endotoxins, and < 75 pg/mg bacterial DNA. The fraction of HSPB5 that was contained in the appropriate multimeric (>400 kDa) complexes was > 99.9%. As previously documented (van Noort, J Exp Neuropathol Neurol 2010; 69:694-703), the preparation did not activate HEK293 reporter cells expressing either of the individual human TLRs 1-10 (without CD14) at concentrations up to 200 μg/mL, confirming the absence of confounding bacterial contaminants.

Macrophage isolation

For the analysis of macrophage responses, human monocyte-derived macrophages were obtained by isolating CD14+ monocytes from peripheral blood-mononuclear cells (PBMC) using EasySep® magnetic beads (Stemcell Technologies, Grenoble, France), and differentiating these at 1.5 x 10^6 cells/mL with 50 ng/mL macrophage-colony stimulating factor (Preprotech, Rocky Hill, NJ) for 5-6 days. Microglia from post-mortem human white matter were isolated and verified for purity as described previously (van Noort et al, 2010) Cultured macrophages and microglia were stimulated with HSPB5 at a final concentration of 50 μg/mL in all cases.

Microarray analysis

Microarray data on the HSPB5-induced changes in macrophage transcripts were collected as described previously [van Noort et al, Biomaterials 2013; 34:831-840]. After 1 and 4 h following activation of macrophages with HSPB5, as well as from untreated cells, total RNA was isolated in 0.75 mL of TRIzol®, and extracted according to the manufacturer's protocol (Invitrogen, Breda, The Netherlands). RNA integrity and concentrations were measured on a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA) and a Nanodrop spectrophotometer ND-1000 (Fisher Scientific,

Waltham, MA). All samples were of high quality with an RNA Integrity Number (RIN-value) between 7.6 and 8.7 and 260/280 ratios greater than 2.00. Samples of 500 ng total RNA were used as input for amplification and labeling with the Quick Amp Labeling kit (Agilent Technologies, Palo Alto, CA), according to the manufacturer's guidelines including control spikes. Labeled RNA was purified using the RNeasy Mini Kit (Qiagen) yielding 7.5 µg or more of labeled cRNA and specific activities greater than 15.3 pg Cye3 dye/µg cRNA and 18.9 pg Cy5 dye/µg cRNA. Labeled samples were hybridized onto whole human genome GE 4 x 44K GF4112F microarrays according to the manufacturer's protocol (Agilent Technologies, Palo Alto, CA). Scanning was performed using a microarray scanner G2505C (Agilent Technologies) and Feature Extraction v10.7 using the manufacturers protocols (Agilent Technologies). Processing of microarray data was performed with project R and BioConductor package Limma. Normexp background correction was done with an offset of 50. Global Loess within array normalization and a quantile between array normalization were performed on both datasets separately. Log-expression values were extracted from the MA-list.

Production of IL-10 transcripts was further verified by real-time PCR. Secretion of IL-10 or IL-12p70 into the culture medium of macrophages was quantified using commercially available ELISA kits.

Pharmacokinetics of intravenous HSPB5

Quantitation of HSPB5 in human serum samples following intravenous administration was performed using mass spectrometry-based analysis of trypsin-digested serum samples, and comparing the signal of tryptic HSPB5 fragments with those of predefined quantities of C13-labelled reference peptides representing an identical amino acid sequence, that had been added as internal reference.

Quantitation of antigen-specific T cell responses

Quantitation of the HSPB5- or tetanus toxoid-induced proliferation of either CD4 or CD45RO T cells in human samples of peripheral blood was performed using the carboxyfluorescein succinimidyl ester (CFSE)-based method. Briefly, peripheral blood mononuclear cells were isolated from fresh blood samples by centrifugation on a Histopaque layer, and labelled in serum-free PBS with 1 μM CFSE for 5 min at room temperature at a cell density of 1 x 10^7 cells/mL. Labelled PBMC were subsequently seeded in ceel culture plates at 2 x 10^6/mL, and supplied with either 200 μg/mL HSPB5, or 0.2 μg/mL tetanus toxoid. After 9 days, all cells were harvested, washed and labelled with either fluorescently labelled anti-CD4 or anti-CD45RO antibodies (Beckton Dickinson), and analyzed by flowcytometry as illustrated in Fig. 5, for a quantitation of the absolute percentage of either $CD4^+$ or $CD45RO^+$ cells with reduced CFSE labelling intensity. Figures 7-10 reflect these absolute percentages without any further data processing.

EXAMPLE 1

First, we focused on the tolerogenic factors produced by antigen-presenting cells in response to HSPB5 that are critical for the development of iTreg. As stated above, it is well known that important tolerogenic macrophage factors include IL-10, CD274, kynureninase, IDO1, and the adenosine A2A receptor [reviewed by Bilate & Lafaille, Annu Rev Immunol. 2012; 30: 733-758]. Additional factors that promote the tolerogenic state of macrophages, often by suppressing the production of pro-inflammatory mediators by macrophages, include ZC3H12A (also known as monocyte chemoattractant protein-induced protein, or MCPIP) [Mizgalska et al, FEBS J. 2009; 276: 7386-7399], TNF receptor-associated factor (TRAF) 1 [Folco et al, J Biol Chem. 2009; 284: 25569-25575], the chloride intracellular channel (CLIC) 4 [Malik et al. Proc Natl Acad Sci USA. 2012; 109: 6130-6135], TNF-α-stimulated gene 6 protein (TNFAIP6, or TSG-6) [Choi et al. Blood. 2011;

118: 330-338], adrenomedullin (ADM [Wu et al. Regul Pept. 2003; 112: 19-26], and the chemokine CCL18 [Schraufstatter et al, Immunology 2012; 135: 287-298].

We therefore examined at which concentrations of HSPB5, human macrophages start to produce these factors. By first performing genome-wide microarray profiling of human macrophages in cell culture, and analyzing the entire transcriptome at two different points in time after activation with highly purified HSPB5, it was found that the above tolerogenic factors all accumulate over the first 4 hours, with IL-10 being a relatively rapidly developing response marker (Fig 1) [see also van Noort et al. Biomaterials. 2013; 34: 831-840]. Thus, apart from being a key tolerogenic factor on its own, secretion of IL-10 over the first 48 h after stimulation can be suitably used as a representative marker to monitor macrophage production of many other tolerogenic factors in response to HSPB5.

Considering this, we examined using an enzyme-linked immunosorbent assay (ELISA) in a cell culture model of human monocyte-derived macrophages the relationship between the concentration of HSPB5 that was supplied to the culture medium, and the amounts of IL-10 that accumulated over a 4- to 8-hour period in the culture medium. The results were remarkably consistent, despite the use of a range of different isolates of primary human monocyte-derived macrophages. In a highly reproducible manner, macrophage IL-10 responses started to become readily detectable in the presence of HSPB5 at about 10 μg/mL, as illustrated in Fig. 2. IL-10 production gradually increased with increasing concentrations of HSPB5, reaching a maximum at about 40 μg/mL.

EXAMPLE 2

As a next step, it was verified that IFN-γ, a known activator of the classical state of macrophage activation, does indeed block and subvert

HSPB5-induced production of IL-10 by macrophages. Exposure of macrophages to IFN-γ for 24 h, though not yet after 2 h, fully abrogates the HSPB5-induced appearance of IL-10-encoding transcripts, as well as IL-10 protein secretion into the culture medium (Fig. 3). IFN-γ on its own had no impact on IL-10 production.

Macrophages do not only stop producing IL-10 in response to HSPB5 due to IFN-γ, they start to produce the strongly pro-inflammatory factor IL-12 instead (Fig. 4). While neither HSPB5 nor IFN-γ on their own lead to any detectable IL-12 production, the combination of the two factors clearly does. IL-12 is well known to strongly promote the activity of pro-inflammatory T cells, and inhibit development of Tregs. These data validate IFN-γ production as a suitable marker to monitor the subversive, pro-inflammatory response by HSPB5-reactive memory T cells in humans that counteracts the tolerogenic effects of HSPB5.

EXAMPLE 3

As confirmed in Fig. 4, IFN-γ is a critical factor that subverts the HSPB5-induced tolerogenic state of macrophages. To examine the concentration of HSPB5 at which human memory T cells start to produce IFN-γ, the HSPB5-induced production of IFN-γ in the culture medium of human peripheral blood mononuclear cells was monitored by ELISA. Again, a highly consistent relationship was observed between the concentration of HSPB5 added to the culture medium, and amounts of IFN-γ being secreted by responsive T cells. As a rule, HSPB5 concentrations of up to 20 μg/mL failed to induce any detectable production of IFN-γ, but at concentrations beyond this threshold, IFN-γ induction was prominent (FIG. 2). While absolute maximum levels of IFN-γ that were found in the culture media did differ somewhat between different T-cell isolates, the dose-response relationships as such were highly reproducible. The same dose-response relationship was observed for the proliferative response by memory T cells,

as was examined using the so-called CFSE assay. By flowcytometry, this assay measures the number of T cells with reduced levels of the cellular tracer carboxyfluorescein succinimidyl ester (CFSE), indicative of cellular division (illustrated in Fig. 5). Again, HSPB5 up to concentrations of about 20 μg/mL did not trigger any detectable levels of T-cell proliferation, but at higher concentrations such proliferation becomes clearly detectable. Thus, proliferation and IFN-γ production in response to HSPB5 are closely linked in having the exact same antigen dose dependence *in vitro*.

EXAMPLE 4

By comparing the concentrations of HSPB5 required to activate the production of tolerogenic factors by macrophages, with HSPB5 concentrations required to induce T-cell-derived IFN-γ, a concentration window was identified in which HSPB5 triggers tolerogenic macrophage signals without already inducing IFN-γ production (or proliferation) by human T cells (Fig. 2). This concentration window is surprisingly narrow, which again emphasizes the importance of antigen concentration for establishment of tolerance. The data in Fig. 3 clarify that in cell culture, a concentration between 10 and 20 μg/mL HSPB5 is optimal in inducing beneficial macrophage activation without inducing detrimental T-cell activation. Importantly, a deviation from this optimal concentration by a factor of only 3 is already sufficient to fully abolish the effect. At concentrations below the effective range, HSPB5 fails to activate tolerogenic macrophage signals such as IL-10. At concentration above it, high levels of IFN-γ released by memory T-cell will abrogate tolerance induction by abolishing secretion of IL-10 and promoting IL-12 production instead.

EXAMPLE 5

Finally, it was explored how the concentration window of HSPB5 that proved adequate in cell culture models to induce tolerogenic macrophage

signals while avoiding concomitant pro-inflammatory T-cell signals, translates to absolute intravenous doses in humans. These clinical studies were based on the notion that at least to some extent, intravenous HSPB5 is likely to predominantly accumulate in the spleen as the result of capture by marginal zone macrophages. Not only is splenic absorption a well-known fate of all intravenously administered proteins, marginal zone macrophages in the human spleen are also known to express macrophage express CD14, TLR1 and TLR2, the receptors required for HSPB5-mediated activation of the tolerogenic state of macrophages. In addition, marginal zone macrophages express scavenger receptor A and MARCO, both of which are relevant for capturing HSPB5. Direct excretion of circulating HSPB5 via the kidneys after intravenous administration is unlikely, given the large size of the natural 24-mer HSPB5 complex, which is around 14 nm in diameter, well over the general 6-nm size limit of nanoparticles that can be excreted via the kidney.

Since accumulation of HSPB5 in the spleen will lead to a local concentration of HSPB5 that will inevitably be somewhat higher than in serum, the lowest intravenous dose tested was a single dose of 4 mg. Pharmacokinetic analyses of serum concentrations of HSPB5 in the first hours after intravenous administration revealed that peak serum concentrations were reached within 10 min after administration (Fig. 6). For all dose levels examined, and using single as well as multiple administration schemes, normalized HSPB5 serum concentrations measured after 10 min turned out to be consistently between 0.26 and 0.32 μg/mL per mg of administered HSPB5. Thus, after administering the lowest dose of 4 mg, peak concentrations in serum of around 1 μg/mL were found, well below the optimum "cell culture" concentration of HSPB5 of about 10-20 μg/ml. Additional intravenous doses of HSPB5 doses were 12.5, 25 and 37.5 mg. In this manner, we the HSPB5 dose was increased stepwise, but not with a factor of more than about 3, to avoid missing out on the

presumably narrow effective dose window. At the maximum intravenous dose of 37.5 mg, peak serum concentrations of HSPB5 were approximately 12.5 μg/mL, which is around the lower limit of effective "cell culture" concentrations. In this way, doses were tested that should lead to peak serum concentrations of HSPB5 between approximately 1 and 12 μg/mL.

