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ORIGINAL PAPER

Mycobacterial and mouse HSP70 have immuno-modulatory effects on dendritic cells

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Abstract Previously, it has been shown that heat shock protein 70 (HSP70) can prevent inflammatory damage in experimental autoimmune disease models. Various possible underlying working mechanisms have been proposed. One possibility is that HSP70 induces a tolerogenic phenotype in dendritic cells (DCs) as a result of the direct interaction of the antigen with the DC. Tolerogenic DCs can induce antigen-specific regulatory T cells and dampen pathogenic T cell responses. We show that treatment of murine DCs with either *mycobacterial* (Mt) or mouse HSP70 and pulsed with the disease-inducing antigen induced suppression of proteoglycan-induced arthritis (PGIA), although mouse HSP70-treated DCs could ameliorate PGIA to a greater extent. In addition, while murine DCs treated with Mt- or mouse HSP70 had no significantly altered phenotype as compared to untreated DCs, HSP70-treated DCs pulsed with pOVA (ovalbumin peptide 323–339) induced a significantly increased production of IL-10 in pOVA-specific T cells. IL-10-producing T cells were earlier shown to be involved in Mt HSP70-induced suppression of PGIA. In conclusion, this study indicates that Mt- and mouse HSP70-treated BMDC can suppress PGIA via an IL-10-producing T cell-dependent manner.

Keywords Dendritic cell · HSP70 · Arthritis · Mouse/murine · Tolerance

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Introduction

Heat shock proteins (HSPs) are intracellular molecular chaperones that support folding and transport of a large variety of proteins under normal physiological conditions and following stress stimuli like hypothermia, oxidative stress or exposure to inflammatory mediators. HSPs are evolutionarily ancient and can be divided into several families based on their monomeric molecular weight, like the HSP10, HSP40, HSP60, HSP70 and HSP90 families (Li and Srivastava 2004). The HSP60 and HSP70 families are highly conserved in evolutionarily distant organisms such as bacteria and mammals. However, despite high homology between different species, the effects on the immune system can be markedly different and even within the same HSP, inhibitory as well as stimulatory sequences have been described (Wang et al. 2005).

From literature, it becomes clear that HSPs can trigger innate as well as adaptive arms of the immune system and the final outcome of the immune responses induced by HSPs can be either pro- or anti-inflammatory. Especially in early reports, HSP70 was described as an immune stimulatory mediator that induced rapid intracellular calcium flux and pro-inflammatory cytokine production via binding receptors like toll-like receptor (TLR)-2, TLR-4, CD91, lectin-like oxidized low-density lipoprotein receptor 1 (LOX)-1 or CD14 (Asea et al. 2000; Basu et al. 2001; Delneste et al. 2002; Panjwani et al. 2002; Vabulas et al. 2002; Wang et al. 2001). However, later on, there was some controversy in the area as non-protein products such as lipopolysaccharide (LPS), lectins and ADP/ATP nucleotides were found to copurify with HSP70. These substances are ligands for TLRs or can induce calcium signals in dendritic cells (DCs) (Bausinger et al. 2002; Bendz et al. 2008; Tsan and Gao 2004). In contrast, some groups have observed an uptake of HSP70 by DCs via endocytotic mechanisms

without involvement of any cell surface receptors (Wang et al. 2006). As opposed to stimulating immune responses, anti-inflammatory effects of HSP70 have also been described. Treatment of DCs with *Mycobacterium tuberculosis* (Mt) HSP70 inhibited maturation and induced interleukin (IL)-10 production in DCs (Detanico et al. 2004; Motta et al. 2007). In addition, exogenous administration of Mt HSP70 was able to suppress experimental arthritis in different mouse and rat models as reviewed by van Eden et al. (2005).

In the present study, we explored the immunological effect of treatment with a eukaryotic versus a prokaryotic HSP70 family member on the phenotype and function of DCs. The stress-inducible form of mouse HSP70, HSPA1A and Mt HSP70 induced a functionally tolerogenic DC that suppressed proteoglycan-induced arthritis (PGIA), a T cell-dependent, antibody-mediated murine model for rheumatoid arthritis. Although the direct effects of HSP70 treatment on DCs were small, the function of DCs had been changed and treated DCs were able to induce an altered phenotype in T cells. An important difference in the phenotype of the T cells was the significant increase in the production of the anti-inflammatory cytokine IL-10. A cytokine earlier described as essential in the suppression of PGIA induced by Mt HSP70 (Wieten et al. 2009).

