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Badanie immunomodulacyjnych właściwości zewnątrzkomórkowych białek szoku termicznego w kontekście rozwoju i terapii wybranych chorób autoimmunologicznych

The study of the immunomodulatory properties of extracellular heat shock proteins in the context of the development and therapy of selected autoimmune diseases

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Streszczenie

Choroby autoimmunologiczne dotykają 5-8% światowej populacji, stanowiąc globalny problem społeczno-ekonomiczny. Obecnie scharakteryzowano około 80 chorób autoimmunologicznych, od ogólnoustrojowych, takich jak reumatoidalne zapalenie stawów (RZS), po narządowo-specyficzne, takie jak łuszczyca oraz autoimmunologiczne choroby pęcherzowe skóry. Choroby autoimmunologiczne charakteryzują się utratą tolerancji immunologicznej na własne antygeny (autoantygeny) prowadząc do przewlekłego stanu zapalnego, w którym pośredniczą elementy pierwotnej i nabytej (wtórnej) odpowiedzi immunologicznej. Choroby te cechuje szczególnie zaburzona równowaga pomiędzy prozapalnymi limfocytami T pomocniczymi (Th) (np. Th1 lub Th17) a limfocytami T regulatorowymi (Treg) oraz obecność autoreaktywnych limfocytów T lub B (Sarhan i wsp. 2018; Rydzewska i wsp. 2018; Geng i wsp. 2020; Yan i wsp. 2020; Fugger i wsp. 2020). Ponieważ niewystarczająca aktywność lub liczba limfocytów Treg jest jedną z przyczyn utraty tolerancji immunologicznej (Long i Buckner, 2011), uzasadnione są strategie terapeutyczne mające na celu indukcję immunosupresyjnych komórek regulatorowych lub zapobieganie niekontrolowanej aktywacji komórek autoreaktywnych i efektorowych.

Chociaż poczyniono znaczne postępy w identyfikacji kluczowych komórek układu immunologicznego zaangażowanych w rozwoju lub progresji licznych chorób autoimmunologicznych, ich terapie pozostają wyzwaniem i nadal bazują na konwencjonalnym leczeniu immunosupresyjnym polegającym na zastosowaniu kortykosteroidów, a także zaawansowanych terapii z wykorzystaniem leków biologicznych. Terapie te skupiają się głównie na wyciszeniu procesu zapalnego. Niestety, rzadko pozwalają na osiągnięcie trwałej immunotolerancji i równowagi między ochronnym działaniem układu immunologicznego (tj. przeciwko mikroorganizmom) a reakcją autoimmunologiczną. Ponadto nie każda terapia konwencjonalna jest skuteczna i może prowadzić do poważnych skutków ubocznych. Dlatego uzasadnione jest poszukiwanie nowszych, bezpieczniejszych i skuteczniejszych terapii chorób autoimmunologicznych.

Białka szoku termicznego (ang. *heat shock proteins*, Hsp) uważane są za potencjalne cele terapii wybranych chorób autoimmunologicznych. Wysoce zachowane w ewolucji białka Hsp są niezbędne do przeżycia komórek, dlatego znajdują się we wszystkich organizmach prokariotycznych i eukariotycznych. Białka Hsp wykazujące aktywność opiekuńczą

(chaperonową) odpowiedzialne są za fałdowanie wewnątrzkomórkowych polipeptydów, stabilizację i translokację białek natywnych. W oparciu o masę cząsteczkową i funkcje, Hsp podzielono na kilka rodzin, w tym Hsp100, Hsp90, Hsp70, Hsp60, Hsp40 i małe Hsp. W tkankach objętych stanem zapalnym, niektóre z białek opiekuńczych ulegają silnej ekspresji lub są wydzielane do przestrzeni pozakomórkowej. W związku z powyższym, Hsp stały się przedmiotem zainteresowania naukowców w kontekście procesu zapalnego i autoimmunizacyjnego. Dokładna rola zewnątrzkomórkowych Hsp w przebiegu chorób autoimmunologicznych oraz zapalnych pozostaje jednak niewyjaśniona.

Niniejsza rozprawa doktorska miała na celu zbadanie immunomodulacyjnych właściwości zewnątrzkomórkowych białek Hsp w rozwoju i terapii RZS, łuszczycy i należącego do grupy autoimmunologicznych chorób pęcherzowych skóry, nabytego pęcherzowego oddzielania się naskórka (łac. *epidermolysis bullosa acquisita*, EBA). W przypadku łuszczycy i EBA wykorzystano dobrze poznane i opisane w literaturze eksperymentalne mysie modele tych schorzeń. Ponadto w związku z zaproponowanym (na podstawie badań *in silico*) rozwojem procesu autoimmunizacji poprzez występowanie podobieństw strukturalnych (teoria mimikry molekularnej) między białkami wirusa SARS-CoV-2 wywołującego COVID-19 a immunogennymi białkami Hsp (Marino Gammazza i wsp. 2020), postanowiono zweryfikować tę hipotezę eksperymentalnie.

Wyniki badań uzyskane w ramach realizacji niniejszej rozprawy doktorskiej zostały opublikowane w czterech oryginalnych artykułach:

1. Wiele dowodów wskazuje na to, że Hsp mogą być aktywnie i pasywnie uwalniane odpowiednio z komórek żywych i nekrotycznych do przestrzeni pozakomórkowej. Wysoce immunogenne białka Hsp mogą aktywować odpowiedź immunologiczną i tym samym stymulować wytwarzanie autoprzeciwciał anty-Hsp. Stwierdzono, że poziomy tych autoprzeciwciał są podwyższone u pacjentów z chorobami autoimmunologicznymi, takimi jak celiakia lub opryszczkowe zapalenie skóry (Tukaj i Kamiński, 2019; Tukaj, 2020). Celem mojej pracy było zbadanie humoralnej odpowiedzi autoimmunologicznej na ludzkie białka Hsp60, Hsp70 i Hsp90 u pacjentów z RZS (n = 39). W porównaniu z osobami zdrowymi (n = 40), poziomy krążących autoprzeciwciał (IgG, IgM i IgA) anty-Hsp60, -Hsp70 i -Hsp90 były znacząco wyższe u pacjentów z RZS, co zmierzono za pomocą wcześniej opracowanego testu immunoenzymatycznego (ELISA). Analiza statystyczna nie

wykazała istotnych korelacji między immunoglobulinami anty-Hsp a aktywnością lub progresją choroby. Z drugiej strony, wykazano dodatnie korelacje między poziomami surowiczych przeciwciał anty-Hsp90 IgG i prozapalnej cytokiny IFN-ɣ (Th1) u pacjentów z RZS. Ponadto stwierdzono odwrotną korelację między poziomami przeciwciał anty-Hsp70 IgM a prozapalną cytokiną TNF-α (Th1) u pacjentów. Wyniki te sugerują, że humoralna reakcja autoimmunologiczna anty-Hsp60, -Hsp70 i -Hsp90 u pacjentów z RZS nie jest bezpośrednio związana z aktywnością lub progresją choroby, ale może mieć potencjalny wpływ modulujący na mediatory stanu zapalnego (**Mantej i wsp. 2019).**

2. Wraz z rozwojem pandemii koronawirusa COVID-19, postawiono hipotezę, że wirus SARS-CoV-2 może potencjalnie wywoływać autoimmunizację poprzez podobieństwo molekularne pomiędzy immunogennymi białkami wirusa a ludzkimi zewnątrzkomórkowymi białkami Hsp (Marino Gammazza i wsp. 2020). Aby zweryfikować tę hipotezę, określono poziomy krążących autoprzeciwciał skierowanych przeciwko Hsp60, Hsp70 i Hsp90 u osób posiadających przeciwciała skierowane przeciwko wirusowi SARS-CoV-2 (seropozytywni) za pomocą testu ELISA. Do tego badania zostało włączonych 15 seropozytywnych osób po przebytym COVID-19, 26 seropozytywnych zdrowych ochotników, którzy otrzymali dwie dawki szczepionki mRNA COVID-19 firmy Pfizer-BioNTech, oraz 51 zdrowych osób z ujemnym wynikiem na obecność przeciwciał anty-SARS-CoV-2 IgG. Stwierdzono, że poziomy surowiczych autoprzeciwciał anty-Hsp60, anty-Hsp70 i anty-Hsp90 klasy IgG, IgM lub IgA nie uległy zmianie u pacjentów immunizowanych szczepionką anty-COVID-19 oraz osób po przebytej chorobie (seropozytywni) w porównaniu ze zdrowymi osobami, u których nie potwierdzono obecności przeciwciał skierowanych przeciwko białkom wirusa. Obserwacje te przemawiają przeciwko związkowi między przebytą infekcją lub szczepieniem przeciwko COVID-19 prowadzącą do wytworzenia przeciwciał anty-SARS-CoV-2 IgG a immunologiczną reakcją krzyżową z ludzkimi białkami Hsp (**Mantej i wsp. 2021**).

3. Z niektórych badań przedklinicznych wynika, że immunizacja białkami/peptydami Hsp70 może stać się potencjalnym celem terapii RZS poprzez m.in. indukcję limfocytów Treg. Ponadto, pierwsze testy kliniczne z wykorzystaniem białek Hsp

w leczeniu pacjentów RZS i cukrzycy typu I wykazały ich potencjał terapeutyczny (van Herwijnen i wsp. 2012; Tukaj i Kamiński, 2019; Tukaj, 2020). W celu rozwinięcia poprzednich doniesień dotyczących immunosupresyjnych właściwości Hsp70, oceniono jego potencjał terapeutyczny w mysim modelu łuszczycy, aby dowiedzieć się, czy ta metoda leczenia jest skuteczna również w innych chorobach autoimmunologicznych. Łuszczyca jest jedną z najczęstszych organospecyficznych chorób autoimmunizacyjnych charakteryzujących się nadmierną proliferacją oraz nieprawidłowym różnicowaniem keratynocytów w naskórku oraz nadaktywnością m.in. subpopulacji komórek Th17 i niedostateczną aktywnością limfocytów Treg. W tej pracy, po raz pierwszy pokazano, że immunizacja myszy wysoce czystym, wolnym od substratów i endotoksyn bakteryjnych, rekombinowanym Hsp70, szczególnie pochodzenia roślinnego, chroniła zwierzęta przed klinicznymi i histologicznymi objawami łuszczycy indukowanej imikwimodem (IMQ). Towarzyszyła temu również indukcja dwóch populacji komórek Treg ($CD4^+CD25^+$ i $CD4^+FoxP3^+$) oraz wzrost stosunku populacji $CD4^+FoxP3^+$ do Th17. Podobnie, leczenie przeciwciałem anty-Hsp70 IgG skutkowało niższą aktywnością choroby i związane było ze spadkiem liczby prozapalnych komórek Th17 w śledzionie. Bezpośrednią stymulację komórek Treg przez roślinne Hsp70 i jego działanie antyproliferacyjne na keratynocyty potwierdzono w modelu *in vitro*. Te przedkliniczne obserwacje sugerują, że Hsp70 pochodzenia roślinnego może być w przyszłości obiecującym sposobem leczenia łuszczycy (**Tukaj i wsp. 2021**).