In a second series of tests, three consecutive daily doses of 10 and 25 mg, respectively, were tested as well. We compared consecutive daily doses along with single doses of HSPB5.

In these clinical tests, data were collected on clinical responses and other aspects relevant to evaluate safety and tolerability of intravenous HSPB5 administration to humans. Together, these indicated that at all doses tested, HSPB5 was safe and well tolerated. Furthermore, tolerance induction in all subjects was monitored by examining the extent of HSPB5-induced proliferation of human memory T cells isolated at various points in time from samples of peripheral blood. The results of these tests, performed using the CFSE assay (Fig. 5), are illustrated in Figs. 7-10.

In Fig. 7, it is shown how of all four single dose levels tested, only the 12.5 mg-dose led to a statistically significant suppression of HSPB5-reactive responses of both CD4 and CD45RO (memory CD4 and CD8) T cells. This suppressive effect was readily detectable after 8 days following administration, and persisted for at least 28 days. After 8 days, T-cell responsiveness for both types of T cells had been reduced by the 12.5-mg dose of HSPB5 to levels very close to background levels. The other dose levels, involving either lower (4 mg) or higher (25 and 37.5 mg) doses, all failed to induce tolerance, with higher doses tending to promote T-cell responses.

To establish the antigen specificity of the tolerizing effects of HSPB5, $CD45RO^+$ memory T-cell responses in groups 1-4 were also monitored to tetanus toxoid, an antigen that most humans have established immunity against as the result of routine vaccination programmes (Fig. 8). T-cell

responses to either HSPB5 or tetanus were monitored in parallel, using the same fresh blood samples, culture plates, CFSE labelling conditions, antibodies and flowcytometric conditions. The data obtained confirmed the absence of significant suppressive or stimulatory effect of HSPB5 at any single dose level on the T-cell responses to tetanus). This result confirms the antigen-specificity of the suppressive effects induced by a 12.5 mg dose of HSPB5.

To verify that the tolerizing effect was indeed the result of HSPB5 being administered, responses were also monitored after administration of an equal volume of phosphate-buffered saline injected intravenously in control subjects as a placebo (Fig. 9). Again, the data confirm that the T-cell suppressive effects seen in Group 2 were not due to the mere act of injecting a fluid intravenously, but did specifically depend on the fluid containing HSPB5. To examine whether any changes would occur in levels of serum antibodies, these levels were additionally monitored (data not shown). Consistent with the idea that tolerance achieved by intravenous HSPB5 is mediated by iTreg development, serum antibody titers to HSPB5 did not change in any of the dose groups. It should be noted that this result is critically different from what had been observed in the single mouse study in which high-dose tolerance to HSPB5 was induced by three consecutive daily administrations of very high doses of HSPB5 [Verbeek et al. Immunology. 2007; 121: 416-426]. In this study, a massive increase in anti-HSPB5 serum antibodies was observed as the result of the repeated intravenous administrations.

Finally, in Fig. 10 it is shown how three consecutive daily doses of either 10 or 25 mg led to a statistically significant and strong increase in T cell reactivity to HSPB5 within 28 days. This illustrates that the application of this strategy that had been consistently applied in mouse models, led to an immune-stimulatory rather than tolerogenic effect in humans. As explained above, persistence of processed forms of HSPB5 on splenic

antigen-presenting cells is probably the reason why repeated daily administration causes HSPB5 to become immunogenic rather than tolerogenic. Clearly, even dosages of HSPB5 that are tolerogenic when given once, are no longer sub-immunogenic when given on three consecutive days.

Collectively, these data disclose that HSPB5 can be safely administered intravenously to humans at the dose levels examined here. Surprisingly, however, of the dosages tested, only the single dose of 12.5 mg HSPB5 led to the statistically significant and antigen-specific inhibition of the proliferative response by HSPB5-specific memory T cells within 8 days, an effect which persisted for at least 28 days, while leaving the T-cell response to the control antigen tetanus toxoid unaffected. Either lower or higher doses, or similar doses of 10 mg given on three consecutive days all failed to induce tolerance.

EXAMPLE 6

Following up on data described in Example 5, it was examined what the impact is of total body weight on the relationship between the absolute amount of HSPB5 administered intravenously to adult human subjects, and the subsequent peak serum concentration of HSPB5, *i.e.* the concentration monitored about 10 min following administration. After all, in the field of therapeutic drug dosing it is of general interest to establish whether or not it is useful to correct for body weight in determining the optimal dose of a therapeutic compound. In the case of intravenous HSPB5 as a tolerogen, the therapeutic protein is distributed over total volume of circulatory blood before being absorbed in the spleen (and perhaps also liver), and does not penetrate into all tissues and organs. The relationship between the total blood volume and body weight of adult humans is not necessarily very strong. Indeed, and as shown in Fig. 13, while body weight does significantly correlate with the peak serum concentration of HSPB5 following intravenous administration, the goodness-of-fit is relatively poor, as

evidenced by a correlation coefficient of only 0.2774. A value of 1.0 would have indicated a perfect association between body weight and dose-normalized peak serum levels, and a value of 0 the total absence of such an association. These data indicate that body weight is not a critical factor in controlling peak serum concentrations of HSPB5 and, consequently, in controlling tolerance induction.

EXAMPLE 7

Next, it was verified whether controlling the absolute amount of intravenous HSPB5 alone is adequate as a strategy to control the peak serum concentration of HSPB5 in humans, irrespective of body weight. This was tested in patients suffering from relapsing-remitting multiple sclerosis (RR-MS). In the context of a Phase IIa clinical study, three groups of 8 RR-MS patients were given varying doses of HSPB5, and peak serum concentrations of HSPB5 were determined in blood samples collected 10 min after dosing. In Fig. 14, the relationship is shown between the absolute dose administered and the peak serum concentration. The results confirms that this relationship for doses of HSPB5 between 7.5 and 17.5 mg is highly significant ($p<0.0001$) and linear. These data confirm that controlling the absolute dose of intravenous HSPB5 alone is adequate in controlling peak serum concentrations, and that there is no advantage in correcting such intravenous HSPB5 doses for body weight.

EXAMPLE 8

Having established that not daily repeated doses but a single dose of between 5 and 20 mg intravenous HSPB5 is effective in establishing antigen-specific tolerance in humans, and after having established that such a dose need not be corrected for body weight, the clinical effects of HSPB5-targeted tolerance induction was examined in multiple sclerosis patients. In a randomized, double blind, placebo-controlled Phase IIa clinical study,

groups of 8 RR-MS patients were given HSPB5 at three different intravenous dose levels of 7.5, 12.5 and 17.5 mg, respectively. A further group of 8 RR-MS patients was given phosphate-buffered saline (PBS) as a placebo.

In this study, it was also tested whether or not a repeat of the single administration at some point in time would pose any safety issue. A state of antigen-specific tolerance as induced by intravenous antigen (and illustrated in Fig. 7) is not a permanent one, since the influx of new T cells into the repertoire, and renewed priming of these T cells, will gradually restore responsiveness in humans. While it is currently unknown what the life span is of human antigen-induced regulatory T cells following their induction in the periphery, it is likely to be in the order of several months. To secure a permanent state of tolerance in adult humans, a repeat of the tolerizing procedure at some point during this period will therefore be essential. In the present Phase IIa study, an interval of 2 months was chosen for scrutiny, also for practical reasons. Standard Phase IIa studies in RR-MS extend over a period of 24 week (with a follow-up period up to 48 weeks), and repeated dosing with an interval of many months is therefore impossible to accommodate in such a time span. For this reason, an interval of 2 months was chosen, and RR-MS patients were given intravenous HSPB5 or placebo at the beginning of the study period, after 8 weeks, and after 16 weeks. It is important to emphasize that repeated administration with a 2-month interval should be considered as repeated single administrations, given the dynamics of the adaptive immune system. It takes up to several weeks for an adaptive response to fully develop. For this reason, consecutive daily administrations (or similar high-frequency administrations) have an essentially different effect from administrations with much longer intervals, such as 2 months. This notion is emphasized by the data below, which show such infrequent dosing (as opposed to

consecutive daily dosing) to not lead to any increase in disease activity due to enhanced T-cell reactivity.

To evaluate the clinical effects of what therefore should be considered as repeated single administrations of HSPB5, RR-MS patients in the above-mentioned Phase IIa study were monitored by magnetic resonance imaging (MRI) at baseline, 2 weeks after the first dose, and monthly for the first 24 weeks, followed by additional monitoring at 36 and 48 weeks in the follow-up period. Using MRI, the presence of active inflammatory lesions in the brain is visualized following intravenous administration of a contrast agent called gadolinium (Gd). This contrast agent can pass the blood-brain barrier when a local inflammatory reaction occurs, since this renders this barrier more permeable. Thus, the presence of Gd in brain tissue can be taken as a reflection of such an inflammatory reaction. Active disease in multiple sclerosis is therefore reflected, and can be quantified by the number and the volume of these so-called gadolinium-enhancing (Gd^+) MRI lesions. MRI data are generally accepted as valid and sufficiently sensitive reflections of disease activity in multiple sclerosis. Since it is more sensitive than monitoring clinical relapses, which tend to occur much less frequently than MRI lesions, it is the optimal approach to monitor and quantify disease activity with relatively small groups of patients, and over a relatively short period of time of 0.5-1 year. The sensitivity and specificity of so-called T1-weighted MRI lesions is the highest, and this mode of analysis is therefore generally used for the most sensitive quantification of disease activity in Phase II studies of multiple sclerosis.

In Fig. 15 and 16, the MRI parameters are summarized as evaluated at 36 weeks during the first follow-up evaluation, 5 months after the third and final intravenous administration of HSPB5. In Fig. 15, the mean number of active Gd^+ T1 weighted MRI are illustrated, while in Fig. 16, the mean volumes of such lesions are illustrated. As clarified by the data in Figs. 15 and 16, injection of RR-MS patients with HSPB5 generally led to a

progressive decline in both the number and the total volume of active MS lesions, while administration of placebo had no such effect. The progressive HSPB5-induced decline in MS lesions is illustrated in Figs. 15 and 16 by linear regression analysis. For the patient groups receiving either 7.5 mg or 12.5 mg of HSPB5, the decline persists over 36 weeks, 5 months after the final administration, leading to a reduction in number and volume of MS lesions of 73 and 77%, respectively. In the patient group receiving 17.5 of HSPB5, a similar decline in disease activity is seen at the early stage of the study, but this decline persists only up to week 20, and reverses after this time point. The more rapid rebound of disease activity in this particular group of patients is likely caused by an overall markedly higher level of disease activity in this group from the very start of the study. This is evidenced by a 2- to 3-fold higher number and larger volume of active MS lesions at baseline as compared to the placebo group, or the other actively treated groups (see Figs. 15 and 16). Most likely, therefore, disease suppression in this unusually active group would have required continued treatment beyond week 16. For this reason, aggregated analysis as shown in Fig 15 and 16 focuses on the two groups with the more durable decline in disease activity, showing that at 36 weeks, the average reduction in MRI lesion load in these two low-dose groups is approximately 75%. Remarkably, with regard to both lesion number and lesion volume in the patient groups treated with the low doses of 7.5 and 12.5 mg of HSPB5, the decline in disease activity as evaluated by linear regression analysis reaches statistical significance, despite the only small number of patients involved in the study. An evaluation at earlier time points up to 20 weeks would have identified the 17.5-mg dose group as similarly suppressed in their disease activity.

Importantly, therefore, these data confirm that intravenous administration of HSPB5 in single doses of 7.5, 12.5 and 17.5 mg exerts a significant beneficial effect in relapsing-remitting multiple sclerosis

patients. The data also demonstrate that optimal disease suppression, especially in patients with highly active disease, depends on a periodic repeat of single administrations. The current data indicate that when repeated only twice, the treatment results in a reduction of MRI lesion load by around 75%. It is conceivable that even higher levels of disease suppression may be obtained upon continued administration once every two months.