Materials and methods

HSP70 treatment of BMDC

Bone marrow cells were isolated from 10–15-week-old BALB/c mice and cultured in IMDM (Gibco) supplemented with 10 % FBS (Lonza), 100 units/ml penicillin, 100 μg/ml streptomycin and 5×10^{-5} M β-mercaptoethanol in the presence of 20 ng/ml GM-CSF (Cytogen) to obtain bone marrow-derived dendritic cells (BMDC) (Lutz et al. 1999). On day 8, BMDC were seeded in 12-wells plates at 1×10^{6} cells/ml. The next day, 10 μg/ml *Mycobacterium tuberculosis* HSP70 (LIONEX Diagnostics & Therapeutics GmbH; less than 2.1 EU LPS/mg), 10 μg/ml mouse HSP70 (HSP1A1; homemade; ~0.3 EU LPS/mg according to LAL assay) or 10 ng/ml LPS (*Escherichia coli* 0127:B8; Sigma) was added to the wells. Cells or supernatants were collected after different time points. In addition, BMDC were stimulated with 10 μg/ml HSP70, 10 ng/ml LPS or were left untreated followed by addition of 10 ng/ml LPS after 6 h. Supernatants of cells were collected after 48 h of culture at 37 °C.

Proteinase K treatment of mouse and Mt HSP70

To digest mouse and Mt HSP70 and as a control LPS, 100 μg/ml proteinase K (Sigma) was added to the samples and incubation was carried out for 3 hr at 37 °C. Digestion was terminated by adding 5 mM (end concentration) PMSF (Sigma). As a control 100 μg/ml proteinase K was added to the same volume of PBS and 3 h later 5 mM (end concentration) PMSF was added.

Mice

Female BALB/c mice were purchased from Charles River Laboratories. DO11.10 mice, which are transgenic for the pOVA (ovalbumin peptide 323–339) specific T cell receptor (TCR) were bred and kept under standard conditions and received water and food ad libitum. Experiments were approved by the Utrecht University Animal Experimental Committee.

In vivo effect of HSP70 treated BMDC on antigen-specific T cells

$CD4^{+}$ T cells were isolated from spleens of DO11.10 mice and labelled with 5,6-carboxy-succinimidyl-fluorescein-ester (CFSE) as described before (Broere et al. 2008). Acceptor BALB/c mice received intravenously (i.v.) 1×10^{7} $CD4^{+}$ CFSE-labelled cells in 100 μl PBS. On the following day, BMDC treated as described above were allowed to recover for 4 h and then pulsed with 20 μg/ml pOVA at 37 °C. Two hours later, 1×10^{6} BMDC were washed and resuspended in 200 μl PBS and intraperitoneally (i.p.) injected into acceptor mice. Three days later, mice were sacrificed and spleen and mesenteric lymph nodes (mLN) were isolated.

In vivo treatment and arthritis induction

Arthritis was induced in retired female mice or naïve female mice aged between 16 and 26 weeks by i.p. injection of human PG (250 μg) and 2 mg dimethyldioctadecylammonium bromide (DDA) (Sigma) emulsified in 200 μl PBS on days 0 and 21. In the second experiment, a third immunization was given on day 50 (Hanyecz et al. 2004). One day before the second PG/DDA immunization, BMDC treated as described above were allowed to recover for 4 h and then pulsed with 250 μg/ml PG. Two hours later, 1×10^{6} BMDC were washed and resuspended in 200 μl PBS and i.p. injected into acceptor mice. As a control, 200 μl PBS only was used. Onset and severity of arthritis was determined using a visual scoring system based on swelling and redness of paws (Hanyecz et al. 2004). On maximal day 100, animals were killed and spleens and draining LN (dLN) of the paws (brachial, axillary, popliteal) were isolated.