4. Na koniec oceniono rolę zewnątrzkomórkowego białka Hsp70 w rozwoju EBA,w którym pośredniczą autoprzeciwciała skierowane przeciwko kolagenowi typu VII. Rolę tego pozakomórkowego białka opiekuńczego zbadano w mysim modelu EBA wywołanym transferem przeciwciał anty-kolagen typu VII. Stwierdzono, że poziomy Hsp70 we krwi były znacznie podwyższone u myszy z EBA w porównaniu do zwierząt naiwnych. Ponadto myszy z EBA immunizowane Hsp70 wykazywały cięższą kliniczną postać choroby w porównaniu z myszami nietraktowanymi. Towarzyszył temu zwiększony poziom ekspresji skórnej metaloproteinazy 9 (MMP-9) i nadtlenku wodoru w osoczu. Prozapalną aktywność Hsp70 w EBA potwierdzono w teście uwalniania reaktywnych form tlenu z wykorzystaniem kompleksów immunologicznych specyficznych dla tej choroby. Ponadto

eksperymenty z użyciem ludzkich komórek jednojądrzastych krwi obwodowej (PBMC) pozyskanych od osób zdrowych stymulowanych przeciwciałami anty-CD3 ujawniły, że autologiczne Hsp70 stymuluje wydzielanie dwóch głównych cytokin prozapalnych IL-6 i IL-8. Praca ta sugeruje, że w przeciwieństwie do łuszczycy, zewnątrzkomórkowe białko Hsp70 bierze udział w rozwoju EBA stanowiąc potencjalny cel leczenia tej choroby (**Tukaj i wsp. 2022**).

Podsumowując, niniejsza praca doktorska poszerza wiedzę na temat roli zewnątrzkomórkowych białek Hsp w rozwoju RZS, łuszczycy i EBA. Moje badania sugerują, że zewnątrzkomórkowe białka Hsp mogą stanowić zarówno czynnik patofizjologiczny lub terapeutyczny w rozwoju chorób autoimmunologicznych.

Literatura

1. Fugger L, Jensen L.T, Rossjohn J. (2020) Challenges, Progress, and Prospects of Developing Therapies to Treat Autoimmune Diseases. Cell 2;181(1):63-80.
2. Gammazza A.M, Légaré S, Bosco G.L, Fucarino A, Angileri F, Conway E, Macario A.JL, Cappello F. (2020) Human molecular chaperones share with SARS-CoV-2 antigenic epitopes potentially capable of eliciting autoimmunit against endothelial cells: possible role of molecular mimicry in COVID-19. Cell Stress and Chaperones 25:737–741.
3. Geng L, Tang X, Wang S, Sun Y, Wang D, Tsao B.P, Feng X, Lingyun. (2020) Reduced Let-7f in Bone Marrow-Derived Mesenchymal Stem Cells Triggers Treg/Th17 Imbalance in Patients With Systemic Lupus Erythematosus Sun 1 Front Immunol. 18;11:233.
4. Herwijnen MJ, Wieten L, Zee R. et al. (2012) Regulatory T cells that recognize a ubiquitous stress-inducible self-antigen are long-lived suppressors of autoimmune arthritis. Proc Natl Acad Sci U S A 109:14134–14139.
5. Long S.A, Buckner J.H. (2011) CD4+FOXP3+ T Regulatory Cells in Human Autoimmunity: More Than a Numbers Game. J Immunol 187:2061-2066.
6. **Mantej J*, Bednarek M*, Sitko K, Świętoń M, Tukaj S. (2021) Autoantibodies to heat shock protein 60, 70, and 90 are not altered in the anti-SARS-CoV-2 IgG-seropositive humans without or with mild symptoms. Cell Stress Chaperones 26(4): 735–740.**
7. **Mantej J, Polasik K, Piotrowska E, Tukaj S. (2019) Autoantibodies to heat shock proteins 60, 70, and 90 in patients with rheumatoid arthritis. Cell Stress Chaperones 24(1):283-287.**
8. Rydzewska M, Jaromin M, Pasierowska I.E, Stożek K, Bossowski A. (2018) Role of the T and B lymphocytes in pathogenesis of autoimmune thyroid diseases. Thyroid Res. 11: 2.18
9. Sarhan D, Hippen K.L, Lemire A, Hying S, Luo X, Lenvik T, Curtsinger J, Davis Z, Zhang B, Cooley S, Cichocki F, Blazar B.R, Miller J.S. (2018) Adaptive NK Cells Resist Regulatory T-cell Suppression Driven by IL37. Cancer Immunol Res. 6(7):766-775.
10. Tukaj S. (2020) Heat Shock Protein 70 as a Double Agent Acting Inside and Outside the Cell: Insights into Autoimmunity. Int J Mol Sci. 26;21(15):5298.

11. Tukaj S, Kaminski M. (2019) Heat shock proteins in the therapy of autoimmune diseases: too simple to be true? Cell Stress Chaperones 24(3):475-479.
12. **Tukaj S*, Mantej J*, Sitko K, Bednarek M, Zillikens D, Ludwig R.J, Bieber K, Kasperkiewicz M. (2022) Evidence for a role of extracellular heat shock protein 70 in epidermolysis bullosa acquisita. Exp Dermatol. 31(4):528-534.2021.**
13. **Tukaj S*, Mantej J*, Sobala M, Potrykus K, Tukaj Z, Zillikens D, Ludwig R.J, Bieber K, Kasperkiewicz M. (2021) Therapeutic Implications of Targeting Heat Shock Protein 70 by Immunization or Antibodies in Experimental Skin Inflammation. Front Immunol. 12: 614320.**
14. Yan J, Luo M, Chen Z, He B. (2020) The Function and Role of the Th17/Treg Cell Balance in Inflammatory Bowel Disease. J Immunol Res 15;2020:8813558.

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Abstract

Autoimmune diseases affect up to 5–8% of the world's population, thus representing a global socio-economic issue. Currently, there are approximately 80 autoimmune diseases that have been characterized ranging from systemic diseases like rheumatoid arthritis (RA) to organ-specific diseases such as psoriasis and autoimmune blistering skin diseases. In general, autoimmune diseases are characterized by the loss of immune tolerance to self-antigens (autoantigens) leading to chronic inflammation involving cells of the innate and adaptive immune systems. Autoimmune diseases are often characterized by impaired immunological cell function, an altered balance between pro-inflammatory T helper cells (Th) (e.g., Th1 or Th17)/regulatory T cell (Treg), and the presence of autoreactive T or B cells (Sarhan et al. 2018; Rydzewska et al. 2018; Geng et al. 2020; Yan et al. 2020; Fugger et al. 2020). Since the inadequate activity or number of Tregs are one characteristic feature of autoimmune diseases (Long and Buckner, 2011), therapeutic strategies aimed to induce immunosuppressive Tregs or prevent uncontrolled activation of autoreactive and effector cells are warranted.

Although significant progress has been made in identifying the key immune cells responsible for autoimmune diseases, therapy remains a challenge and consists of conventional immunosuppressive treatments such as corticosteroids and advanced biological therapies. These therapies focus mainly on silencing the inflammatory process. However, a permanent immunotolerance and the balance between being protective (i.e., against microorganisms) and an autoimmune response is seldom achieved. In addition, some conventional therapies are, in certain cases, ineffective and may lead to serious side effects. Therefore, newer, safer, and more effective therapies are needed to treat autoimmune diseases.

Based on some pre-clinical studies, heat shock proteins (Hsps) are considered potential treatment targets for autoimmune diseases. Highly conserved during evolution, Hsps are present in all prokaryotic and eukaryotic organisms and are essential for cell survival. Mammalian Hsps with chaperone activity are responsible for intracellular polypeptide folding, native protein stabilization, and translocation. Based on molecular weight and functionality, Hsps are categorized into several families, including Hsp100, Hsp90, Hsp70, Hsp60, Hsp40, and small Hsps. Since some of these chaperones are overexpressed in inflamed tissues and seen in the extracellular space, they have been linked to the inflammation

process. The exact role of extracellular Hsps in autoimmune diseases and other pathological conditions remains enigmatic.

In light of the above-mentioned limitations, this doctoral dissertation aims to study the immunomodulatory properties of extracellular Hsps in the development and therapy of RA, psoriasis, and epidermolysis bullosa acquisita (EBA), the latter belonging to autoimmune blistering skin diseases. In the case of psoriasis and EBA, well-established experimental mouse models were used. In addition, since a link between COVID-19 and the evolution of autoimmunity *via* molecular mimicry between immunogenic proteins of the virus and human extracellular Hsps have been recently proposed (Marino Gammazza et al. 2020), I also decided to verify the above hypothesis experimentally.

The research conducted as part of this doctoral dissertation has been published in four original articles.