Table 1: Sequences of the alpha-crystallin domain and of the CRYAB proteins referred to herein above.

SEQ ID NO:1 The alpha-crystallin domain of CRYAB (corresponding to residues 68-148 of SEQ ID NO:2)

```
  1 MRLEKDRFSV NLDVKHFSPE ELKVKVLGDV IEVHGKHEER QDEHGFISRE
 51 FHRKYRIPAD VDPLTITSSL SSDGVLTVNG P
```

SEQ ID NO:2 CRYAB

```
  1 MDIAIHHPWI RRPFFPFHSP SRLFDQFFGE HLLESDLFPT STSLSPFYLR
 51 PPSFLRAPSW FDTGLSEMRL EKDRFSVNLD VKHFSPEELK VKVLGDVIEV
101 HGKHEERQDE HGFISREFHR KYRIPADVDP LTITSSLSSD GVLTVNGPRK
151 QVSGPERTIP ITREEKPAVT AAPKK
```

SEQ ID NO:3 CRYAA

```
  1 MDVTIQHPWF KRTLGPFYPS RLFDQFFGEG LFEYDLLPFL SSTISPYYRQ
 51 SLFRTVLDSG ISEVRSDRDK FVIFLDVKHF SPEDLTVKVQ DDFVEIHGKH
101 NERQDDHGYI SREFHRRYRL PSNVDQSALS CSLSADGMLT FCGPKIQTGL
151 DATHAERAIP VSREEKPTSA PSS
```

SEQ ID NO:4 HSPB1

```
  1 MTERRVPFSL LRGPSWDPFR DWYPHSRLFD QAFGLPRLPE EWSQWLGGSS
 51 WPGYVRPLPP AAIESPAVAA PAYSRALSRQ LSSGVSEIRH TADRWRVSLD
101 VNHFAPDELT VKTKDGVVEI TGKHEERQDE HGYISRCFTR KYTLPPGVDP
151 TQVSSSLSPE GTLTVEAPMP KLATQSNEIT IPVTFESRAQ LGGPEAAKSD
201 ETAAK
```

SEQ ID NO:5 HSPB2

```
  1 MSGRSVPHAH PATAEYEFAN PSRLGEQRFG EGLLPEEILT PTLYHGYYVR
 51 PRAAPAGEGS RAGASELRLS EGKFQAFLDV SHFTPDEVTV RTVDNLLEVS
101 ARHPQRLDRH GFVSREFCRT YVLPADVDPW RVRAALSHDG ILNLEAPRGG
```

151 RHLDTEVNEV YISLLPAPPD PEEEEEAAIV EP

SEQ ID NO:6 HSPB3

1 MAKIILRHLI EIPVRYQEEF EARGLEDCRL DHALYALPGP TIVDLRKTRA
51 AQSPPVDSAA ETPPREGKSH FQILLDVVQF LPEDIIIQTF EGWLLIKAQH
101 GTRMDEHGFI SRSFTRQYKL PDGVEIKDLS AVLCHDGILV VEVKDPVGTK

SEQ ID NO:7 HSPB6

1 MEIPVPVQPS WLRRASAPLP GLSAPGRLFD QRFGEGLLEA ELAALCPTTL
51 APYYLRAPSV ALPVAQVPTD PGHFSVLLDV KHFSPEEIAV KVVGEHVEVH
101 ARHEERPDEH GFVAREFHRR YRLPPGVDPA AVTSALSPEG VLSIQAAPAS
151 AQAPPPAAAK

SEQ ID NO:8 HSPB7

1 MSHRTSSTFR AERSFHSSSS SSSSSTSSSA SRALPAQDPP MEKALSMFSD
51 DFGSFMRPHS EPLAFPARPG GAGNIKTLGD AYEFAVDVRD FSPEDIIVTT
101 SNNHIEVRAE KLAADGTVMN TFAHKCQLPE DVDPTSVTSA LREDGSLTIR
151 ARRHPHTEHV QQTFRTEIKI

SEQ ID NO:9 HSPB8

1 MADGQMPFSC HYPSRLRRDP FRDSPLSSRL LDDGFGMDPF PDDLTASWPD
51 WALPRLSSAW PGTLRSGMVP RGPTATARFG VPAEGRTPPP FPGEPWKVCV
101 NVHSFKPEEL MVKTKDGYVE VSGKHEEKQQ EGGIVSKNFT KKIQLPAEVD
151 PVTVFASLSP EGLLIIEAPQ VPPYSTFGES SFNNELPQDS QEVTCT

SEQ ID NO:10 HSPB9

1 MQRVGNTFSN ESRVASRCPS VGLAERNRVA TMPVRLLRDS PAAQEDNDHA
51 RDGFQMKLDA HGFAPEELVV QVDGQWLMVT GQQQLDVRDP ERVSYRMSQK
101 VHRKMLPSNL SPTAMTCCLT PSGQLWVRGQ CVALALPEAQ TGPSPRLGSL
151 GSKASNLTR

SEQ ID NO:11 HSPB10

```
  1 MAALSCLLDS VRRDIKKVDR ELRQLRCIDE FSTRCLCDLY MHPYCCCDLH
 51 PYPYCLCYSK RSRSCGLCDL YPCCLCDYKL YCLRPSLRSL ERKAIRAIED
101 EKRELAKLRR TTNRILASSC CSSNILGSVN VCGFEPDQVK VRVKDGKVCV
151 SAERENRYDC LGSKKYSYMN ICKEFSLPPC VDEKDVTYSY GLGSCVKIES
201 PCYPCTSPCS PCSPCSPCNP CSPCNPCSPY DPCNPCYPCG SRFSCRKMIL
```

Claims

1. HSPB5 for use in a method of inducing tolerance to HSPB5 in humans, wherein HSPB5 is administered at a dosage range that results in a serum concentration of HSPB5 that induces a tolerogenic response in macrophages without inducing a pro-inflammatory response in T cells.

2. HSPB5 for use in a method of inducing tolerance to HSPB5 in humans, wherein HSPB5 is prepared for administration at a dosage range that results in a serum concentration of HSPB5 that induces a tolerogenic response in macrophages without inducing a pro-inflammatory response in T cells.

3. HSPB5 for use in a method according to claim 1 or 2, wherein said tolerogenic response in macrophages is characterized by secretion of IL-10 by macrophages, and wherein said pro-inflammatory response in T cells is characterized by secretion of IFN-γ by human memory T cells.

4. HSPB5 for use in a method according to any one of claims 1-3, wherein the medicament is administered or prepared for administration in a single dosage regime.

5 HSPB5 for use in a method according to any one of claims 1-4, wherein the medicament is administered intravenously or prepared for intravenous administration.

6. HSPB5 for use in a method according to any one of claims 1-5, wherein said HSPB5 has the sequence of SEQ ID NO:2.

7. HSPB5 for use in a method according to any one of claims 1-6, wherein said dosage range results in a peak serum concentration of HSPB5 of 1-10 μg/ml.

8. HSPB5 for use in a method according to any one of claims 1-7, wherein said dosage range is 5-20 mg, preferably 12.5 mg of HSPB5.

9. HSPB5 for use in a method according to any one of claims 1-8, wherein said induction of tolerance to HSPB5 provides anti-inflammatory effects in the prophylactic and therapeutic treatment of multiple sclerosis.

10. Use of HSPB5 for the preparation of a medicament for inducing tolerance to HSPB5 in humans, wherein the medicament is prepared for intravenous administration in a single dosage regime and at a dosage range that results in a serum concentration of HSPB5 that induces a tolerogenic response in macrophages but that does not induce a pro-inflammatory response in T cells.

11. HSPB5 for use in a method of inducing an anti-inflammatory response by inducing tolerance to HSPB5 in humans suffering from an inflammatory disease,
wherein HSPB5 is administered intravenously or prepared for administration by a single dosage regime at a dosage range that results in a serum concentration of HSPB5 that induces a tolerogenic response in macrophages without inducing a pro-inflammatory response in T cells, wherein said inflammatory disease is multiple sclerosis.

12. HSPB5 for use according to claim 11, wherein said tolerogenic response in macrophages is characterized by secretion of IL-10 by

macrophages, and wherein said pro-inflammatory response in T cells is characterised by secretion of IFN-γ by human memory T cells.

13. HSPB5 for use in a method according to claim 11 or 12, wherein said HSPB5 has the sequence of SEQ ID NO:2.

14. HSPB5 for use in a method according to any one of claims 11-13, wherein said dosage range results in a serum concentration of HSPB5 of 1-10 μg/ml and/or wherein said dosage range is 5-20 mg, preferably 12.5 mg of HSPB5 given via a single intravenous injection.

15. A method of treating multiple sclerosis in a subject, comprising the step of administering HSPB5 to a subject suffering from multiple sclerosis at a dosage range that results in a serum concentration of HSPB5 in said subject that induces a tolerogenic response in macrophages without inducing a pro-inflammatory response in T cells.

16. The method according to claim 15, wherein said tolerogenic response in macrophages is characterized by secretion of IL-10 by macrophages, and wherein said pro-inflammatory response in T cells is characterized by secretion of IFN-γ by human memory T cells.

17. The method according to any one of claims 15-16, wherein the medicament is administered or prepared for administration in a single dosage regime.

18. The method according to any one of claims 15-17, wherein the medicament is administered intravenously or prepared for intravenous administration.

19. The method according to any one of claims 15-18, wherein said HSPB5 has the sequence of SEQ ID NO:2.

20. The method according to any one of claims 15-19, wherein said dosage range results in a peak serum concentration of HSPB5 of 1-10 μg/ml.

21. The method according to any one of claims 15-20, wherein said dosage range is 5-20 mg, preferably 12.5 mg of HSPB5.

Fig. 1

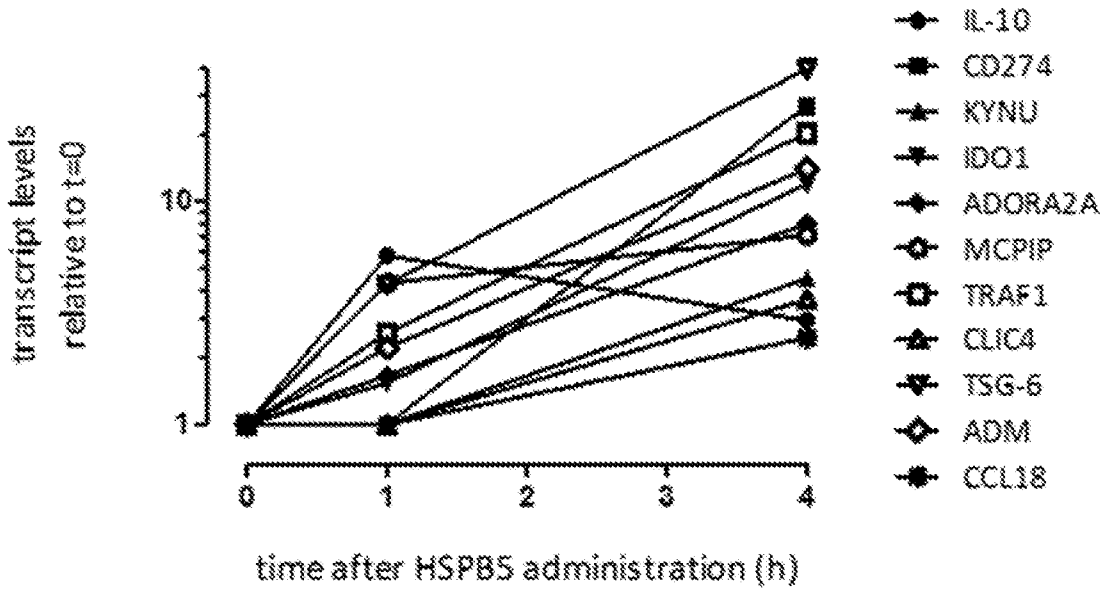

transcript levels
relative to t=0
10
1
0
1
2
3
4
time after HSPB5 administration (h)
IL-10
CD274
KYNU
IDO1
ADORA2A
MCPIP
TRAF1
CLIC4
TSG-6
ADM
CCL18