Flow cytometric analysis of surface markers and FoxP3

BMDC were treated as described above. After overnight recovery, cells were stained with APC-anti-CD11c (HL3)

plus one of the following antibodies: PE-anti-CD40 (3/23), FITC-anti-CD86 (GL1) or PE-anti-I-Ad/I-Ed (M5/114) (BD Biosciences). Single cell suspensions of spleen and mLN were stained with pacific-blue labelled anti-CD4 (RM4-5) and APC-anti-KJ1.26 (OVA-TCR) (BD Biosciences). Additionally, FoxP3 staining was carried out with a FoxP3 (FJK-16S; PE-labelled) staining kit as instructed (eBioscience). Flow cytometry was performed on a FACS-Canto (BD Biosciences).

Analysis of antigen-specific T cell responses

BMDC were treated as described above. After 48 h, recovery supernatants were collected and analysed for cytokine secretion. Single cell suspensions of spleen and mLN were cultured in complete medium in flat-bottom plates (Corning) at 2×10^5 cells per well, in the presence/absence of pOVA (20 μg/ml). After 72 h, supernatants were collected for cytokine assays. Fluoresceinated microbeads coated with capture antibodies for simultaneous detection of IL-6 (MP5-20 F3), IL-10 (JES5-2A5) (BD Bioscience) and IFNγ (AN-18) (homemade) or IL-6 (MP5-20 F3), TNFα (G281-2626) and IL-12p70 (9A5) were added to 50 μl of culture supernatant. Cytokines were detected by biotinylated antibodies IL-6 (MP5-32C11), IL-10 (SXC-1), IFNγ (XMG1.2), TNFα (MP6-XT3), IL-12p70 (C17.8) and PE-labelled streptavidin (BD Biosciences) and analysed on a Luminex model 100 XYP (Luminex).

Statistical analysis

Statistical analysis was carried out using Prism software (version 4.00, Graphpad software Inc.). Significance level was set at $p\leq0.05$ and two-tailed Student's t test or Mann–Whitney U test was applied.

Results

HSP70 treated BMDC induce suppression of PGIA

Earlier, we have shown that Mt HSP70 immunization suppressed PGIA in an IL-10-dependent fashion (Wieten et al. 2009). In addition, as shown by others, treatment of DCs with Mt HSP70 inhibited DC maturation and induced the production of the anti-inflammatory cytokine IL-10 (Detanico et al. 2004; Motta et al. 2007). We now studied if at least part of the suppressive capacity of HSP70 in PGIA is induced via DCs. Mice were i.p.-injected with 1×10^6 Mt HSP70-treated BMDC, LPS-treated BMDC or PBS alone 1 day prior to the second PG immunization. All BMDC had been pulsed with PG. Previously, we have shown that untreated BMDC pulsed with PG, that usually have an unstable tolerogenic phenotype, do not ameliorate or enhance PGIA (Spiering et al. 2012). As shown in Fig. 1a, Mt HSP70-treated BMDC tended to fully suppress PGIA in retired breeders until day 47, followed by a mild form of arthritis. LPS-treated BMDC showed rapid progression of arthritis and disease symptoms were more severe compared to PBS controls (Fig. 1a). To study whether the inducible form of mouse HSP70, HSPA1A and also suppressed experimental arthritis, we i.p. injected 18-week-old mice with 1×10^6 mouse or Mt HSP70-treated BMDC, LPS-treated BMDC or PBS in a manner similar to the first experiment. Eighteen-week-old naïve mice are immunologically more homogenous than retired breeders (obtained with unspecified age) and appeared less susceptible to disease.