1. Multiple lines of evidence reveal that Hsps can be actively and passively released from living and dying cells into the extracellular milieu, respectively. These highly immunogenic proteins can activate the immune response and drive the generation of anti-Hsps autoantibodies. These autoantibodies have been found to be elevated in patients with autoimmune diseases such as celiac diseases or dermatitis herpetiformis (Tukaj and Kaminski, 2019; Tukaj, 2020). I aimed to investigate the humoral autoimmune response to Hsp60, Hsp70, and Hsp90 in RA patients (n=39). In comparison with healthy controls (n=40), circulating IgG, IgM, and IgA autoantibodies against Hsp60, Hsp70, and Hsp90 were significantly increased in RA patients as measured by ‘home-made’ enzyme-linked immunosorbent assays (ELISA). However, statistical analysis revealed no significant correlation between anti-Hsps immunoglobulins and disease activity \or progression. On the other hand, positive correlations between serum levels of anti-Hsp90 IgG and pro-inflammatory IFN-ɣ (Th1-like cytokine) were statistically significant in RA. In addition, a significant inverse correlation was found between serum levels of anti-Hsp70 IgM and TNF-α (Th1-like cytokine) in RA. These results suggest that a pronounced anti-Hsp60, anti-Hsp70, and anti-Hsp90 humoral autoimmune reaction in RA patients is not directly associated with disease activity or progression but may have a potential modulatory impact on inflammatory mediators (**Mantej et al. 2019**).

2. It has been hypothesized that SARS-CoV-2 has the potential to elicit autoimmunity through molecular mimicry between immunogenic proteins of the virus and human extracellular Hsps (Marino Gammazza et al. 2020). To verify this hypothesis, levels of circulating autoantibodies directed to Hsp60, Hsp70, and Hsp90 in anti-SARS-CoV-2 IgG-seropositive participants were evaluated by 'home-made' ELISA tests. Fifteen seropositive post-COVID-19 individuals, 26 seropositive healthy volunteers who received two doses of the mRNA COVID-19 vaccine by Pfizer-BioNTech, and 51 healthy, naïve (anti-SARS-CoV-2 IgG-negative) volunteers were included in this study. The serum levels of IgG, IgM, or IgA isotypes of anti-Hsp60, anti-Hsp70, and anti-Hsp90 autoantibodies were found to be unchanged in the anti-COVID-19-immunized patients and the anti-SARS-CoV-2 IgG-positive convalescence patients when compared to healthy seronegative individuals. These findings argue against a relationship between SARS-CoV-2 infection/vaccines and cross-reactivity to human Hsps (**Mantej et al. 2021**).

3. Some preclinical studies have shown that immunization with Hsp70 peptides/ proteins could be a potential treatment for RA *via* induction of Tregs. In addition, clinical trials evaluating some Hsps in RA and type I diabetes have shown therapeutic potential (van Herwijnen et al. 2012; Tukaj and Kamiński, 2019; Tukaj, 2020). To expand on previous reports on the therapeutic potential of targeting Hsp70 through vaccination, I evaluated a psoriasis mouse model to find out whether this therapy is effective in other autoimmune-like diseases. Psoriasis is one of the most common organ-specific autoimmune diseases characterized by excessive proliferation and abnormal differentiation of keratinocytes in the epidermis as well as overactivity of the Th17 cell subpopulation and inadequate activity of Treg. For the first time, I found that the immunization of mice with a highly pure, substrate- and endotoxin-free recombinant Hsp70, particularly the plant-derived form, protected animals from clinical and histological features of imiquimod (IMQ)-induced psoriasis. It was also paralleled with the induction of two Treg populations ($CD4^+CD25^+$ and $CD4^+FoxP3^+$) and a significant increase in the $CD4^+FoxP3^+$:Th17 ratio. Likewise, anti-Hsp70 IgG antibody treatment resulted in a lower disease activity associated with the down-regulation of pro-inflammatory Th17 cells. Direct stimulation of Tregs by Hsp70 and its anti-proliferative effect on keratinocytes was confirmed *in vitro*. These pre-

clinical observations suggest that plant-derived Hsp70 may be a promising treatment for psoriasis (**Tukaj et al. 2021**).

4. Finally, the role of extracellular Hsp70 in EBA, an anti-type VII collagen autoantibody-mediated autoimmune blistering skin disease, was evaluated. The role of this extracellular chaperone was investigated in an anti-type VII collagen antibody transfer-induced EBA mouse model. It was found that blood levels of Hsp70 were significantly elevated in EBA mice as compared to naive animals. In addition, Hsp70-treated EBA mice had a more severe clinical disease compared to untreated EBA mice. This was paralleled by an increased level of cutaneous matrix metalloproteinase 9 and plasma hydrogen peroxide. The pro-inflammatory activity of Hsp70 in EBA was confirmed by a reactive oxygen species release assay using EBA-specific immune complexes. In addition, experiments using cell cultures of anti-CD3-activated human naive peripheral blood mononuclear cells (PBMCs) revealed that autologous Hsp70 stimulated the secretion of two main pro-inflammatory cytokines, IL-6 and IL-8, that are implicated in autoimmune blistering skin diseases. This work suggests, in contrast to psoriasis, that extracellular Hsp70 acts as a pathophysiological factor and potential treatment target in EBA (**Tukaj et al. 2022**).

Taken together, my work expands knowledge about the role of extracellular Hsps in RA, psoriasis, and EBA. My studies suggest that these extracellular chaperones may represent either a pathophysiological or therapeutic factor in autoimmune diseases.

References

1. Fugger L, Jensen L.T, Rossjohn J. (2020) Challenges, Progress, and Prospects of Developing Therapies to Treat Autoimmune Diseases. Cell 2;181(1):63-80.

2. Gammazza A.M, Légaré S, Bosco G.L, Fucarino A, Angileri F, Conway E, Macario A.JL, Cappello F. (2020) Human molecular chaperones share with SARS-CoV-2 antigenic epitopes potentially capable of eliciting autoimmunit against endothelial cells: possible role of molecular mimicry in COVID-19. Cell Stress and Chaperones 25:737–741.

3. Geng L, Tang X, Wang S, Sun Y, Wang D, Tsao B.P, Feng X, Lingyun. (2020) Reduced Let-7f in Bone Marrow-Derived Mesenchymal Stem Cells Triggers Treg/Th17 Imbalance in Patients With Systemic Lupus Erythematosus Sun 1 Front Immunol. 18;11:233.

4. Herwijnen MJ, Wieten L, Zee R. et al. (2012) Regulatory T cells that recognize a ubiquitous stress-inducible self-antigen are long-lived suppressors of autoimmune arthritis. Proc Natl Acad Sci U S A 109:14134–14139.

5. Long S.A, Buckner J.H. (2011) CD4+FOXP3+ T Regulatory Cells in Human Autoimmunity: More Than a Numbers Game. J Immunol 187:2061-2066.

7. Mantej J*, Bednarek M*, Sitko K, Świętoń M, Tukaj S. (2021) Autoantibodies to heat shock protein 60, 70, and 90 are not altered in the anti-SARS-CoV-2 IgG-seropositive humans without or with mild symptoms. Cell Stress Chaperones 26(4): 735–740.

8. Mantej J, Polasik K, Piotrowska E, Tukaj S. (2019) Autoantibodies to heat shock proteins 60, 70, and 90 in patients with rheumatoid arthritis. Cell Stress Chaperones 24(1):283-287.

9. Rydzewska M, Jaromin M, Pasierowska I.E, Stożek K, Bossowski A. (2018) Role of the T and B lymphocytes in pathogenesis of autoimmune thyroid diseases. Thyroid Res. 11: 2.18

10. Sarhan D, Hippen K.L, Lemire A, Hying S, Luo X, Lenvik T, Curtsinger J, Davis Z, Zhang B, Cooley S, Cichocki F, Blazar B.R, Miller J.S. (2018) Adaptive NK Cells Resist Regulatory T-cell Suppression Driven by IL37. Cancer Immunol Res. 6(7):766-775.

11. Tukaj S. (2020) Heat Shock Protein 70 as a Double Agent Acting Inside and Outside the Cell: Insights into Autoimmunity. Int J Mol Sci. 26;21(15):5298.

12. Tukaj S, Kaminski M. (2019) Heat shock proteins in the therapy of autoimmune diseases: too simple to be true? Cell Stress Chaperones. 24(3):475-479.

13. Tukaj S*, Mantej J*, Sitko K, Bednarek M, Zillikens D, Ludwig R.J, Bieber K, Kasperkiewicz M. (2022) Evidence for a role of extracellular heat shock protein 70 in epidermolysis bullosa acquisita. Exp Dermatol. 31(4):528-534.2021.

14. Tukaj S*, Mantej J*, Sobala M, Potrykus K, Tukaj Z, Zillikens D, Ludwig R.J, Bieber K, Kasperkiewicz M. (2021) Therapeutic Implications of Targeting Heat Shock Protein 70 by Immunization or Antibodies in Experimental Skin Inflammation. Front Immunol. 12: 614320.

15. Yan J, Luo M, Chen Z, He B. (2020) The Function and Role of the Th17/Treg Cell Balance in Inflammatory Bowel Disease. J Immunol Res 15;2020:8813558.

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2. **Mantej J***, Bednarek M*, Sitko K, Świętoń M, Tukaj S. (2021) Autoantibodies to heat shock protein 60, 70, and 90 are not altered in the anti-SARS-CoV-2 IgG-seropositive humans without or with mild symptoms. Cell Stress Chaperones. Jul;26(4):735-740. (**IF = 3,667; MNiSzW = 70**).

3. Tukaj S*, **Mantej J*,** Sobala M, Potrykus K, Tukaj Z, Zillikens D, Ludwig RJ, Bieber K, Kasperkiewicz M. (2021) Therapeutic Implications of Targeting Heat Shock Protein 70 by Immunization or Antibodies in Experimental Skin Inflammation. Front Immunol. Feb 23;12:614320. (**IF = 7,561; MNiSzW = 140**).

4. Tukaj S*, **Mantej J***, Sitko K, Bednarek M, Zillikens D, Ludwig RJ, Bieber K, Kasperkiewicz M. (2022) Evidence for a role of extracellular heat shock protein 70 in epidermolysis bullosa acquisita. Exp Dermatol. Apr;31(4):528-534. (**IF = 3,960; MNiSzW = 100**).