Fig. 2

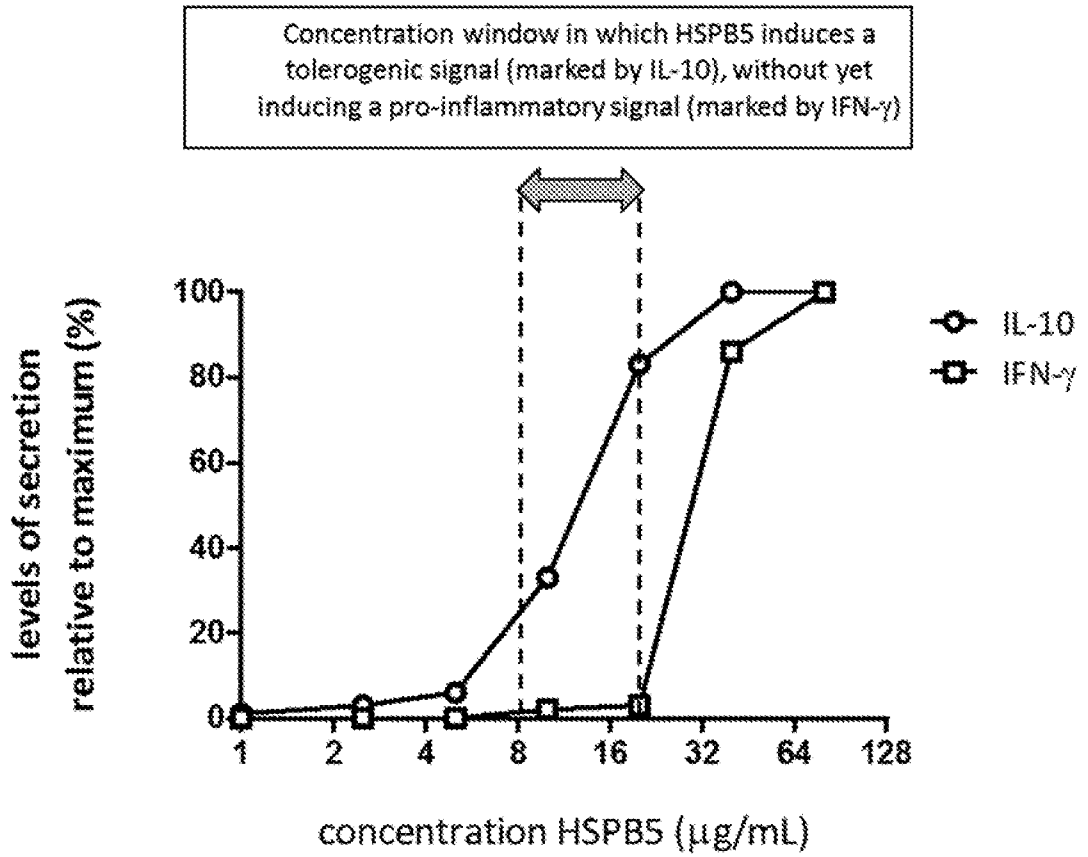

Concentration window in which HSPB5 induces a tolerogenic signal (marked by IL-10), without yet inducing a pro-inflammatory signal (marked by IFN-γ)
levels of secretion relative to maximum (%)
100
80
60
40
20
0
1
2
4
8
16
32
64
128
concentration HSPB5 (µg/mL)
IL-10
IFN-γ

Fig. 3

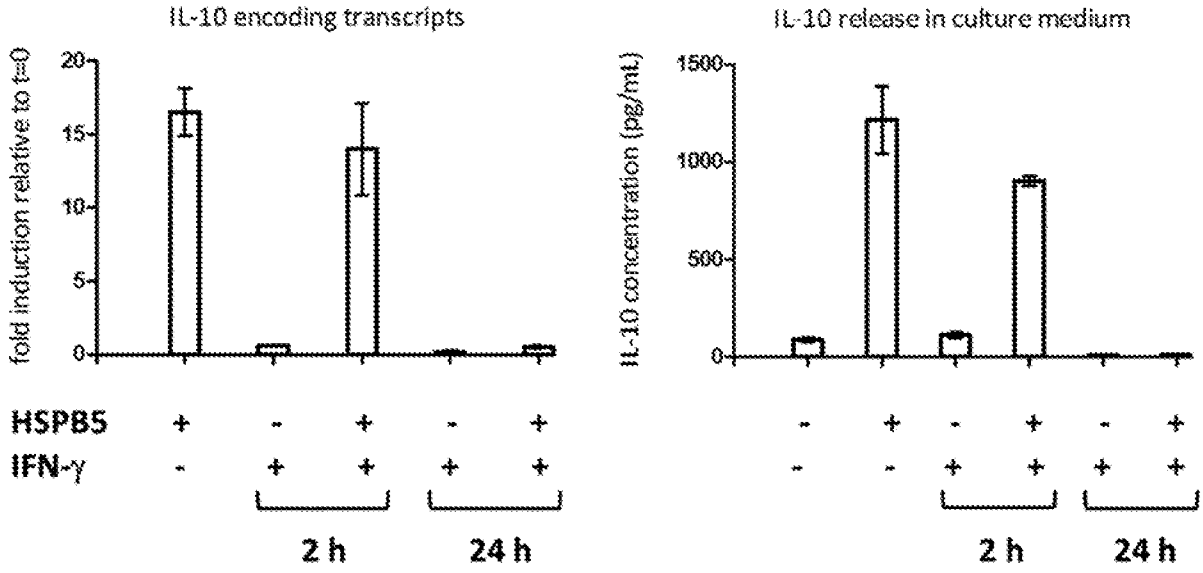

IL-10 encoding transcripts
fold induction relative to t=0
20
15
10
5
0
HSPB5 + - + - +
IFN-γ - + + + +
2 h
24 h
IL-10 release in culture medium
IL-10 concentration (pg/mL)
1500
1000
500
0
- + - + - +
- - + + + +
2 h
24 h

Fig. 4

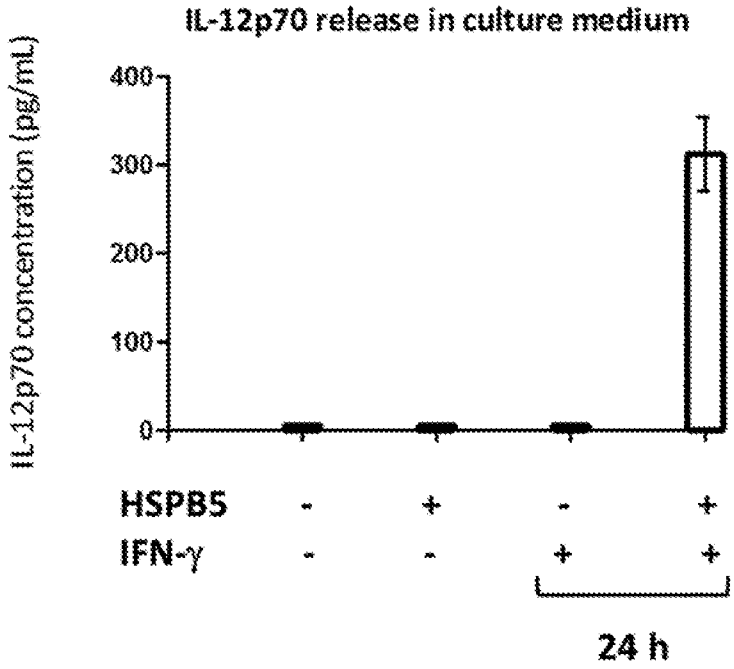

IL-12p70 release in culture medium
IL-12p70 concentration (pg/mL)
400
300
200
100
0
HSPB5 - + - +
IFN-γ - - + +
24 h

Fig. 5

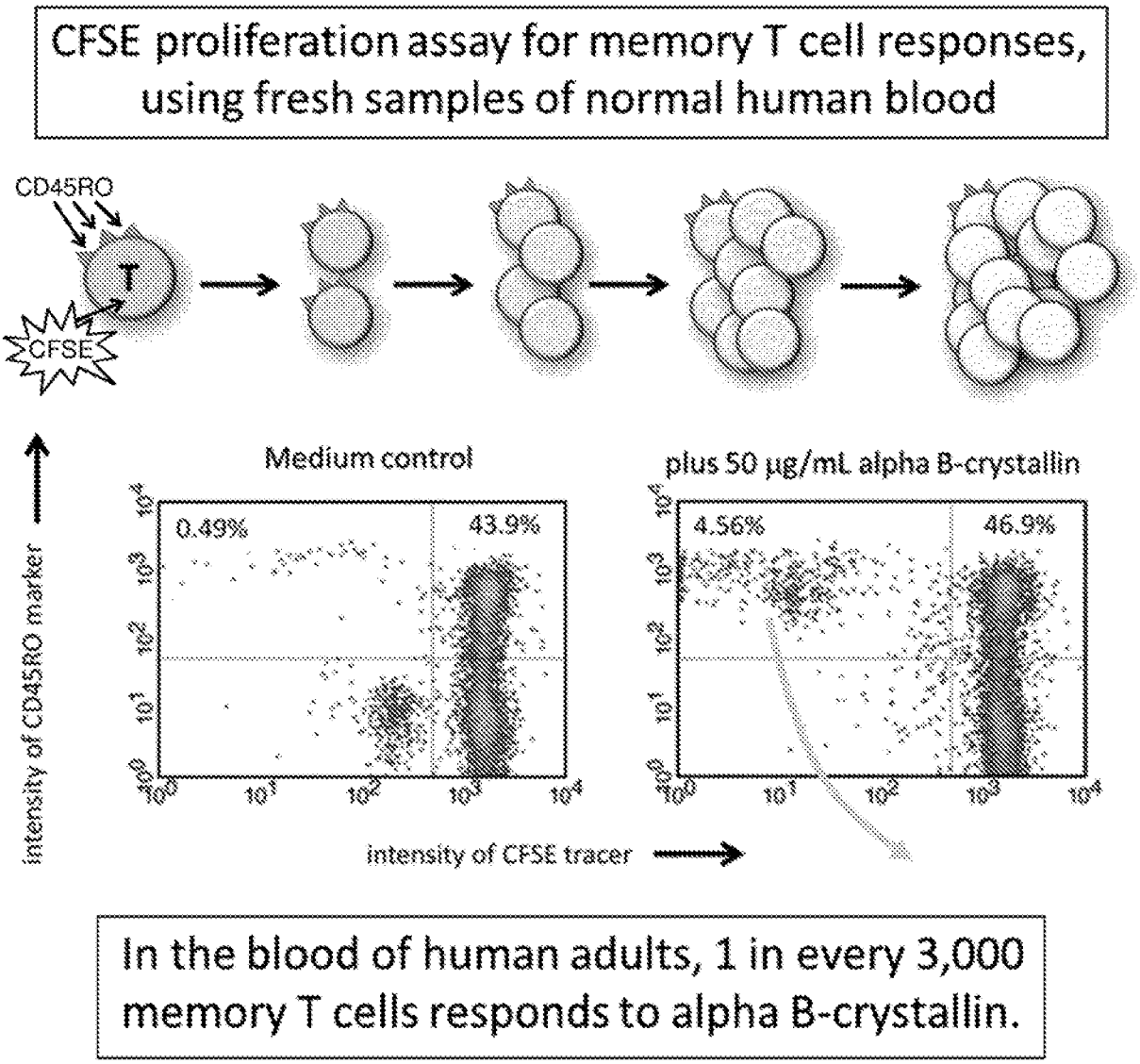

CFSE proliferation assay for memory T cell responses, using fresh samples of normal human blood
CD45RO
T
CFSE
Medium control
plus 50 µg/mL alpha B-crystallin
0.49%
43.9%
4.56%
46.9%
intensity of CD45RO marker
intensity of CFSE tracer
In the blood of human adults, 1 in every 3,000 memory T cells responds to alpha B-crystallin.