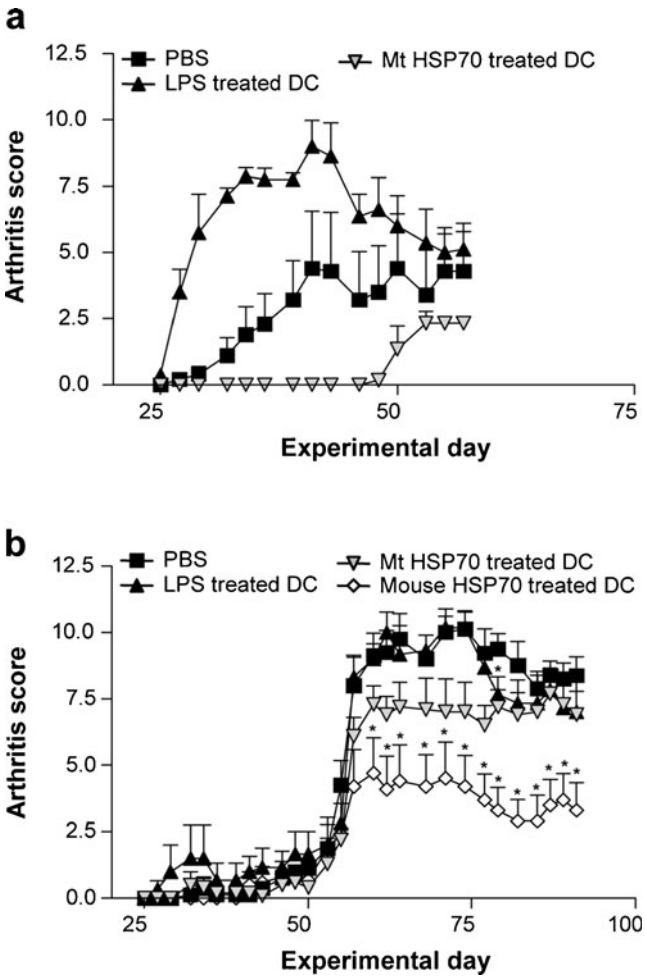

Fig. 1 HSP70 treated BMDC inhibit PGIA. Arthritis was induced by two or three i.p. immunizations with human PG on days 0 and 21 (**a**) or days 0, 21 and 50 (**b**). One day prior to the second PG immunization mice were i.p. injected with 1×10^6 LPS-treated (10 ng/ml), Mt HSP70-treated (10 μg/ml) or mouse HSP70-treated (10 μg/ml) BMDC. All BMDC where pulsed with PG for 2 and 4 h after treatment. As a control, mice received only PBS. Arthritis severity is expressed as the mean and SEM of at least three mice per group. *$p<0.05$ Mt- or mouse HSP70-treated BMDC group as compared to PBS control (Mann–Whitney U test)

Therefore, 18-week-old mice received a third PG immunization on day 50 to induce severe arthritis. Only mice that were administered with LPS-treated BMDC showed mild arthritis symptoms prior to the third PG immunization, indicating the pro-inflammatory activity of the LPS-treated BMDC (Fig. 1b). Mice injected with mouse HSP70-treated BMDC had significantly lower arthritis scores as compared to the control PBS group. This amelioration of disease was maintained during the whole course of arthritis. In addition, Mt HSP70-treated BMDC reduced disease, although not as clearly as mouse HSP70-treated BMDC (Fig. 1b). These data indicated that both Mt- and mouse HSP70-treated BMDC were functionally tolerogenic and were able to suppress PGIA. However, the tolerogenic phenotype of mouse HSP70-treated BMDC appeared more stable since mouse HSP70-treated BMDC ameliorated PGIA to a greater and more enduring extent.

Mt- and mouse HSP70 treatment induce minor phenotypic differences in BMDC

As both Mt- and mouse HSP70 treatment induced a DC that was able to suppress PGIA, we investigated the phenotype of the different HSP70-treated BMDC. Maturation status, cytokine production and tolerogenic DC marker expression of HSP70 or LPS-treated BMDC were investigated and compared with untreated BMDC. As shown in Fig. 2a, mouse HSP70 induced a slight increase in the expression of the co-stimulatory marker CD86 and of MHC class II, whereas Mt HSP70 did not (Fig. 2a). Furthermore, both Mt- and mouse HSP70 induced secretion of IL-6, although the amount produced was much lower as compared to LPS-treated BMDC (Fig. 2b). This secretion was HSP70-dependent as proteinase K-digested HSP70 could not induce this increased secretion of IL-6 seen in HSP70-treated BMDC (Fig. 2b). Other cytokines, like IL-10, TNFα and IL-12p70, were tested but did not reach levels above detection limit (data not shown). An important feature for a functional tolerogenic DC is that its phenotype is stable in a pro-inflammatory milieu. To study the stability of the HSP70-induced phenotype in DCs, HSP70-treated BMDC were additionally stimulated with LPS to see whether a pro-inflammatory phenotype induced by LPS could be prevented. Although the increased expression of CD86 and MHC class II induced by LPS was only slightly less (data not shown), the production of the pro-inflammatory IL-12p70 was markedly reduced by both HSP70 proteins as compared to BMDC solely treated with LPS. This reduction was less pronounced in BMDC treated with proteinase K-digested