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SHORT COMMUNICATION

Autoantibodies to heat shock proteins 60, 70, and 90 in patients with rheumatoid arthritis

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Abstract

Heat shock proteins (HSP) have been reported to impact immune responses and to be associated with rheumatoid arthritis (RA). Recently, we provided evidence for a role of autoantibodies to Hsp40 in patients with RA. In this study, we aimed at investigating the humoral autoimmune response to Hsp60, Hsp70, and Hsp90 in RA patients ($n = 39$). In comparison with healthy controls ($n = 40$), circulating IgG, IgM, and IgA autoantibodies against Hsp60, Hsp70, and Hsp90 were significantly increased in RA patients. Non-parametric statistical analysis, however, revealed no significant association between anti-HSP and disease activity or disease progression. On the other hand, positive correlations between serum levels of anti-Hsp60 IgG and IL-4 (Th2-like cytokine) or between serum levels of anti-Hsp90 IgG and IFN-ɣ (Th1-like cytokine) were found to be statistically significant in RA. In addition, a significant inverse correlation was found for serum levels of anti-Hsp70 IgM and TNF-α (Th1-like cytokine) in RA. Our results suggest a pronounced anti-Hsp60, anti-Hsp70, and anti-Hsp90 humoral autoimmune response in RA patients that seems not to be directly linked to RA pathophysiology, however, may have a potential modulatory impact on inflammatory status in this disease. Further investigations are needed to clarify the role of anti-HSP autoantibodies in RA.

Keywords Heat shock proteins, HSP · Rheumatoid arthritis, RA · Autoantibodies

Introduction

Rheumatoid arthritis (RA) is characterized by chronic inflammation of synovial joints that can lead to cartilage and bone destruction. Although much progress has been made in designing effective therapy, a lot of RA patients do not effectively respond to the current therapies and/or the therapy cause serious side effects (Guo et al. 2018). There is an increasing interest in the role of immunomodulatory heat shock proteins (HSPs) as potential treatment targets for a number of autoimmune diseases, particularly RA (Spierings and van Eden 2017). Highly conserved HSPs are constitutively expressed and/or stress-induced intracellular molecules that participate in proper protein folding and cell signaling (Tukaj 2014). Based on approximate molecular weights, HSPs are classified into several major families including HSP40, HSP60, HSP70, and HSP90 (Kampinga et al. 2009). Beyond the classical role of intracellular HSPs, recent data showed that bacterial and self-HSP epitopes interact with both innate and adaptive arms of the immune cell components (van Eden et al. 2017). Interestingly, these interactions may lead to either stimulation of humoral (auto)immune response including production of HSP (auto)antibodies and/or activation of immune regulatory mechanisms. Although some independent observations have found that autoantibodies against HSP are present in the sera of healthy individuals (Pockley et al. 1998, 1999) and at significantly elevated levels in subjects with various autoimmune diseases including organ-specific coeliac disease (CD) and dermatitis herpetiformis, the last representing a cutaneous manifestation of CD (Kasperkiewicz et al. 2014; Tukaj et al. 2017), their role in autoimmune process is not completely understood.

Recently, we provided evidence for a role of autoantibodies to Hsp40 in patients with RA, a systemic autoimmune disease (Tukaj et al. 2010a, b; Kotlarz et al. 2013). In this study, we aimed at investigating the humoral autoimmune response to Hsp60, Hsp70, and Hsp90 in RA patients.

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Materials and methods

Patients and controls

Thirty-nine patients with RA (mean age 46.02 ± 16.40) with the mean disease duration = 8.47 ± 8.39 years and the mean disease activity score (DAS 28) = 4.13 ± 1.04 fulfilling ACR criteria for the classification of RA and 40 healthy controls (mean age 51.90 ± 6.70) were included in this study. The disease activity was assessed according to the Disease Activity Score including 28 joint counts (DAS 28) and joint damage was evaluated based on the Steinbrocker radiographic criteria. The use of human biological material was approved by the Ethics Committee of the Medical University of Gdańsk, Poland, and written informed consent was obtained according to the Declaration of Helsinki.

Detection of circulating autoantibodies to heat shock proteins

Serum IgG, IgM, and IgA autoantibodies against human Hsp60 (Abcam), human Hsp70 (Abcam), and human Hsp90 (Enzo Life Sciences) were evaluated by home-made ELISA as described previously (Tukaj et al. 2017). Briefly, medium-binding 96-well plates were coated with the respective HSP at a concentration of 0.5 μg/ml in 0.1 M bicarbonate buffer at 4 °C for 18 h. The wells were blocked with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) at room temperature (RT) for 2 h. After being washed with PBS + 0.05% Tween 20, sera diluted 1:100 in PBS + 0.1% BSA were incubated at RT for 1 h. Plates were then incubated with horseradish peroxidase (HRP)-conjugated anti-human IgG (Sigma)–, anti-human IgM (Abcam)–, or anti-human IgA (Biolegend)–specific secondary antibodies diluted 1:5000 in PBS containing 0.1% BSA at RT for 1 h. TMB substrate solution (Sigma) was used to visualize HRP enzymatic reaction, and the reaction was stopped by 1 M H_2SO_4. Optical density measurements were performed at 450 nm with an ELISA plate reader.

Cytokine detection

Serum levels of IFN-ɣ, TNF-α, IL-10, IL-6, IL-4, and IL-2 were measured by a flow cytometric bead array (Becton Dickinson).

Statistical analysis

Statistical analyses were performed using GraphPad Prism 5 (San Diego, CA). The Shapiro-Wilk test was used to verify whether the data had normal distribution. Non-normal distributed data was analyzed by the Mann–Whitney *U* test and Spearman's rank correlation test. *P* values less than 0.05 were considered as significant.

Results

Circulating anti-Hsp60, anti-Hsp70, and anti-Hsp90 autoantibodies are increased in RA patients

In comparison with sera from healthy individuals, we found that sera of patients with RA contained significantly higher levels of IgG, IgM, and IgA autoantibodies against Hsp60, Hsp70, and Hsp90, as measured by ELISA (Fig. 1).

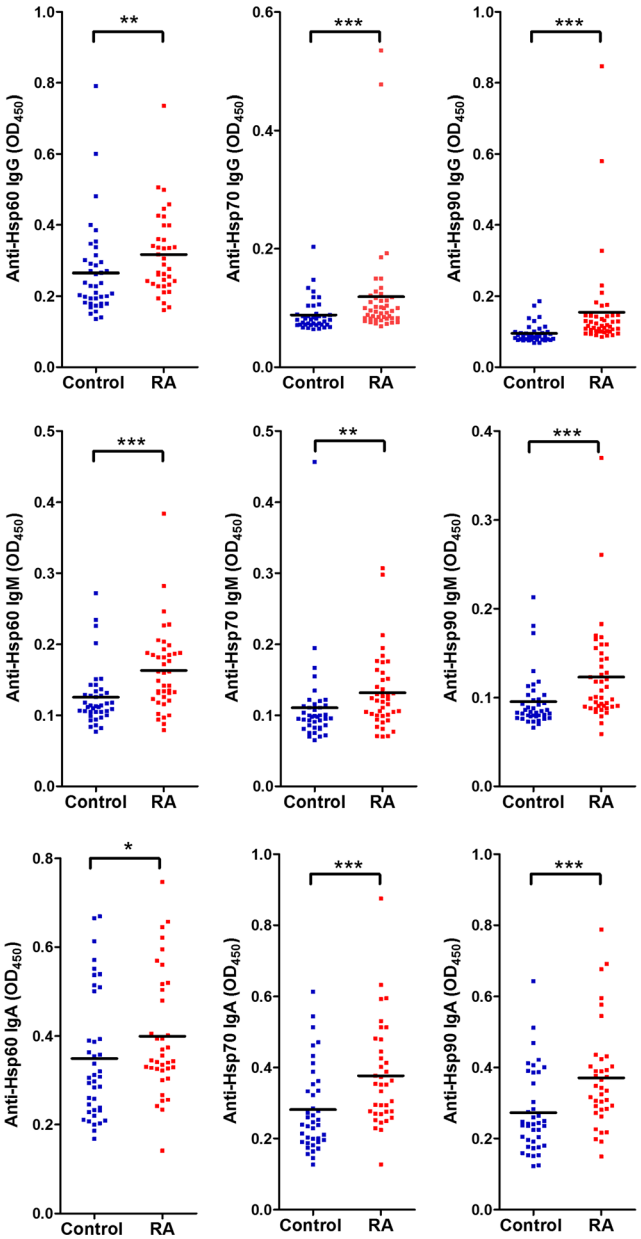

Fig. 1 Levels of anti-Hsp60, anti-Hsp70, and anti-Hsp90 IgG, IgM, and IgA autoantibodies in sera of patients with rheumatoid arthritis (RA), as well as of age- and gender-matched healthy controls measured by enzyme-linked immunosorbent assay. The squares and horizontal bars indicate individual and mean values in each group, respectively. *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$

Autoantibodies to HSP are associated with Th1-like and Th2-like cytokine levels in RA

Although the levels of circulating anti-Hsp60, anti-Hsp70, and anti-Hsp90 autoantibodies revealed no significant correlations with disease activity (DAS 28) and joint damage based on the Steinbrocker criteria (Table 1), some significant associations between anti-HSP and serum levels of Th1-like and Th2-like cytokine have been found in RA (Table 1). Positive correlations between serum levels of anti-Hsp60 IgG and IL-4 (Th2-like cytokine) and between serum levels of anti-Hsp90 IgG and IFN-ɣ (Th1-like cytokine), as well as anti-Hsp90 IgA and rheumatoid factor (RF), were found to be statistically significant in RA (Table 1). In addition, a significant inverse correlation was found for serum levels of anti-Hsp70 IgM and TNF-α (Th1-like cytokine) in RA (Table 1). No significant associations between serum levels of anti-HSP and the remaining cytokines IL-2, IL-6, and IL-10 were found (data not shown).