Fig. 6

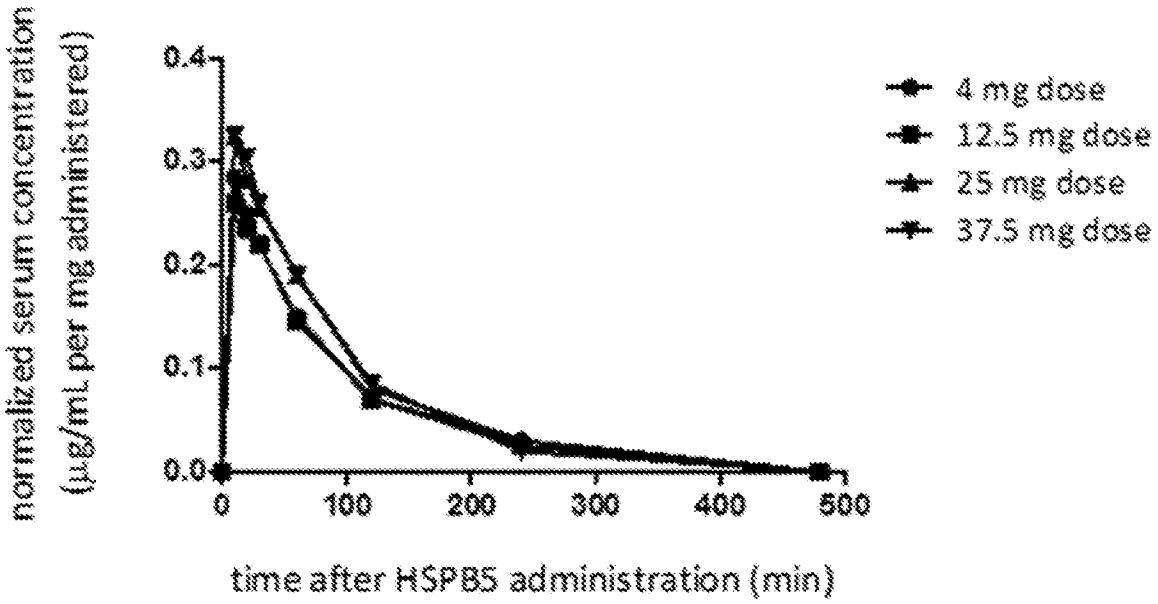

normalized serum concentration
(µg/mL per mg administered)
0.4
0.3
0.2
0.1
0.0
0
100
200
300
400
500
time after HSPB5 administration (min)
4 mg dose
12.5 mg dose
25 mg dose
37.5 mg dose

Fig. 7

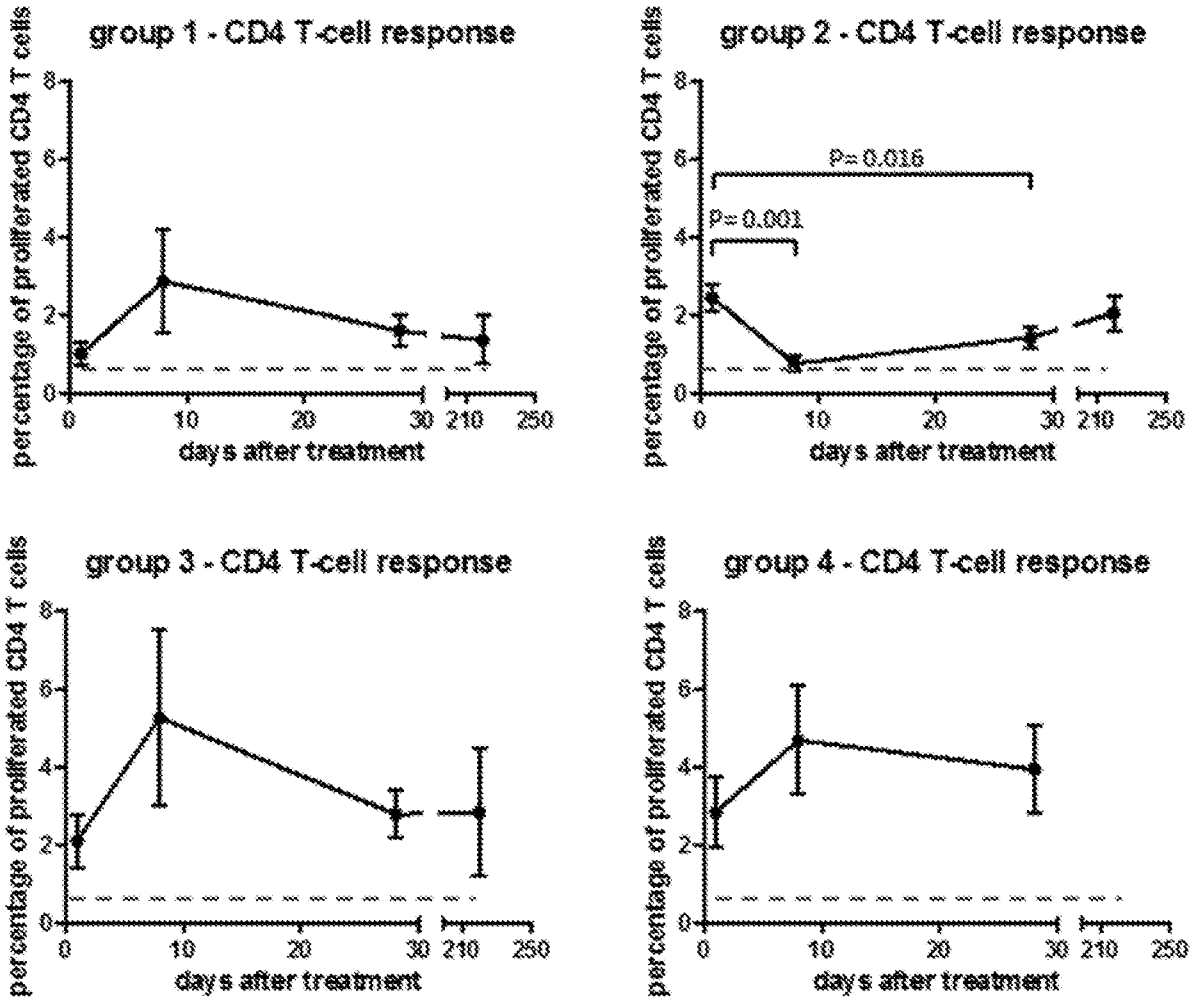

group 1 - CD4 T-cell response
group 2 - CD4 T-cell response
group 3 - CD4 T-cell response
group 4 - CD4 T-cell response
percentage of proliferated CD4 T cells
days after treatment
P= 0.016
P= 0.001
0
2
4
6
8
0
10
20
30
210
250

Fig. 7, cont'd

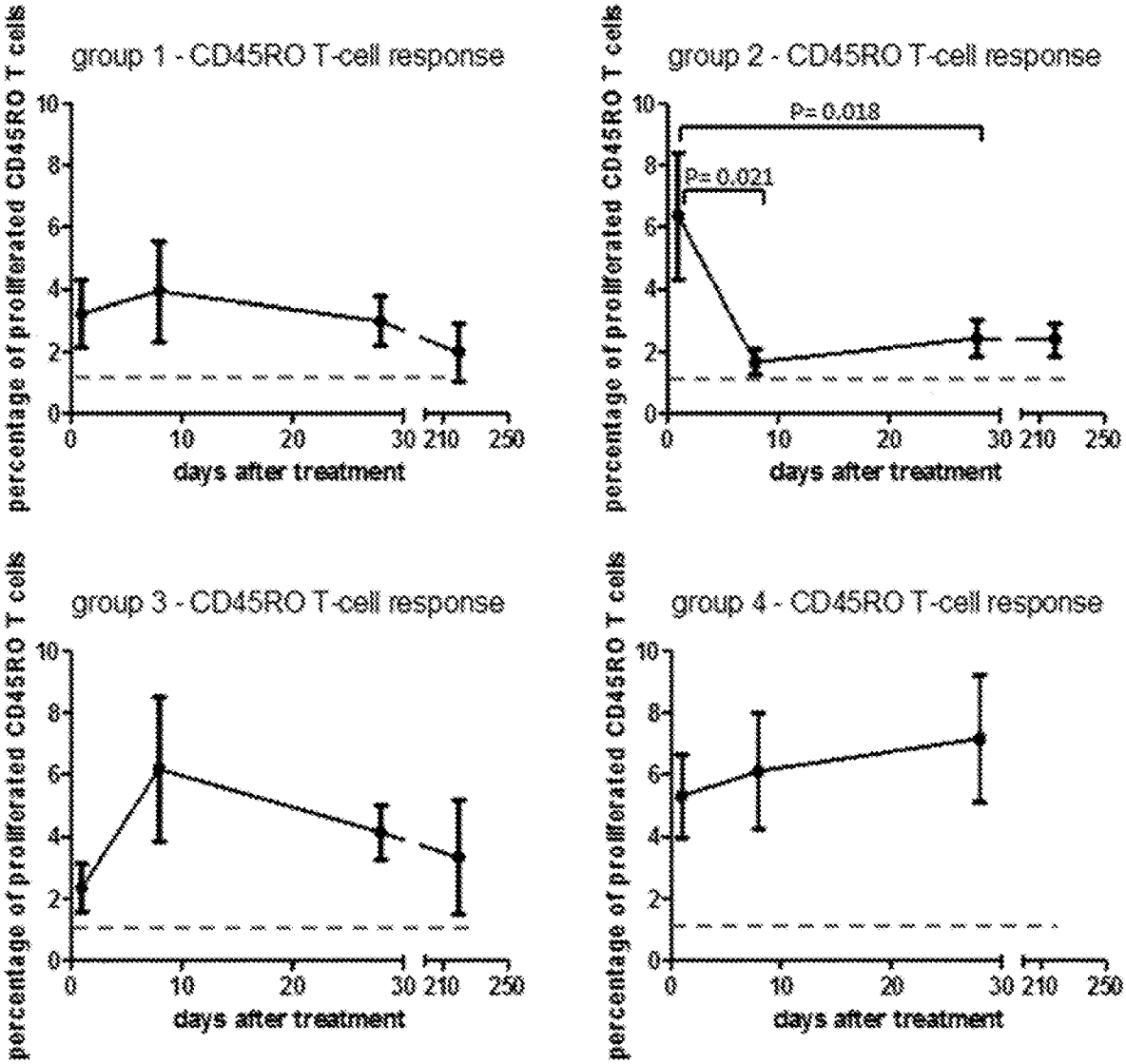

group 1 - CD45RO T-cell response
group 2 - CD45RO T-cell response
group 3 - CD45RO T-cell response
group 4 - CD45RO T-cell response
percentage of proliferated CD45RO T cells
days after treatment
P= 0.018
P= 0.021
0
2
4
6
8
10
0
10
20
30
210
250