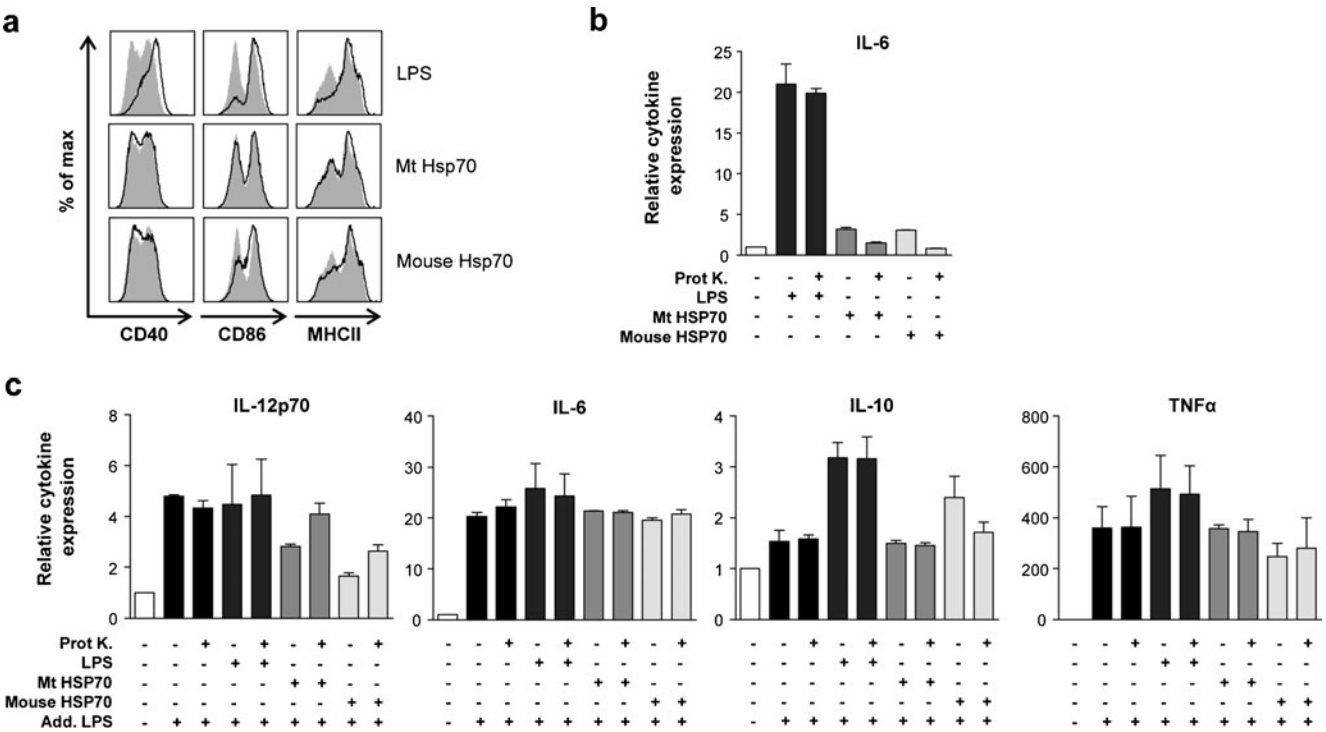

Fig. 2 Phenotypical changes in BMDC induced by HSP70 treatment. Mouse BMDC were incubated with 10 μg/ml Mt HSP70, 10 μg/ml mouse HSP70, 10 ng/ml LPS or were left untreated. **a** After overnight recovery at 37 °C CD40, CD86 and MHCII were analysed. *Grey solid* untreated BMDC; *black line* treated BMDC. **b** After 48 h of incubation, IL-6 secretion was measured by Luminex. Production levels relative to non-stimulated cells are shown. **c** BMDC were additionally treated with 10 ng/ml LPS 6 h after treatment to induce maturation. Forty-eight hours later, IL-12p70, IL-6, IL-10 and TNFα secretion was measured by Luminex. Production levels relative to non-stimulated cells are shown. Values are the mean and SEM of two independent experiments

HSP70 indicating a HSP70-dependent decrease of cytokine production (Fig. 2c). Pre-exposure to both HSP70 proteins did not reduce the LPS-induced levels of IL10 and TNFα. In fact, enhancement of the IL10 level was seen after pre-exposure to mouse HSP70 (Fig. 2c). The tolerogenic DC markers IDO, ILT3 and GILZ (Cohen et al. 2006; Manavalan et al. 2003; Mellor and Munn 2004) were not altered compared to untreated BMDC on mRNA level (data not shown).