Discussion

Rheumatoid arthritis is a chronic systemic autoimmune disease characterized by dysregulation of the balance between pro- and anti-inflammatory immune reactions. Therefore, therapeutic strategies which aim at the induction of immunoregulatory mechanisms and blockade of uncontrolled activation of effector cell populations are highly warranted. There is an increasing interest in the role of heat shock proteins (HSP) as potential treatment targets for a number of autoimmune diseases including RA. Both bacterial and autologous HSP interactions with host immune cell components lead to stimulation of humoral (auto)immune response including production of HSP (auto)antibodies and/or activation of immune regulatory mechanisms (van Eden et al. 2017). Recently, we found higher titers of IgG autoantibodies to Hsp40 in sera of RA patients which positively correlated with the stages of joint damage (Steinbrocker's criteria), disease activity according to Disease Activity Score (DAS 28), and serum levels of pro-inflammatory IL-6 (Tukaj et al. 2010a, b). In parallel, we found that Hsp40 modulates T cell response in RA, manifested by the reduction of T cell proliferation and stimulation of anti-inflammatory IL-10 in PBMC cultures isolated from RA patients (Tukaj et al. 2010a). It seems that despite the activation of the humoral immune response and concomitant positive correlations with disease activity and disease progression, as well as pivotal cytokine involved in the pathophysiology of RA, Hsp40 displays immunosuppressive properties, as was

Table 1 Correlation coefficients for the relationships between levels of circulating anti-Hsp60, anti-Hsp70, and anti-Hsp90 IgG, IgM, and IgA autoantibodies and selected parameters of RA patients including disease activity (DAS 28), Steinbrocker radiographic criteria (RTG), rheumatoid factor (RF), and serum cytokine levels. *P* values ≤ 0.05 were considered significant

	DAS 28	RTG	RF	IFN-ɣ	TNF-α	IL-4
IgG						
Anti-Hsp60	− 0.081	0.339	0.267	0.253	0.117	0.328
	P = 0.622	*P* = 0.058	*P* = 0.188	*P* = 0.121	*P* = 0.479	*P* = 0.042
Anti-Hsp70	− 0.004	− 0.220	− 0.111	0.113	− 0.022	− 0.005
	P = 0.982	*P* = 0.226	*P* = 0.590	*P* = 0.493	*P* = 0.896	*P* = 0.977
Anti-Hsp90	− 0.277	− 0.249	− 0.085	0.372	0.183	0.147
	P = 0.088	*P* = 0.169	*P* = 0.681	*P* = 0.020	*P* = 0.264	*P* = 0.373
IgM						
Anti-Hsp60	0.179	0.093	0.199	− 0.073	− 0.161	− 0.290
	P = 0.297	*P* = 0.633	*P* = 0.363	*P* = 0.673	*P* = 0.349	*P* = 0.086
Anti-Hsp70	0.142	0.047	0.121	− 0.213	− 0.353	− 0.273
	P = 0.409	*P* = 0.809	*P* = 0.582	*P* = 0.212	*P* = 0.035	*P* = 0.107
Anti-Hsp90	− 0.076	− 0.046	0.087	0.006	− 0.184	− 0.307
	P = 0.660	*P* = 0.811	*P* = 0.694	*P* = 0.971	*P* = 0.282	*P* = 0.068
IgA						
Anti-Hsp60	0.050	0.245	0.390	0.014	− 0.049	0.175
	P = 0.785	*P* = 0.210	*P* = 0.089	P = 0.971	*P* = 0.790	*P* = 0.339
Anti-Hsp70	0.102	0.182	0.390	− 0.156	− 0.075	− 0.018
	P = 0.579	*P* = 0.353	P = 0.089	*P* = 0.394	*P* = 0.685	*P* = 0.921
Anti-Hsp90	0.047	0.248	0.477	− 0.095	− 0.035	− 0.037
	P = 0.796	*P* = 0.203	*P* = 0.033	*P* = 0.603	*P* = 0.850	*P* = 0.840

earlier proved in clinical trials with DnaJp1 (bacterial Hsp40-derived epitope) in RA (Koffeman et al. 2009).

In this study, we found that sera of RA patients contained significantly higher levels of anti-Hsp60, anti-Hsp70, and anti-Hsp90 autoantibodies as compared to healthy individuals. There was no significant association, however, between these autoantibodies and diseases activity or stages of joint damage (Steinbrocker's criteria) in RA. Autoantibodies to HSP, however, were associated with Th1-like and Th2-like cytokine levels in RA suggesting the immunomodulatory role of HSP/anti-HSP in RA patients.

A majority of studies regarding immunosuppressive HSP actions concern Hsp60 and Hsp70, despite higher titer of (auto)antibodies directed to these molecules under physiological and pathological conditions (van Eden et al. 2005, 2017; Tukaj 2014). For instance, naturally occurring as well as acquired *Mycobacterium tuberculosis* anti-Hsp60 antibodies protect against the induction of murine autoimmune inflammatory arthritis (Ulmansky et al. 2002) and humanized anti-Hsp60 mAb was found to be effective in protecting and suppressing autoimmune arthritis and colitis experimental models (Ulmansky et al. 2015). In human PBMC cultures, anti-Hsp60 mAb inhibited the secretion of pro-inflammatory IL-6 and IFN-ɣ (Th1-like cytokine) (Ulmansky et al. 2015), the last representing antagonist cytokine to the Th2 subpopulation. This is in line with our results since positive correlation between anti-Hsp60 IgG and IL-4 (Th2-like cytokine) was found to be statistically significant in RA patients.

Further, active immunization with Hsp70, which generates Hsp70-specific IgG, can prevent or arrest inflammatory damage in murine arthritis models via IL-10 and Treg induction (Wieten et al. 2009; van Herwijnen et al. 2012). This is also partly in line with the results obtained herein since a significant inverse correlation between serum levels of anti-Hsp70 IgM and TNF-α (Th1-like cytokine) was found in RA patients.

Hsp90 plays an important role in activation of innate and adaptive cells of the immune system (Srivastava 2002). *It has been shown that Hsp90 participates in stabilizing and activating the key signaling molecules including transcription factors* (e.g., *NF-κB*) *or kinases* (e.g., *p38/MAPK*) *which regulate multiple cellular processes like inflammation* (Trepel et al. 2010). *Moreover, its pharmacological inhibition has increasingly become the focus of research in RA and other autoimmune diseases* (Tukaj and Węgrzyn 2016). *On the other hand, several independent observations have revealed immunosuppressive activity of Hsp90 or its endoplasmic reticulum analog gp96 in preclinical studies* (Quintana et al. 2004; Chandawarkar et al. 2004; Liu et al. 2009). *In this study, however, the presence of positive correlations between serum levels of anti-Hsp90 IgG and IFN-ɣ (Th1-like cytokine), as well as between serum levels of anti-Hsp90 IgA and rheumatoid factor in RA patients, may suggest possible contribution of Hsp90/anti-Hsp90 in this disease.*

In summary, although further research is needed to confirm the present data, based on our report and previous observations, humoral immune response against autologous Hsp60 and Hsp70 is not in contradiction with the known immunosuppressive activity of these chaperones in RA.

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Compliance with ethical standards

The use of human biological material was approved by the Ethics Committee of the Medical University of Gdańsk, Poland, and written informed consent was obtained according to the Declaration of Helsinki.

References

Chandawarkar RY, Wagh MS, Kovalchin JT, Srivastava P (2004) Immune modulation with high-dose heat-shock protein gp96: therapy of murine autoimmune diabetes and encephalomyelitis. Int Immunol 16:615–624

Guo Q, Wang Y, Xu D, Nossent J, Pavlos NJ, Xu J (2018) Rheumatoid arthritis: pathological mechanisms and modern pharmacologic therapies. Bone Res 6:15. https://doi.org/10.1038/s41413-018-0016-9

Kampinga HH, Hageman J, Vos MJ, Kubota H, Tanguay RM, Bruford EA, Cheetham ME, Chen B, Hightower LE (2009) Guidelines for the nomenclature of the human heat shock proteins. Cell Stress Chaperones 14:105–111. https://doi.org/10.1007/s12192-009-0106-0

Kasperkiewicz M, Tukaj S, Gembicki AJ, Silló P, Görög A, Zillikens D, Kárpáti S (2014) Evidence for a role of autoantibodies to heat shock protein 60, 70, and 90 in patients with dermatitis herpetiformis. Cell Stress Chaperones 9:837–843. https://doi.org/10.1007/s12192-014-0507-6

Koffeman EC, Genovese M, Amox D, Keogh E, Santana E, Matteson EL, Kavanaugh A, Molitor JA, Schiff MH, Posever JO, Bathon JM, Kivitz AJ, Samodal R, Belardi F, Dennehey C, van den Broek T, van Wijk F, Zhang X, Zieseniss P, le T, Prakken BA, Cutter GC, Albani S (2009) Epitope-specific immunotherapy of rheumatoid arthritis: clinical responsiveness occurs with immune deviation and relies on the expression of a cluster of molecules associated with T cell tolerance in a double-blind, placebo-controlled, pilot phase II trial. Arthritis Rheum 60:3207–3216. https://doi.org/10.1002/art.24916

Kotlarz A, Tukaj S, Krzewski K, Brycka E, Lipinska B (2013) Human Hsp40 proteins, DNAJA1 and DNAJA2, as potential targets of the immune response triggered by bacterial DnaJ in rheumatoid arthritis. Cell Stress Chaperones 18:653–659. https://doi.org/10.1007/s12192-013-0407-1

Liu Z, Li X, Qiu L, Zhang X, Chen L, Cao S, Wang F, Meng S (2009) Treg suppress CTL responses upon immunization with HSP gp96. Eur J Immunol 39:3110–3120. https://doi.org/10.1002/eji.200939593

Pockley AG, Shepherd J, Corton JM (1998) Detection of heat shock protein 70 (Hsp70) and anti-Hsp70 antibodies in the serum of normal individuals. Immunol Investig 27:367–377

Pockley AG, Bulmer J, Hanks BM, Wright BH (1999) Identification of human heat shock protein 60 (Hsp60) and anti-Hsp60 antibodies in the peripheral circulation of normal individuals. Cell Stress Chaperones 4:29–35