Fig. 8

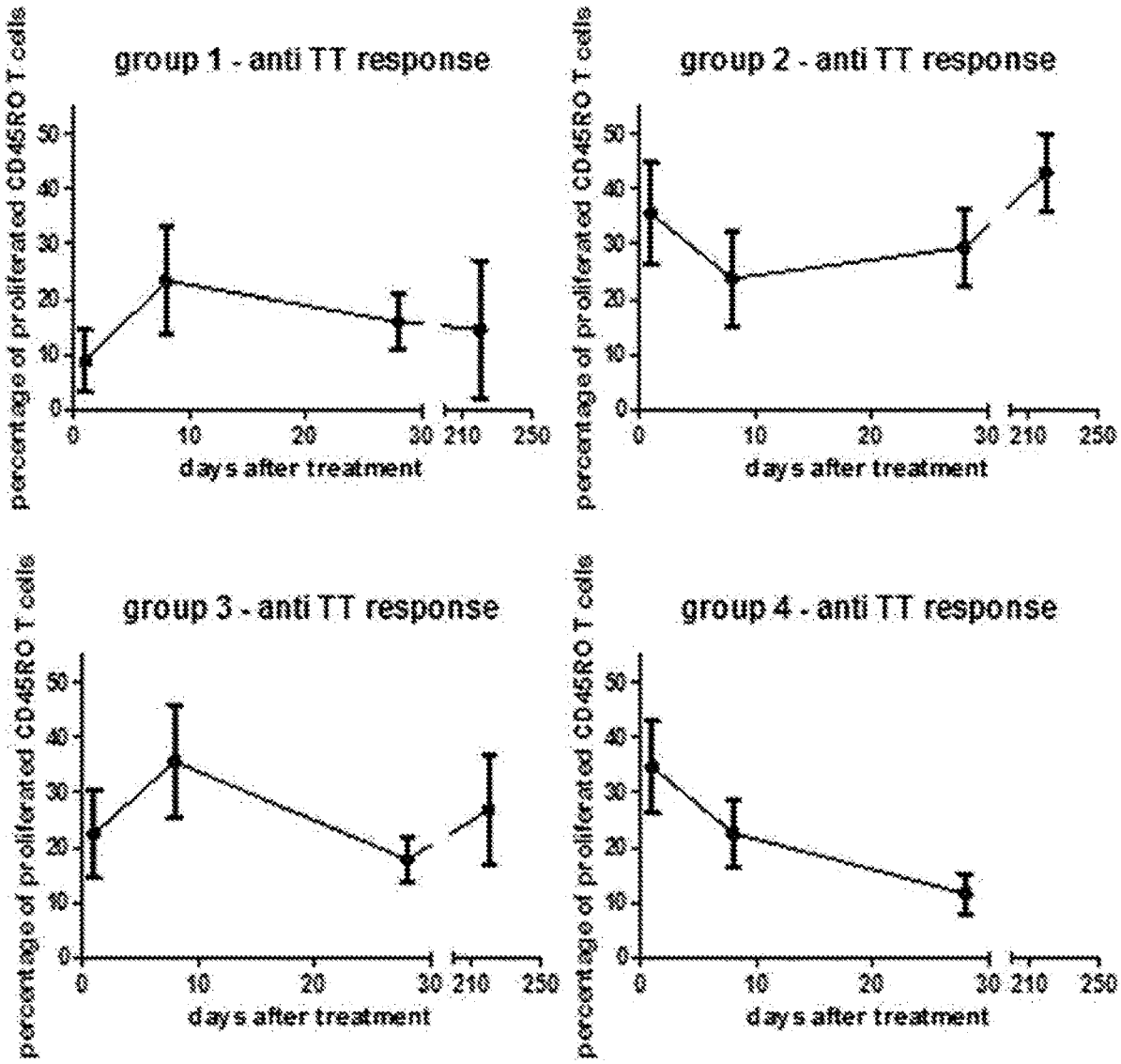

group 1 - anti TT response
group 2 - anti TT response
group 3 - anti TT response
group 4 - anti TT response
percentage of proliferated CD45RO T cells
days after treatment
0
10
20
30
210
250
0
10
20
30
40
50

Fig. 9

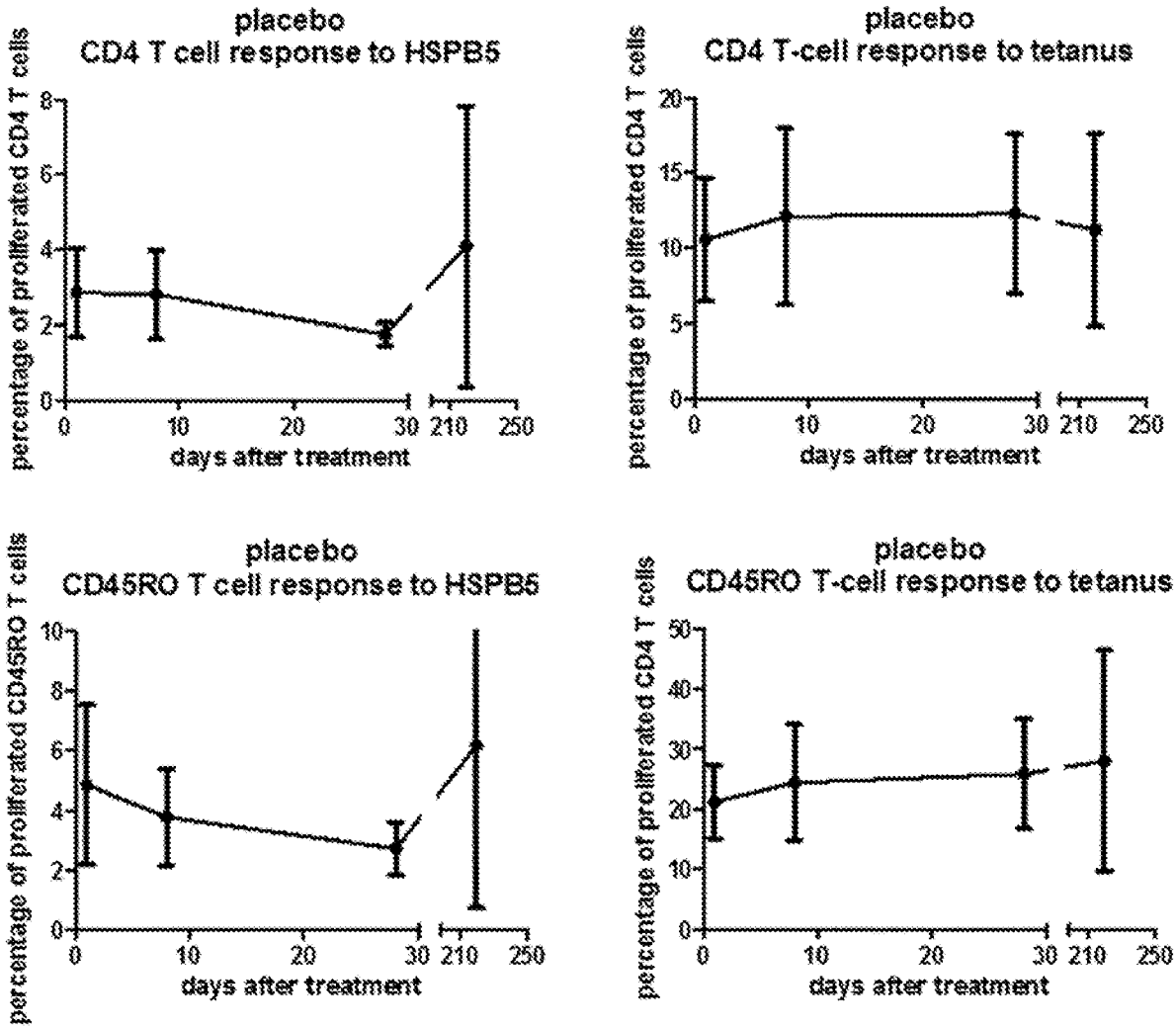

placebo
CD4 T cell response to HSPB5
percentage of proliferated CD4 T cells
days after treatment
placebo
CD4 T-cell response to tetanus
percentage of proliferated CD4 T cells
days after treatment
placebo
CD45RO T cell response to HSPB5
percentage of proliferated CD45RO T cells
days after treatment
placebo
CD45RO T-cell response to tetanus
percentage of proliferated CD4 T cells
days after treatment

Fig. 10

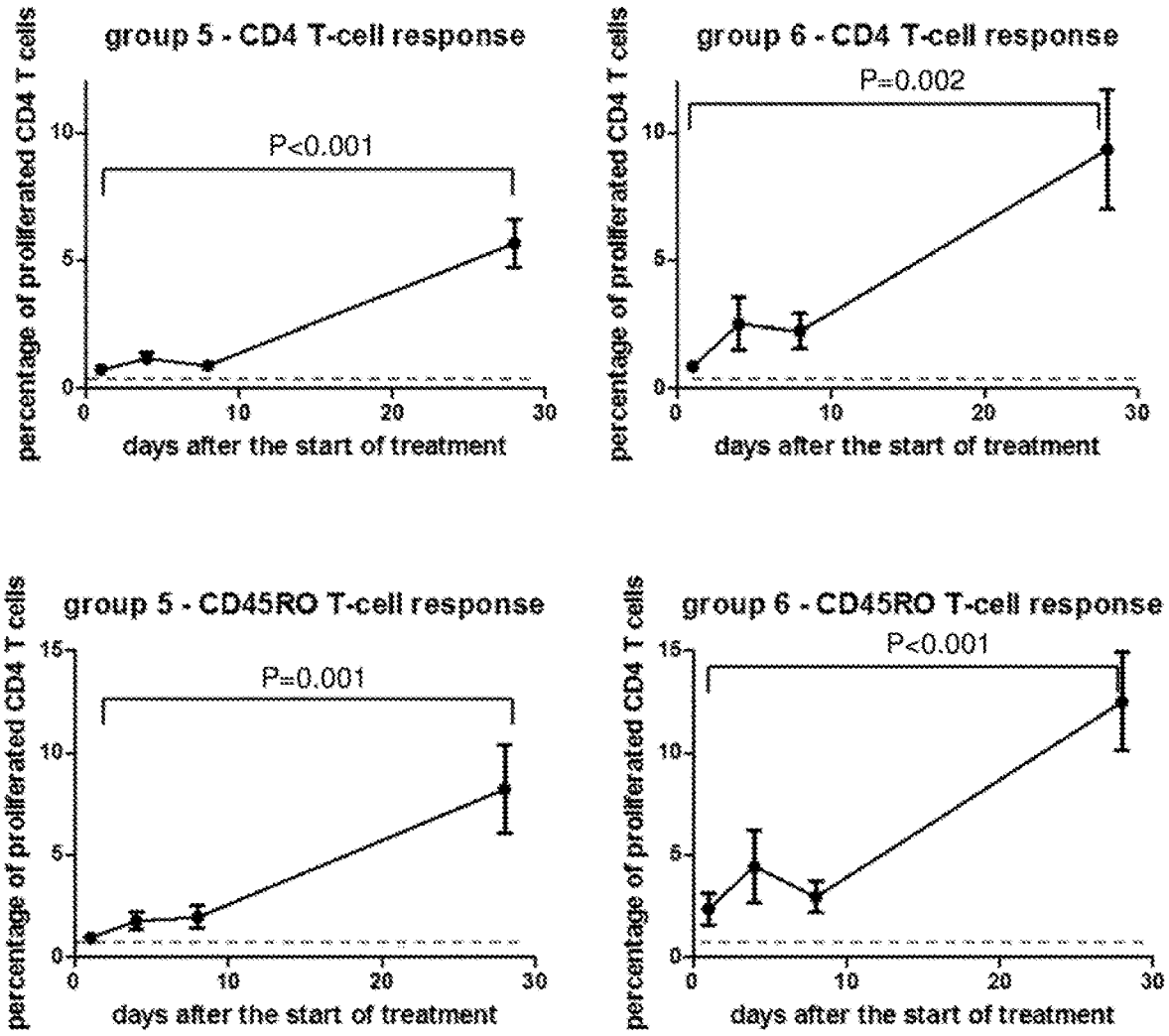

group 5 - CD4 T-cell response
P<0.001
percentage of proliferated CD4 T cells
days after the start of treatment
group 6 - CD4 T-cell response
P=0.002
percentage of proliferated CD4 T cells
days after the start of treatment
group 5 - CD45RO T-cell response
P=0.001
percentage of proliferated CD4 T cells
days after the start of treatment
group 6 - CD45RO T-cell response
P<0.001
percentage of proliferated CD4 T cells
days after the start of treatment