In sum, mouse HSP70 treatment induced a semi-mature phenotype in BMDC, whereas the phenotype of Mt HSP70-treated BMDC was barely changed as compared to untreated BMDC. However, although mouse HSP70-treated BMDC appeared more stable in the PGIA model as compared to Mt HSP70-treated BMDC, both HSP70 treatments were able to prevent the induction of LPS-induced IL-12p70 production. This indicated a more stable phenotype of the HSP70-treated BMDC as compared to untreated BMDC.

HSP70-treated BMDC induce an altered phenotype in T cells

As small phenotypic changes in BMDC were observed after HSP70 treatment, we set out to analyse the antigen-presenting capacity of HSP70-treated BMDC and the induction of functional changes in responding T cells. HSP70-treated BMDC were pulsed with pOVA and transferred by i.p. injection into recipient mice 1 day after i.v. injection of pOVA-specific CFSE-labelled $CD4^+$ T cells. As controls, untreated and LPS-treated BMDC pulsed with pOVA were transferred. Three days after injection of BMDC, proliferation and phenotype of the pOVA-specific T cells was measured in spleen and mLN of the recipient mice. No differences were observed in T cell proliferation when Mt- or mouse HSP70-treated BMDCs were transferred compared to untreated BMDC (Fig. 3a and b). This indicated that HSP70-treated BMDC did not induce antigen-specific T cell anergy. Furthermore, expression of the regulatory T cell marker FoxP3 was not altered (Fig. 3c), indicating that no classic regulatory T cell phenotype was induced. To further investigate functional characteristics of the DC primed pOVA-specific T cells in terms of cytokine production, splenic cell suspensions were restimulated with pOVA. Secretion of IL-6 and the anti-inflammatory cytokine IL-10 were significantly increased in splenic cell suspensions after 72 h of culture obtained from mice injected with Mt- or mouse HSP-treated BMDC. Moreover, production of IL-10 had more than doubled after HSP70-treated BMDC injection (Fig. 3d). In conclusion, these data indicated that Mt- and mouse HSP70-treated BMDC did not induce antigen-specific T cell anergy or a classic regulatory T cell phenotype. Nevertheless, HSP70-treated BMDC induced a possibly regulatory T cell phenotype as production of IL-10 had more than doubled in restimulated splenic cell suspensions obtained from mice injected with HSP70-treated BMDC.