Quintana FJ, Carmi P, Mor F, Cohen IR (2004) Inhibition of adjuvant-induced arthritis by DNA vaccination with the 70-kd or the 90-kd human heat-shock protein: immune cross-regulation with the 60-kd heat-shock protein. Arthritis Rheum 50:3712–3720. https://doi.org/10.1002/art.20635

Spierings J, van Eden W (2017) Heat shock proteins and their immunomodulatory role in inflammatory arthritis. Rheumatology (Oxford) 56:198–208. https://doi.org/10.1093/rheumatology/kew266

Srivastava P (2002) Roles of heat-shock proteins in innate and adaptive immunity. Nat Rev Immunol 2:185–194. https://doi.org/10.1038/nri749

Trepel J, Mollapour M, Giaccone G, Neckers L (2010) Targeting the dynamic HSP90 complex in cancer. Nat Rev Cancer 10:537–549. https://doi.org/10.1038/nrc2887

Tukaj S (2014) Immunoregulatory properties of Hsp70. Postepy Hig Med Dosw 68:722–727

Tukaj S, Węgrzyn G (2016) Anti-Hsp90 therapy in autoimmune and inflammatory diseases: a review of preclinical studies. Cell Stress Chaperones 21:213–218. https://doi.org/10.1007/s12192-016-0670-z

Tukaj S, Kotlarz A, Jozwik A, Smolenska Z, Bryl E, Witkowski JM, Lipinska B (2010a) Hsp40 proteins modulate humoral and cellular immune response in rheumatoid arthritis patients. Cell Stress Chaperones 15:555–566. https://doi.org/10.1007/s12192-010-0168-z

Tukaj S, Kotlarz A, Jóźwik A, Smoleńska Z, Bryl E, Witkowski JM, Lipińska B (2010b) Cytokines of the Th1 and Th2 type in sera of rheumatoid arthritis patients; correlations with anti-Hsp40 immune response and diagnostic markers. Acta Biochim Pol 57:327–332

Tukaj S, Görög A, Kleszczyński K, Zillikens D, Kárpáti S, Kasperkiewicz M (2017) Autoimmunity to heat shock proteins and vitamin D status in patients with celiac disease without associated dermatitis herpetiformis. J Steroid Biochem Mol Biol 173:23–27. https://doi.org/10.1016/j.jsbmb.2016.10.002

Ulmansky R, Cohen CJ, Szafer F, Moallem E, Fridlender ZG, Kashi Y, Naparstek Y (2002) Resistance to adjuvant arthritis is due to protective antibodies against heat shock protein surface epitopes and the induction of IL-10 secretion. J Immunol 168:6463–6469

Ulmansky R, Landstein D, Moallem E, Loeb V, Levin A, Meyuhas R, Katzavian G, Yair S, Naparstek Y (2015) A humanized monoclonal antibody against heat shock protein 60 suppresses murine arthritis and colitis and skews the cytokine balance toward an anti-inflammatory response. J Immunol 194:5103–5109

van Eden W, van der Zee R, Prakken B (2005) Heat-shock proteins induce T-cell regulation of chronic inflammation. Nat Rev Immunol 5:318–330

van Eden W, Jansen MAA, Ludwig I, van Kooten P, van der Zee R, Broere F (2017) The enigma of heat shock proteins in immune tolerance. Front Immunol 8:1599. https://doi.org/10.3389/fimmu.2017.01599

van Herwijnen MJ, Wieten L, van der Zee R et al (2012) Regulatory T cells that recognize a ubiquitous stress-inducible self-antigen are long-lived suppressors of autoimmune arthritis. Proc Natl Acad Sci U S A 109:14134–14139. https://doi.org/10.1073/pnas.1206803109

Wieten L, Berlo SE, Ten Brink CB, van Kooten PJ, Singh M, van der Zee R, Glant TT, Broere F, van Eden W (2009) IL-10 is critically involved in mycobacterial HSP70 induced suppression of proteoglycan-induced arthritis. PLoS One 4:e4186. https://doi.org/10.1371/journal.pone.0004186

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SHORT COMMUNICATION

Autoantibodies to heat shock protein 60, 70, and 90 are not altered in the anti-SARS-CoV-2 IgG-seropositive humans without or with mild symptoms

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Abstract

Highly conserved heat shock proteins (Hsps) are localized in the cytoplasm and cellular organelles, and act as molecular chaperones or proteases. Members of Hsp families are released into the extracellular milieu under both normal and stress conditions. It is hypothesized that the severe acute respiratory syndrome corona virus 2 (SARS-CoV-2) has the potential to elicit autoimmunity due to molecular mimicry between human extracellular Hsps and immunogenic proteins of the virus. To confirm the above hypothesis, levels of circulating autoantibodies directed to the key human chaperones i.e., Hsp60, Hsp70, and Hsp90 in the anti-SARS-CoV-2 IgG-seropositive participants have been evaluated. Twenty-six healthy volunteers who got two doses of the mRNA vaccine encoding the viral spike protein, anti-SARS-CoV-2 IgG-positive participants (n = 15), and healthy naïve (anti-SARS-CoV-2 IgG-negative) volunteers (n = 51) have been included in this study. We found that the serum levels of anti-Hsp60, anti-Hsp70, and anti-Hsp90 autoantibodies of the IgG, IgM, or IgA isotype remained unchanged in either the anti-COVID-19-immunized humans or the anti-SARS-CoV-2 IgG-positive participants when compared to healthy naïve volunteers, as measured by enzyme-linked immunosorbent assay. Our results showing that the humoral immune response to SARS-CoV-2 did not include the production of anti-SARS-CoV-2 antibodies that also recognized extracellular heat shock protein 60, 70, and 90 represent a partial evaluation of the autoimmunity hypothesis stated above. Further testing for cell-based immunity will be necessary to fully evaluate this hypothesis.

Keywords Heat shock proteins, Hsps · COVID-19 · Severe acute respiratory syndrome corona virus 2, SARS-CoV-2 · Autoimmunity · Autoantibodies

Introduction

Highly conserved heat shock proteins (Hsps) are found in the cytoplasm, cellular organelles and extracellular fluids acting as molecular chaperones and proteases. Based on their molecular weight and the presence of characteristic domains, Hsps are categorized into several families, including Hsp60, Hsp70, and Hsp90 chaperones (Kampinga et al. 2009). In fact, these stress proteins can be released to the extracellular milieu and activate both the innate and adaptive immune responses (De Maio 2014; Pockley and Henderson 2018). This activation may drive to the generation of circulating anti-Hsps autoantibodies that are frequently elevated in autoimmune diseases (Tukaj and Kaminski 2019). Nevertheless, we and others have found that the anti-Hsps (auto)antibodies are also present in the serum of normal individuals (Pockley et al. 1998; Tukaj 2020).

Infections/vaccines and autoimmunity are linked fields (Cusick et al. 2012; Guimarães et al. 2015; Rojas et al. 2018). Recently, a link between COVID-19 and the development of autoimmunity has been also proposed (Cappello et al. 2020; Lucchese and Flöel 2020; Ehrenfeld et al. 2020; Kasperkiewicz 2021a, b; Hall 2021). Marino Gammazza et al. (2020) predicted that the severe acute respiratory syndrome corona virus 2 (SARS-CoV-2), the cause of the COVID-19 disease, has the potential to elicit an autoimmune reaction due to molecular mimicry between Hsps and immunogenic viral proteins. Molecular mimicry might occur when peptides derived from pathogens share amino acid sequence

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(linear epitopes) or structural similarities (conformational epitopes) with self-antigens. It has been found that 17 human Hsp proteins belonging to inter alia Hsp60, Hsp70, and Hsp90 chaperones shared immunogenic epitopes (at least six amino acids) with SARS-CoV-2 proteins, as analyzed by the free Immune Epitope Database and Analysis Resource (Marino Gammazza et al. 2020).

To verify the above hypothesis experimentally, levels of circulating autoantibodies directed to the key human chaperones, i.e., Hsp60, Hsp70, or Hsp90 have been evaluated in the anti-SARS-CoV-2 IgG-positive participants.

Materials and methods

Human blood samples

Twenty-six healthy volunteers who got two doses of the mRNA anti-COVID-19 vaccine encoding the viral spike protein (Pfizer-BioNTech COVID-19 Vaccine), anti-SARS-CoV-2 IgG-positive participants (n = 15), and healthy naïve (anti-SARS-CoV-2 IgG-negative) volunteers (n = 51) have been included in this study. Vaccinated participants were monitored for presence of anti-SARS-CoV-2 IgG to the nucleocapsid protein and the S1 domain of the viral spike protein within 3 to 5 weeks of the last dose of the vaccine. All vaccinated participants were positive to the S1 domain of the viral spike protein and additionally one person was positive to the nucleocapsid protein of the virus, as assayed by ELISA. Additional information on unvaccinated anti-SARS-CoV-2 IgG-positive participants is presented in Table 1. Serum samples were collected from blood donors from Northern Poland between December 2020 and February 2021 and stored at −20 °C until analysis. Volunteers who suffered from any (auto)immunological and skin disorders have been excluded from the study. The use of human biological material was approved by a bioethics committee at the regional medical chamber in Gdańsk (Poland) and written informed consents were performed in accordance with the Declaration of Helsinki.