Fig. 11

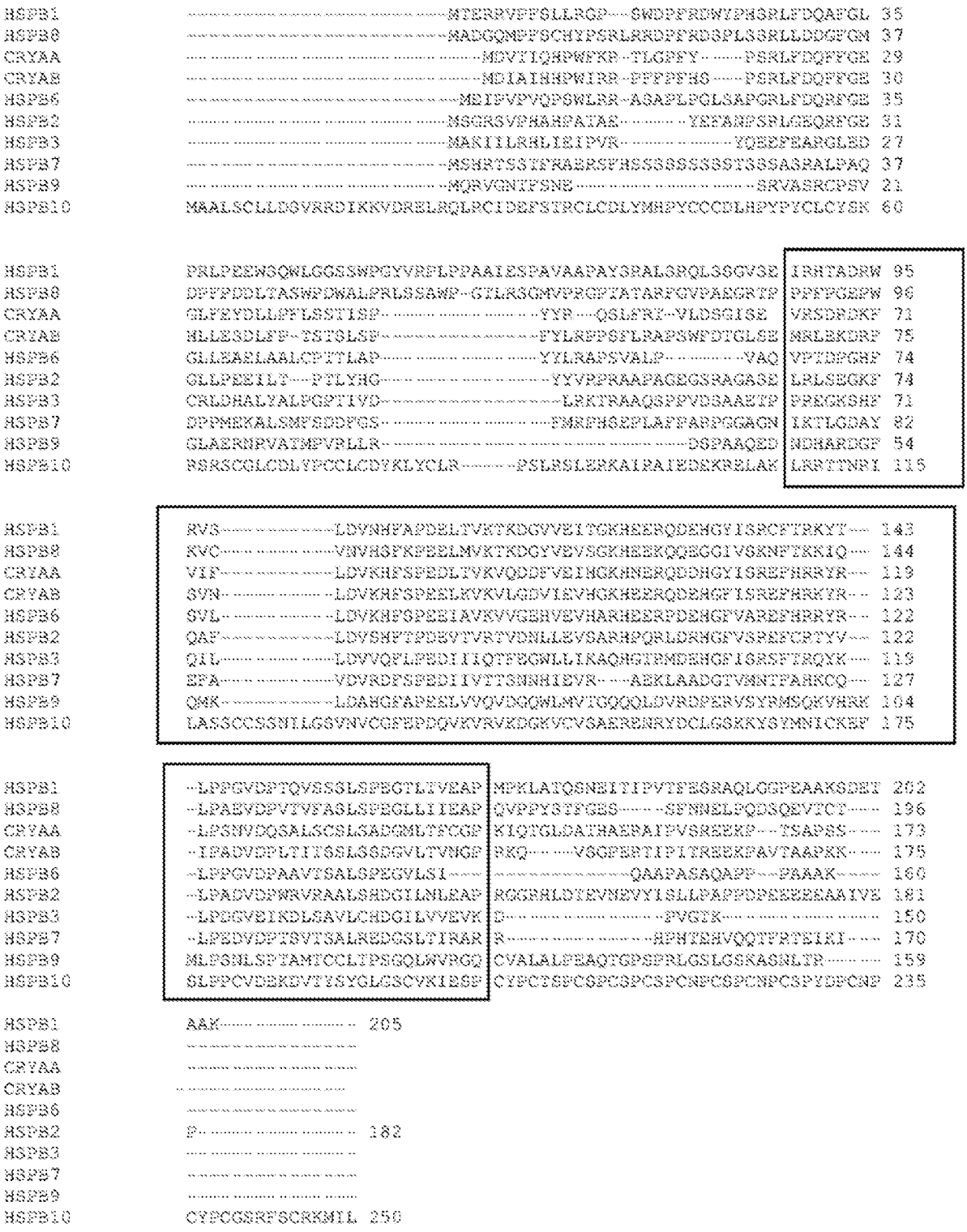

HSPB1
HSPB8
CRYAA
CRYAB
HSPB6
HSPB2
HSPB3
HSPB7
HSPB9
HSPB10

Fig. 12

Homologies between alpha-crystallin domains in CRYAB-like proteins

Between CRYAB (HSPB5) and HSPB1: 78.9% homology in 81 aa overlap:
(Note: residue numbers reflect the position in the alpha-crystallin domain, not in the full-length protein sequence)

```
               10         20         30         40         50         60
HSPB5  MRLEKDRFSVNLDVKHFSPEELKVKVLGDVIEVHGKHEERQDEHGFISREFHRKYRIPAD
       .:   ::. :.:::.::.:.:: ::.   :.:. :::::::::::.::: : ::: .:
HSPB1  IRHTADRWRVSLDVNHFAPDELTVKTKDGVVEITGKHEERQDEHGYISRCFTRKYTLPPG
               10         20         30         40         50         60

               70         80
HSPB5  VDPLTITSSLSSDGVLTVNGP
       :::  ..:::: .:.:::..:
HSPB1  VDPTQVSSSLSPEGTLTVEAP
               70         80
```

Between CRYAB (HSPB5) and HSPB2: 81.5% homology in 81 aa overlap:

```
               10         20         30         40         50         60
HSPB5  MRLEKDRFSVNLDVKHFSPEELKVKVLGDVIEVHGKHEERQDEHGFISREFHRKYRIPAD
       .:: . .:.. :::.::.:.:. :... ...:: ..: .: :.:::.:::: : : .:::
HSPB2  LRLSECKFQAFLDVSHFTPDEVTVRTVDNLLEVSARHPQRLDRHGFVSREFCRTYVLPAD
               10         20         30         40         50         60

               70         80
HSPB5  VDPLTITSSLSSDGVLTVNGP
       :::  . ..:: ::.:....:
HSPB2  VDPWRVRAALSHDGILNLEAP
               70         80
```

Between CRYAB (HSPB5) and HSPB3: 71.4% homology in 77 aa overlap (2-78:2-78);

```
              10         20         30         40         50         60
HSPB5  RLEKDRFSVNLDVKHFSPEELKVKVLGDVIEVHGKHEERQDEHGFISREFHRKYRIPADV
       :  :..:.. ::: .: :::. ....   . ....:  :.:::::::: : :.:..:  :
HSPB3  RECKSHFQILLDVVQFLPEDIIIQTFEGWLLIKAQHGTRMDEHGFISRSFTRQYKLPDGV
              10         20         30         40         50         60

              70
HSPB5  DPLTITSSLSSDGVLTV
       .   ... :  ::.:.:
HSPB3  EIKDLSAVLCHDGILVV
              70
```

Fig. 12 cont'd

Between CRYAB (HSPB5) and HSPB4 (CRYAA): 85.2% homology in 81 aa overlap (1-81:1-81);

```
               10         20         30         40         50         60
HSPB5  MRLEKDRFSVNLDVKHFSPEELKVKVLGDVIEVHGKHEERQDEHGFISREFHRKYRIPAD
       .: ..:.: . :::::::::.: ::: : .:.::::.::::.::.:::::::.::.:..
HSPB4  VRSDRDKFVIFLDVKHFSPEDLTVKVQDDFVEIHGKHNERQDDHGYISREFHRRYRLPSN
               10         20         30         40         50         60

               70         80
HSPB5  VDPLTITSSLSSDGVLTVNGP
       ::  ... :::.::.::  ::
HSPB4  VDQSALSCSLSADGMLTFCGP
               70         80
```

Between CRYAB (HSPB5) and HSPB6: 86.7% homology in 75 aa overlap (4-78:4-78);

```
            10         20         30         40         50         60
HSPB5  EKDRFSVNLDVKHFSPEELKVKVLGDVIEVHGKHEERQDEHGFISREFHRKYRIPADVDP
       .  .::: :::::::::. :::.:. .::..:::: ::::::.::::::.::.:  :::
HSPB6  DPGHFSVLLDVKHFSPEEIAVKVVGEHVEVHARHEERPDEHGFVAREFHRRYRLPPGVDP
            10         20         30         40         50         60

            70
HSPB5  LTITSSLSSDGVLTV
        ..::.:: .:::..
HSPB6  AAVTSALSPEGVLSI
            70
```

Between CRYAB (HSPB5) and HSPB7: 63.0% homology in 73 aa overlap (6-78:6-75);

```
          10         20         30         40         50         60
HSPB5  DRFSVNLDVKHFSPEELKVKVLGDVIEVHGKHEERQDEHGFISREFHRKYRIPADVDPLT
       : .   .::. :::::.. : . .. :::::   :.    : .   : .: ..: :::: .
HSPB7  DAYEFAVDVRDFSPEDIIVTTSNNHIEVRA~~~EKLAADGTVMNTFAHKCQLPEDVDPTS
          10         20         30           40         50         60

          70
HSPB5  ITSSLSSDGVLTV
       .:::.:  :: ::.
HSPB7  VTSALREDGSLTI
            70
```

Fig. 12 cont'd

Between CRYAB (HSPB5) and HSPB8: 81.0% homology in 74 aa overlap (8-81:9-82);

```
         10         20         30         40         50         60
HSPB5  FSVNLDVKHFSPEELKVKVLGDVIEVHGKHEERQDEHGFISREFHRKYRIPADVDPLTIT
       ..: ..:. :.:::: ::.    .:: ::::::.:: :..:.. .: ..::.:::.:.
HSPB8  WKVCVNVHSFKPEELMVKTKDGYVEVSGKHEEKQQEGGIVSKNFTKKIQLPAEVDPVTVF
       10         20         30         40         50         60

         70         80
HSPB5  SSLSSDGVLTVNGP
       .::: .:.: ...:
HSPB8  ASLSPEGLLIIEAP

       70         80
```

Between CRYAB (HSPB5) and HSPB9: 70.0% homology in 80 aa overlap (5-80:5-83);

```
           10         20         30         40         50         60
HSPB5  KDRFSVNLDVKHFSPEELKVKVLGDVIEVHGKHE-ERQDEHGF-----ISREFHRKYRIPAD
       .: :...::.. :.:::: :.: :. . : :... . .: .     .:.. :::. .:..
HSPB9  RDGFQMKLDAHGFAPEELVVQVDGQWLMVTGQQQLDVRDPERVSYRMSQKVHRKM-LPSN
           10         20         30         40         50         60

                 70         80
HSPB5  VDPLTITSSLSSDGVLTVNG
       ..: ..:  :. .: : : :
HSPB9  LSPTAMTCCLTPSGQLWVRG
            70         80
```

Between CRYAB (HSPB5) and HSPB10: 56.7% homology in 74 aa overlap (11-81:21-94);

```
                20         30         40         50           60
HSPB5  NLDVKHFSPEELKVKVLGDVIEVHGKHEERQDEHGFISREFH----RKYRIPADVDPLTIT
       ...:  : :...::.:    . : ...:.: :  :  .  .    ... .:  ::   .:
HSPB10 SVNVCGFEPDQVKVRVKDGKVCVSAERENRYDCLGSKKYSYMNICKEFSLPPCVDEKDVT
                30         40         50         60         70         80

         70         80
HSPB5  SSLSSDGVLTVNGP
        : .  . . ...:
HSPB10 YSYGLGSCVKIESP
                90
```

Fig. 13

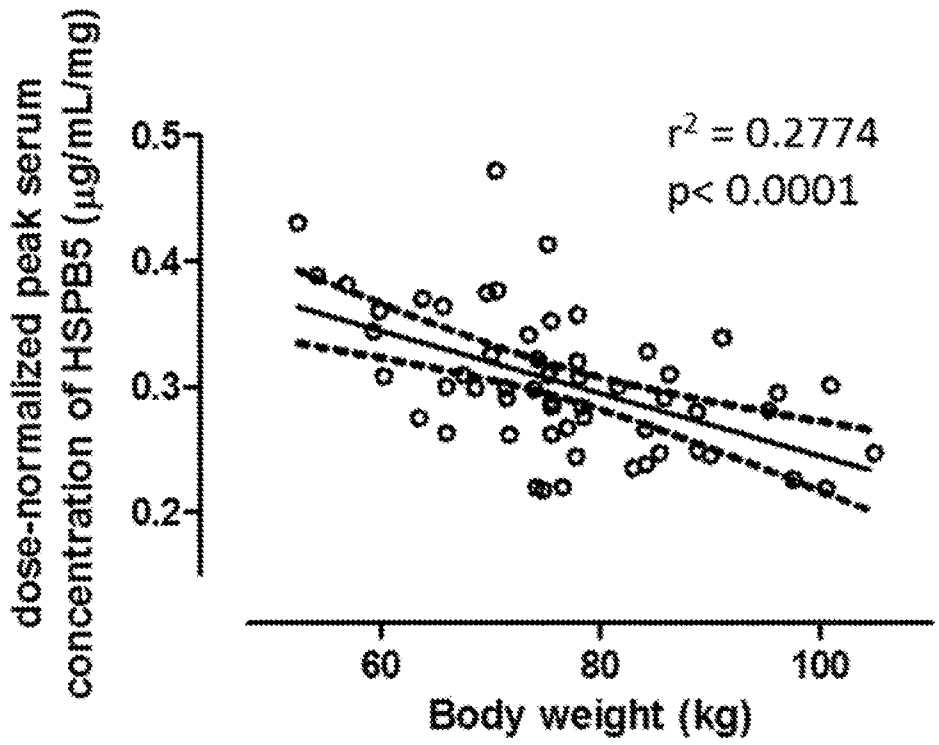

dose-normalized peak serum concentration of HSPB5 (µg/mL/mg)
0.5
0.4
0.3
0.2
r² = 0.2774
p< 0.0001
60
80
100
Body weight (kg)