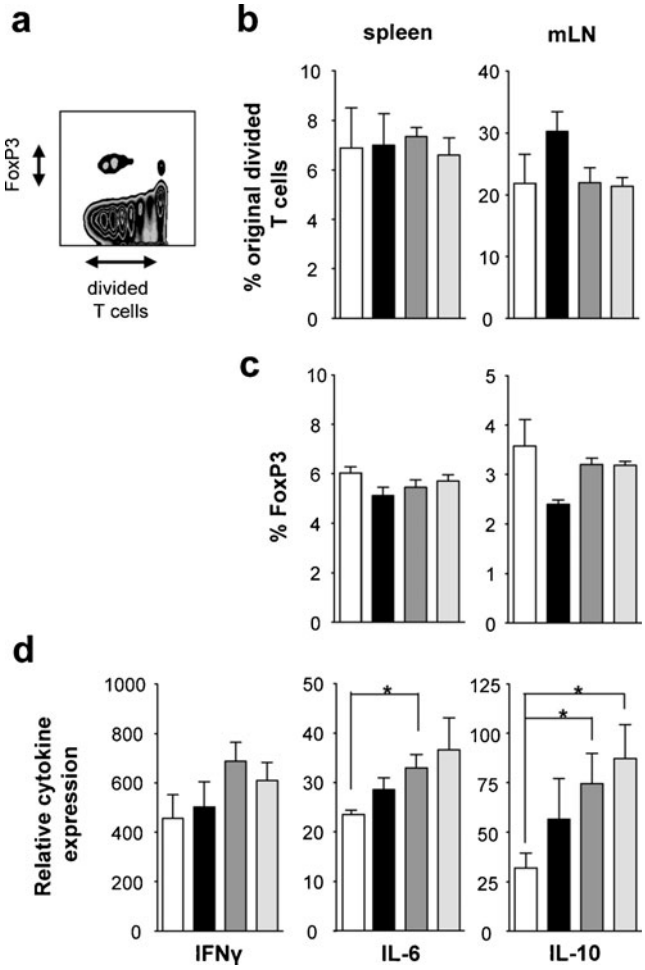

Fig. 3 HSP70-treated BMDC induced IL-6 and IL-10 production in antigen-specific T cells. Mice were i.v. injected with 1×10^7 CFSE-labelled $CD4^+DO11.10^+$ T cells 1 day prior to i.p. injection of 1×10^6 untreated, LPS-treated (10 ng/ml), Mt HSP70-treated (10 μg/ml), or mouse HSP70-treated (10 μg/ml) BMDC. All BMDC were pulsed with pOVA for 2 h, 4 h after initial HSP70 treatment. Three days after BMDC injection, spleen and mLN were harvested. **a** Example of CFSE dilution and FoxP3 expression of $CD4^+DO11.10^+$ T cells in mLN. Percentage of $CD4^+DO11.10^+$ T cells from the original sample that have divided in spleen and mLN (**b**) and FoxP3 expression of $CD4^+DO11.10^+$ T cells (**c**) analysed by flow cytometry. **d** Splenic cell suspensions were restimulated with pOVA for 72 h. Cytokine secretion for IFNγ, IL-6 and IL-10 was measured in supernatant by Luminex. Production levels relative to non-stimulated cells are shown. *White* untreated BMDC, *black* LPS-treated BMDC, *dark grey* Mt HSP70-treated BMDC, *light grey* mouse HSP70-treated BMDC. Values are the mean and SEM of four mice per group. *$p<0.05$ (two-tailed Student's *t* test)

Discussion

The stress protein HSP70 is not only a vitally important chaperone molecule but its involvement has also been

described with respect to several immunological phenomena. For example, it has been reported as a carrier of antigens to antigen-presenting cells (Lussow et al. 1991; Nishikawa et al. 2007), as pro-inflammatory mediator (Kono and Rock 2008), or on the contrary, as an anti-inflammatory molecule that inhibits maturation and induces the secretion of IL-10 in DCs (Detanico et al. 2004; Motta et al. 2007). However, the idea of HSP70 as a pro-inflammatory mediator has been considered controversial since several groups have shown that bacterial products often present in HSP70 protein preparations were responsible for the detected pro-inflammatory phenotype (Bausinger et al. 2002; Bendz et al. 2008; Stocki et al. 2012). Therefore, for the experiments performed here, HSP70 preparation with less than 2.1 EU LPS/mg was used. In addition, administration of Mt HSP70 has been shown to be protective in several experimental autoimmune disease models (Kingston et al. 1996; Prakken et al. 2001; van Eden et al. 2005; Wieten et al. 2009).

Here, we report that Mt- and mouse HSP70-treated BMDC can be functionally tolerogenic and can suppress PGIA. However, mouse HSP70-treated BMDC ameliorated disease to a much larger extent. Differences between mouse and Mt HSP70-treated BMDC are most probably due to differences in protein sequence of the two HSP70 species. Even though both molecules have a sequence conservation of about 50 %, the sequences that interact with the immune system could be very different. As illustrated by two papers of Wang et al., one HSP70 molecule may have stimulatory as well as inhibitory sequences. Stimulatory sequences induced maturation of DCs and the production of pro-inflammatory cytokines. In contrast, inhibitory sequences could inhibit DC maturation and cytokine production after stimulation (Wang et al. 2002; Wang et al. 2005). In our experiments, the balance between inhibitory and stimulatory sequences might tilt towards inhibitory sequences more clearly for mouse HSP70 as it does for Mt HSP70.