Detection of circulating anti-SARS-CoV-2 antibodies

Although SARS-CoV-2 infection is usually verified by PCR, a detection of circulating anti-SARS-CoV-2 IgG antibodies is also an accepted approach to confirm past infection in convalescents (Zhang et al. 2020). Ninety-two serum samples were screened for the presence of anti-SARS-CoV-2 antibodies directed to the S1 domain of the viral spike protein and nucleocapsid protein by a commercially available FDA-approved anti-SARS-CoV-2 ELISA (IgG) kit (EUROIMMUN, Cat. no. EI2606-9601-2 G; sensitivity: 94.4%, specificity: 99.6%)

Table 1 Characteristics of unvaccinated anti-SARS-CoV-2 IgG-positive participants

Patients' ID no.	Anti-SARS-CoV-2 S1 (IgG) by ELISA	Anti-SARS-CoV-2 NCP (IgG) by ELISA	COVID-19 symptoms	COVID-19 verified by PCR
1	+	+	Fever, cough, fatigue, muscle and body aches, headache, loss of taste and smell	+
2	+	+	Cough	+
3	+	+	Fever, cough, fatigue, muscle and body aches	NT
4	+	+	Fever, cough, fatigue, muscle and body aches, sore throat	NT
5	+	+	Fever, fatigue, muscle and body aches	NT
6	+	+	Fever, cough, fatigue, muscle and body aches, headache,	NT
7	+	+	Fever, cough, fatigue, muscle and body aches	NT
8	+	+	Fever, fatigue	NT
9	+	+	Fever, cough, fatigue, muscle and body aches	NT
10	+	+	Loss of taste and smell	NT
11	+	+	Fever, cough, fatigue, muscle and body aches, sore throat	NT
12	+	−	Fatigue	NT
13	+	+	NR	NT
14	−	+	NR	NT
15	+	+	NR	NT

The presence of anti-SARS-CoV-2 antibodies directed to the S1 domain of the viral spike protein and/or nucleocapsid protein (NCP) were analyzed separately by commercially available anti-SARS-CoV-2 ELISA (IgG) kits. Twelve out of 15 positive volunteers reported at least one of the typical COVID-19 symptoms (e.g., fever, cough, fatigue, muscle or body aches, headache, loss of taste or smell, or sore throat) that appeared in the last 12 weeks prior to blood sampling for anti-SARS-CoV-2 IgG analysis. In two donors, the presence of SARS-CoV-2 virus was confirmed by PCR. *NR*, not reported; *NT*, not tested

and anti-SARS-CoV-2 NCP ELISA (IgG) kit (EUROIMMUN, Cat. no. EI2606-9601-1 G; sensitivity: 94.6%, specificity: 99.8%), respectively.

Detection of circulating anti-heat shock protein antibodies

Levels of IgG, IgM, and IgA against human Hsp60, Hsp70, and Hsp90 were evaluated in the serum samples by a home-made enzyme-linked immunosorbent assay (ELISA), as described previously (Mantej et al. 2019). Briefly, medium-binding 96-well plates (Cat. no. **504201**, Nest Scientific Biotechnology) were coated with commercially available full-length recombinant Hsp60 (Cat. no. ab78792; Abcam), Hsp90 (Cat. no. ADI-SPP-770; Enzo Life Science) or previously purified recombinant Hsp70 (Tukaj et al. 2021) proteins at a concentration of 0.5 μg/ml in 0.05 M bicarbonate buffer at 4°C overnight. The wells were blocked with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) at room temperature (RT) for 90 min. After washing, the evaluated sera were diluted in PBS containing 0.1% BSA (Cat. no. 05482-100G, Sigma), added to the wells and were incubated at RT for 90 min. Plates were then incubated with horseradish peroxidase (HRP)-conjugated anti-human IgG (Cat. no. 096M-4809V; Sigma)-, anti-human IgM (Cat. no. ab8507; Abcam)- or anti-human IgA (Cat. no. 41100; BioLegend)-specific secondary antibodies diluted in PBS containing 0.1% BSA at RT for 60 min. The TMB substrate solution (Cat. no. ab171523; Abcam) was used to visualize HRP enzymatic reaction and the reaction was stopped by adding H_2SO_4. Optical density measurements were performed at 450 nm with an ELISA plate reader (VICTOR Multilabel Plate Reader, PerkinElmer).

Statistical analysis

Statistical analyses were performed using the GraphPad Prism (San Diego, CA, USA) software. The Shapiro-Wilk test was used to verify whether the data had normal distribution. Data was analyzed by the Kruskal-Wallis test. P values less than 0.05 were considered as significant.

Results

No reactivity of the anti-SARS-CoV-2-positive serum with heat shock protein 60, 70, and 90

Given the assumption that the circulating anti-SARS-CoV-2 IgG generated during either vaccination or infection might cross-react with human Hsps, we hypothesized that the healthy volunteers who got the anti-COVID-19 vaccine or the anti-SARS-CoV-2 IgG-positive participants have higher titers of anti-Hsp antibodies in their serum. Here, healthy volunteers who got two-doses of the mRNA anti-COVID-19 vaccine encoding the viral spike protein (n = 26), anti-SARS-CoV-2 IgG-positive participants (n = 15), and healthy naïve volunteers (n = 51) have been included in this study. The presence of anti-SARS-CoV-2 antibodies directed to the S1 domain of the viral spike protein and/or nucleocapsid protein were analyzed separately by commercially available anti-SARS-CoV-2 ELISA (IgG) kits (Table 1). We found that the serum levels of anti-Hsp60, anti-Hsp70, and anti-Hsp90 autoantibodies of IgG, IgM, or IgA isotype remained unchanged in either the anti-COVID-19 vaccinated volunteers or the anti-SARS-CoV-2 IgG-positive participants when compared to healthy naïve volunteers (anti-SARS-CoV-2 IgG-negative), as measured by ELISA (Fig. 1).

Discussion

Several mechanisms have been proposed to explain the effects of viral or bacterial infections, as well as vaccines that can initiate and/or exacerbate a pathological autoimmune reaction. One such mechanism is molecular mimicry, where a foreign antigen shares sequence or structural similarities with autoantigens (Cusick et al. 2012; Guimarães et al. 2015; Rojas et al. 2018). Recently, a link between COVID-19 and the development of autoimmunity has also been proposed (Cappello et al. 2020; Lucchese and Flöel 2020; Ehrenfeld et al. 2020; Kasperkiewicz 2021a, 2021b). It is hypothesized that SARS-CoV-2 has the potential to elicit autoimmune reaction due to molecular mimicry between heat shock proteins (Hsps) and immunogenic viral proteins (Marino Gammazza et al. 2020). Hsps are a diverse group of constitutive and/or stress-induced proteins (chaperones and/or proteases) that are categorized into several families on the basis of their molecular weight and the presence of characteristic domains. Hsps mediate a range of essential cellular functions, including proper folding of polypeptides and antigen presentation (Kampinga et al. 2009; Tukaj and Kaminski 2019; Tukaj 2020). Interestingly, various Hsps might be passively or actively released from the necrotic or stressed cells, respectively (De Maio 2014; Pockley and Henderson 2018). Highly immunogenic Hsps released into the extracellular space are able to activate both the innate and adaptive immune responses and could be implicated in the autoimmune reaction (Tukaj 2020). This activation can lead to the generation of circulating anti-Hsps autoantibodies that are frequently elevated in autoimmune diseases, such as rheumatoid arthritis, coeliac disease or dermatitis herpetiformis (Tukaj et al. 2010; Kasperkiewicz et al. 2014; Tukaj et al. 2017; Mantej et al. 2019). Therefore, volunteers who suffered from any (auto)immunological and skin disorders were excluded from this study. Here, we found that the serum levels of anti-Hsp60, anti-Hsp70, and anti-Hsp90 autoantibodies remained unchanged in either the anti-COVID-19-immunized humans or the anti-SARS-CoV-2 IgG-positive participants when compared to healthy naïve volunteers. Even though proposed similarities between epitopes found in the human Hsps and the