Fig. 14

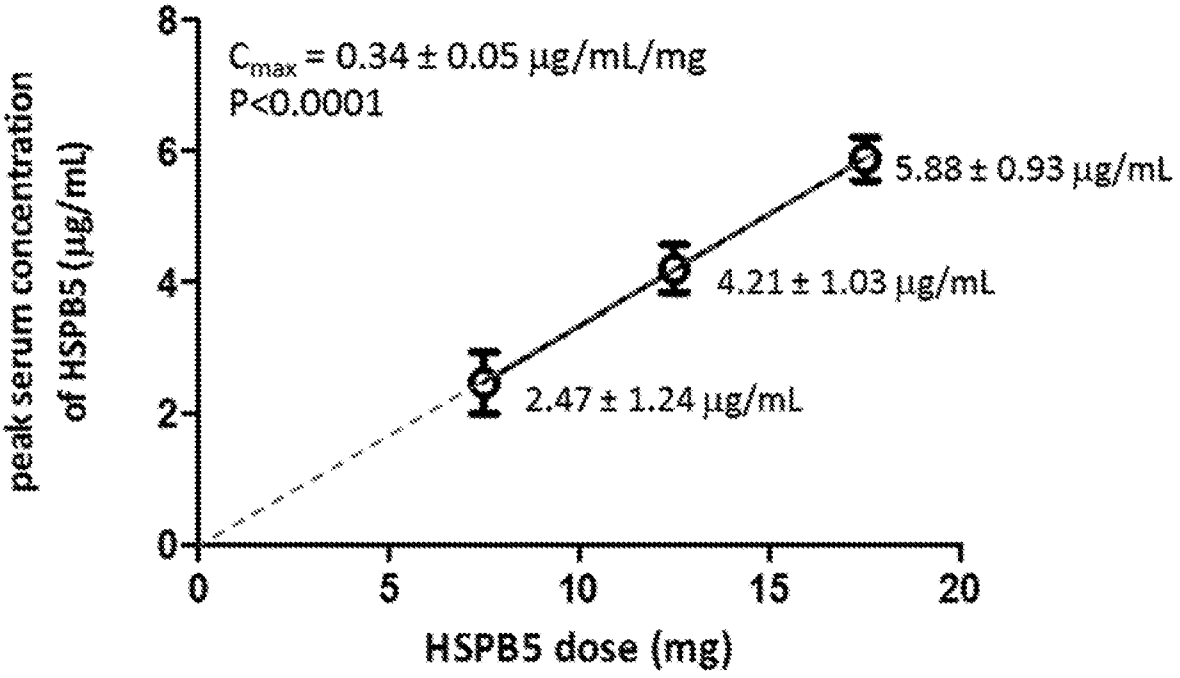

peak serum concentration of HSPB5 (µg/mL)
C_{max} = 0.34 ± 0.05 µg/mL/mg
P<0.0001
5.88 ± 0.93 µg/mL
4.21 ± 1.03 µg/mL
2.47 ± 1.24 µg/mL
0
2
4
6
8
0
5
10
15
20
HSPB5 dose (mg)

Fig. 15

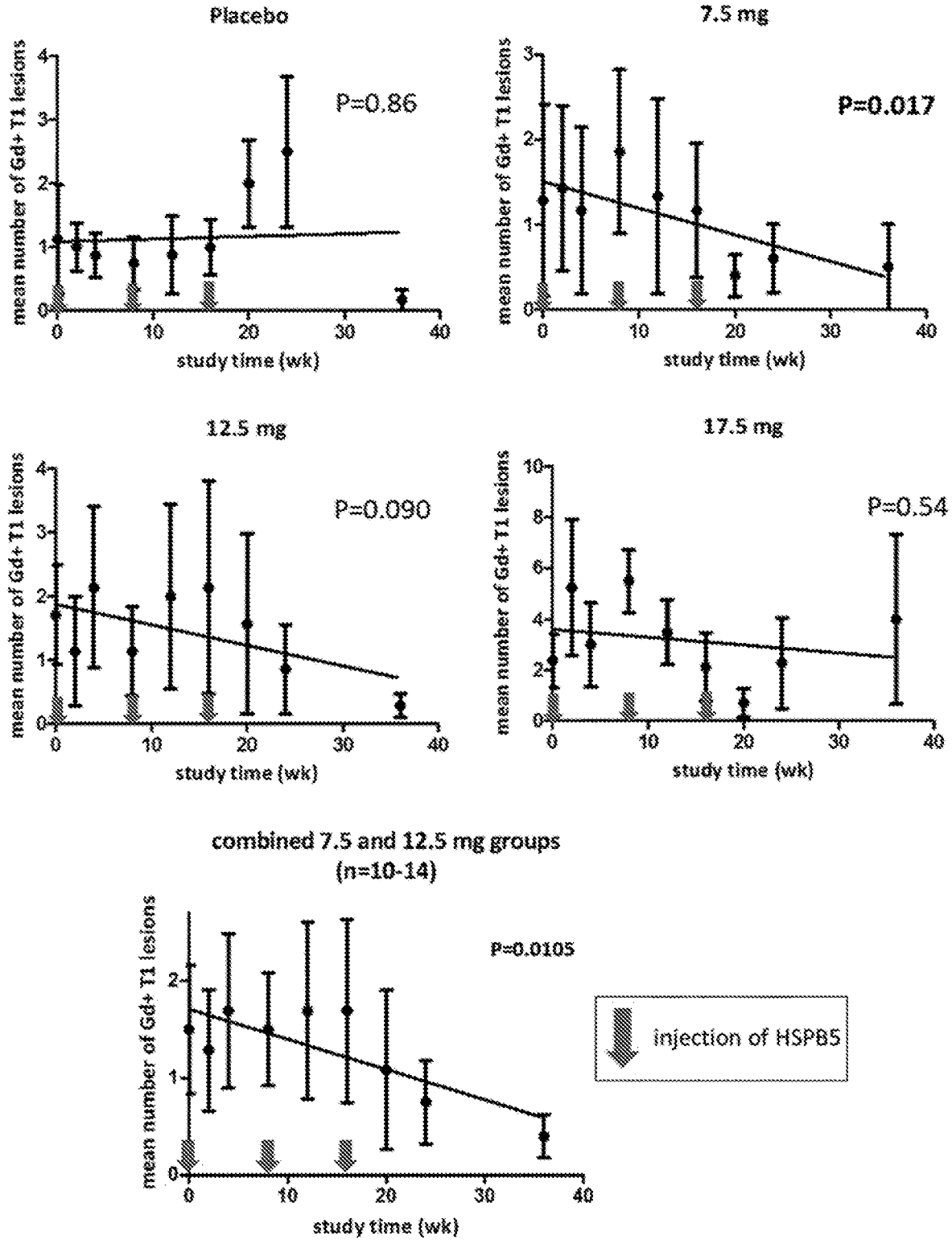
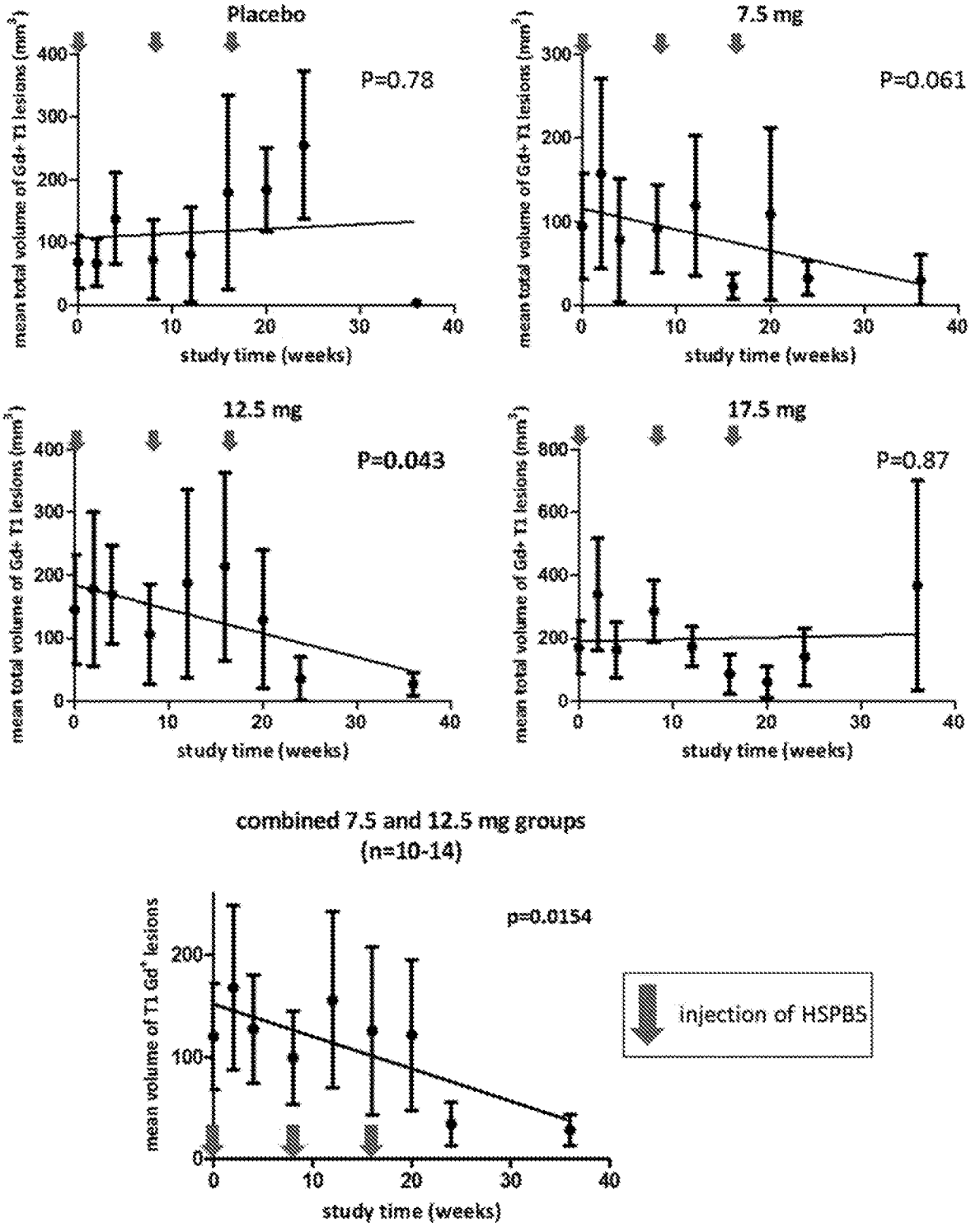

Placebo
P=0.86
7.5 mg
P=0.017
12.5 mg
P=0.090
17.5 mg
P=0.54
combined 7.5 and 12.5 mg groups
(n=10-14)
P=0.0105
mean number of Gd+ T1 lesions
study time (wk)
injection of HSP85

Fig. 16


Placebo
P=0.78
7.5 mg
P=0.061
12.5 mg
P=0.043
17.5 mg
P=0.87
mean total volume of Gd+ T1 lesions (mm3)
study time (weeks)
combined 7.5 and 12.5 mg groups
(n=10-14)
p=0.0154
mean volume of T1 Gd+ lesions
injection of HSPBS

INTERNATIONAL SEARCH REPORT

International application No
PCT/NL2014/050383

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K38/17 A61P37/02
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	LEE J ET AL: "Controlled delivery of heat shock protein using an injectable microsphere/hydrogel combination system for the treatment of myocardial infarction", JOURNAL OF CONTROLLED RELEASE, ELSEVIER, AMSTERDAM, NL, vol. 137, no. 3, 4 August 2009 (2009-08-04), pages 196-202, XP026223531, ISSN: 0168-3659, DOI: 10.1016/J.JCONREL.2009.04.008 [retrieved on 2009-04-14] abstract -----	1-21
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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
22 September 2014	26/09/2014

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Schnack, Anne

INTERNATIONAL SEARCH REPORT

International application No
PCT/NL2014/050383

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/NL2014/050383

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