Since HSP70-treated BMDC had obtained a functionally tolerogenic capacity, we investigated the exact phenotype of Mt- and mouse HSP70-treated BMDC. Although no clear tolerogenic phenotype was induced, mouse HSP70-treated BMDC had obtained a semi-mature phenotype as indicated by the increased expression of CD86 and MHC class II. Furthermore, mouse and Mt HSP70 treatments induced the production of low amounts of IL-6. Both induction of a semi-mature phenotype and production of IL-6 have also been described as tolerogenic DC features (Frick et al. 2010). Furthermore, levels of IL-12p70 were lower after HSP70 pretreatment in LPS-treated BMDC as compared to BMDC solely treated with LPS. These results indicate an inhibition of LPS-induced maturation and a stable tolerogenic phenotype of the HSP70-treated BMDC. Expression of the earlier-described tolerogenic DC markers IDO, ILT3 and GILZ (Cohen et al. 2006; Manavalan et al. 2003; Mellor and Munn 2004) were not altered after HSP70 treatment. In addition, production of the anti-inflammatory cytokine IL-10 did not reach levels above detection limit after HSP70 treatment. The increased IL-6 production after HSP70 treatment was not caused by contaminants like endotoxins or nucleotides (Bendz et al. 2008; Goth et al. 2006; Takeuchi et al. 1999). BMDC treatment with proteinase K-digested Mt- or mouse HSP70 in order to degrade the protein but not the possible contaminants, showed no increased IL-6 secretion compared to untreated BMDC. Furthermore, endotoxin levels of the Mt- and mouse batches were low and our preparation of Mt HSP70 did not trigger the NF-κB pathway in a NF-κB luciferase reporter construct transfected intestinal crypt epithelial m-IC_{cl2} cell-line that expresses TLR-2 or TLR-4 (data not shown) (Hornef et al. 2003). By the group of Bonorino, two papers were published where they showed that Mt HSP70-treated DCs were less mature as shown by CD86 expression, and produced more IL-10 as compared to PBS-treated DCs (Detanico et al. 2004; Motta et al. 2007). They suggest that HSP70 binds a yet undefined endocytic receptor and hereby signal via TLR-2. This would eventually result in the production of IL-10 (Borges et al. 2012). We did not observe the reduced CD86 expression or an induced production of IL-10, although our HSP70 treated BMDC were tolerogenic. However, our DCs were stimulated with a lower HSP70 dose and for a shorter period of time.

As HSP70 treatment induced a semi-mature phenotype in DCs, we investigated possible alterations in the antigen-presenting capacity of HSP70-treated BMDC in vivo. No increase or decrease of pOVA-specific $CD4^+$ T cell proliferation was found. Also, no evidence for an altered expression of the regulatory T cell marker FoxP3 was found in HSP70 BMDC-treated mice when compared with untreated BMDC-treated mice. These results indicated that HSP70-treated BMDC did not induce antigen-specific T cell anergy or the induction of a classic $FoxP3^+$ regulatory T cell phenotype. In contrast, HSP70-treated BMDC induced a small but significant increase of IL-6 secretion and a more pronounced and significant increase of the anti-inflammatory cytokine IL-10 in T cells. As it was earlier demonstrated that administration of Mt HSP70 or a conserved HSP70 sequence was protective in experimental arthritis models in an IL-10-producing T cell-dependent manner (Prakken et al. 2001; Tanaka et al. 1999; Wendling et al. 2000; Wieten et al. 2009), it is feasible that HSP70-treated BMDC-induced suppression of PGIA is mediated by IL-10-producing antigen-specific Tr1-like cells.

We recently showed that immunization with an Mt HSP70-derived peptide induced HSP70-specific regulatory T cells. These regulatory T cells suppressed PGIA when given prophylactically and decreased disease severity therapeutically in established PGIA (van Herwijnen et al. 2012). Thus, another possibility for HSP70-treated BMDC to

suppress PGIA is via the induction of HSP70-specific regulatory T cells. The BMDC in the PGIA experiments are pulsed with PG; however, HSP70-derived peptides will also be loaded onto MHC class II and possibly presented to HSP70-specific T cells. As suggested by a review of van Eden et al. (van Eden et al. 2005), these regulatory HSP70-specific T cells can inhibit effector T cells via the secretion of IL-10.

In conclusion, this study shows that Mt- and mouse HSP70-treated BMDC can suppress PGIA; although mouse HSP70-treated BMDC suppressed PGIA to a greater extent. This could be an indication of a more stable tolerogenic phenotype in mouse HSP70-treated BMDC. Amelioration of disease was likely induced by the induction of regulatory T cells: either by IL-10-producing arthritis antigen-specific T cells or possibly via HSP70-specific regulatory T cells.

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