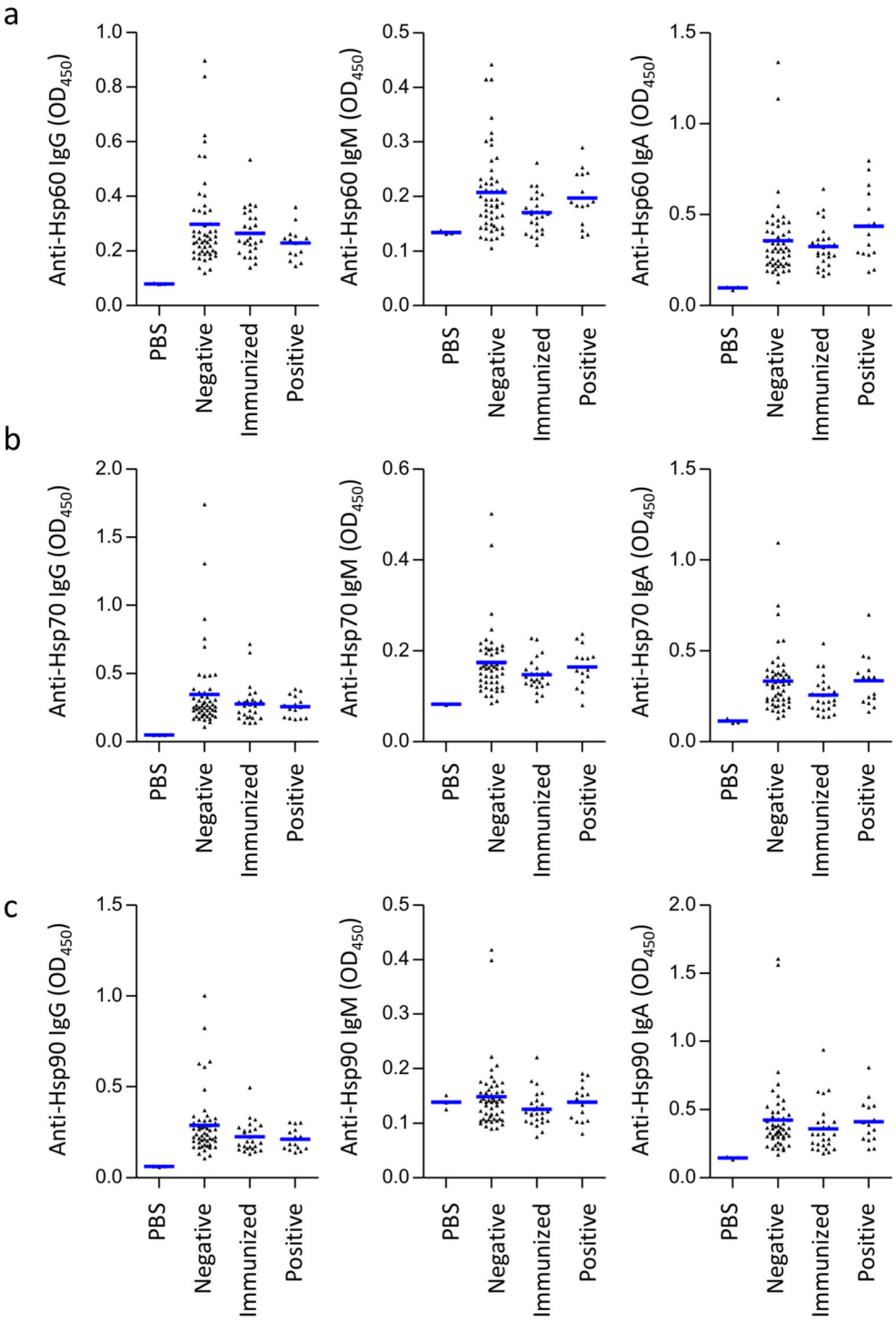

Fig. 1 Levels of circulating anti-heat shock protein autoantibodies in the anti-SARS-CoV-2 IgG-positive patients. Levels of (**a**) anti-Hsp60, (**b**) anti-Hsp70, and (**c**) anti-Hsp90 of the IgG, IgM, and IgA autoantibody isotype in the sera of anti-COVID-19-immunized humans (n = 26), anti-SARS-CoV-2 IgG-positive participants (n = 15), and naïve (anti-SARS-CoV-2 IgG-negative) volunteers (n = 51), assessed by the enzyme-linked immunosorbent assay. Values of sera's reactivity with the respective Hsps above the mean values of BSA reactivity (negative control) were regarded positive, as expressed by optical density measured at 450 nm (OD_{450}). The dots and horizontal bars indicate individual and mean values in each group, respectively

virus (Marino Gammazza et al. 2020; Cappello et al. 2020; Lucchese and Flöel 2020) were not confirmed herein, we are aware of the limitations of our approach. For example, Hsps are subject to conformational changes depending on a huge variety of factors found in vivo and under experimental conditions (Chavez et al. 2016) that might contribute to the final outcome of this study. Also, further testing for cell-based immunity, not limited to the humoral autoimmune reactions, will be necessary to fully evaluate proposed hypothesis and to investigate potential long-term consequences of such immune cross-reactivity.

Finally, more advanced experimental approaches, such as epitope mapping would be necessary to find out whether the proposed shared-epitopes might be cross-recognized by anti-SARS-CoV-2 antibodies generated during infection and could drive autoimmunity via molecular mimicry.

Conclusions

Based on our preliminary results, we can conclude that the extracellular heat shock proteins 60, 70, and 90 are not always targeted by the anti-SARS-CoV-2 antibodies raised in human serum after either infection or immunization. Further investigations with higher number of participants are needed to clarify the role of Hsps and other autoantigens in the course of the SARS-CoV-2 infection and after anti-COVID-19 vaccination.

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Declarations

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

References

Cappello F, Marino Gammazza A, Dieli F, de Macario EC, Macario AJL (2020) Does SARS-cov-2 trigger stress-induced autoimmunity by molecular mimicry? A hypothesis. J Clin Med 9:2038. https://doi.org/10.3390/jcm9072038

Chavez JD, Schweppe DK, Eng JK, Bruce JE (2016) In Vivo Conformational Dynamics of Hsp90 and Its Interactors. Cell Chem Biol 23:716–726. https://doi.org/10.1016/j.chembiol.2016.05.012

Cusick MF, Libbey JE, Fujinami RS (2012) Molecular mimicry as a mechanism of autoimmune disease. Clin Rev Allergy Immunol 42:102–111. https://doi.org/10.1007/s12016-011-8294-7

De Maio A (2014) Extracellular Hsp70: export and function. Curr Protein Pept Sci 15:225–231. https://doi.org/10.2174/1389203715666140331113057

Ehrenfeld M, Tincani A, Andreoli L, Cattalini M, Greenbaum A, Kanduc D, Alijotas-Reig J, Zinserling V, Semenova N, Amital H, Shoenfeld Y (2020) Covid-19 and autoimmunity. Autoimmun Rev 19:102597. https://doi.org/10.1016/j.autrev.2020.102597

Guimarães LE, Baker B, Perricone C, Shoenfeld Y (2015) Vaccines, adjuvants and autoimmunity. Pharmacol Res 100:190–209. https://doi.org/10.1016/j.phrs.2015.08.003

Hall BG (2021) Stress proteins as predictors of COVID-19 outcomes. Cell Stress Chaperones 26:287–288 https://doi.org/10.1007/s12192-020-01186-x

Kampinga HH, Hageman J, Vos MJ, Kubota H, Tanguay RM, Bruford EA, Cheetham ME, Chen B, Hightower LE (2009) Guidelines for the nomenclature of the human heat shock proteins. Cell Stress Chaperones 14:105–111. https://doi.org/10.1007/s12192-008-0068-7

Kasperkiewicz M (2021a) COVID-19 outbreak and autoimmune bullous diseases: a systematic review of published cases. J Am Acad Dermatol 84:563–568. https://doi.org/10.1016/j.jaad.2020.08.012

Kasperkiewicz M (2021b) Covid-19, heat shock proteins, and autoimmune bullous diseases: a potential link deserving further attention. Cell Stress Chaperones 26:1–2. https://doi.org/10.1007/s12192-020-01180-3

Kasperkiewicz M, Tukaj S, Gembicki AJ, Silló P, Görög A, Zillikens D, Kárpáti S (2014) Evidence for a role of autoantibodies to heat shock protein 60, 70, and 90 in patients with dermatitis herpetiformis. Cell Stress Chaperones 19:837–843. https://doi.org/10.1007/s12192-014-0507-6

Lucchese G, Flöel A (2020) SARS-cov-2 and Guillain-Barré syndrome: molecular mimicry with human heat shock proteins as potential pathogenic mechanism. Cell Stress Chaperones 25:731–735. https://doi.org/10.1007/s12192-020-01145-6

Mantej J, Polasik K, Piotrowska E, Tukaj S (2019) Autoantibodies to heat shock proteins 60, 70, and 90 in patients with rheumatoid arthritis. Cell Stress Chaperones 24:283–287. https://doi.org/10.1007/s12192-018-0951-9

Marino Gammazza A, Légaré S, Lo Bosco G, Fucarino A, Angileri F, Conway de Macario E, Macario AJ, Cappello F (2020) Human molecular chaperones share with SARS-cov-2 antigenic epitopes potentially capable of eliciting autoimmunity against endothelial cells: possible role of molecular mimicry in COVID-19. Cell Stress Chaperones 25:737–741. https://doi.org/10.1007/s12192-020-01148-3

Pockley AG, Henderson B (2018) Extracellular cell stress (heat shock) proteins-immune responses and disease: an overview. Philos Trans R Soc Lond Ser B Biol Sci 373:20160522. https://doi.org/10.1098/rstb.2016.0522

Pockley AG, Shepherd J, Corton JM (1998) Detection of heat shock protein 70 (Hsp70) and anti-Hsp70 antibodies in the serum of normal individuals. Immunol Investig 27:367–377. https://doi.org/10.3109/08820139809022710

Rojas M, Restrepo-Jiménez P, Monsalve DM, Pacheco Y, Acosta-Ampudia Y, Ramírez-Santana C, Leung PSC, Ansari AA, Gershwin ME, Anaya JM (2018) Molecular mimicry and autoimmunity. J Autoimmun 95:100–123. https://doi.org/10.1016/j.jaut.2018.10.012

Tukaj S (2020) Heat Shock Protein 70 as a Double Agent Acting Inside and Outside the Cell: Insights into Autoimmunity. Int J Mol Sci 21:5298. https://doi.org/10.3390/ijms21155298

Tukaj S, Kaminski M (2019) Heat shock proteins in the therapy of autoimmune diseases: too simple to be true? Cell Stress Chaperones 24: 475–479. https://doi.org/10.1007/s12192-019-01000-3

Tukaj S, Kotlarz A, Jozwik A, Smolenska Z, Bryl E, Witkowski JM, Lipinska B (2010) Hsp40 proteins modulate humoral and cellular immune response in rheumatoid arthritis patients. Cell Stress Chaperones 15:555–566. https://doi.org/10.1007/s12192-010-0168-z

Tukaj S, Görög A, Kleszczyński K, Zillikens D, Kárpáti S, Kasperkiewicz M (2017) Autoimmunity to heat shock proteins and vitamin D status in patients with celiac disease without associated dermatitis herpetiformis. J Steroid Biochem Mol Biol 173:23–27. https://doi.org/10.1016/j.jsbmb.2016.10.002

Tukaj S, Mantej J, Sobala M, Potrykus K, Tukaj Z, Zillikens D, Ludwig RJ, Bieber K, Kasperkiewicz M (2021) Therapeutic Implications of Targeting Heat Shock Protein 70 by Immunization or Antibodies in Experimental Skin Inflammation. Front Immunol 12:614320. https://doi.org/10.3389/fimmu.2021.614320

Zhang W, Du R-H, Li B, Zheng X-S, Yang X-L, Hu B et al (2020) Molecular and serological investigation of 2019-nCoV infected patients: implication of multiple shedding routes. Emerg Microbes Infect 9:386–389. https://doi.org/10.1080/22221751.2020.1729071

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Therapeutic Implications of Targeting Heat Shock Protein 70 by Immunization or Antibodies in Experimental Skin Inflammation

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Heat shock proteins (Hsp) are constitutive and stress-induced molecules which have been reported to impact innate and adaptive immune responses. Here, we evaluated the role of Hsp70 as a treatment target in the imiquimod-induced, psoriasis-like skin inflammation mouse model and related *in vitro* assays. We found that immunization of mice with Hsp70 resulted in decreased clinical and histological disease severity associated with expansion of T cells in favor of regulatory subtypes ($CD4^+FoxP3^+$/$CD4^+CD25^+$ cells). Similarly, anti-Hsp70 antibody treatment led to lowered disease activity associated with down-regulation of pro-inflammatory Th17 cells. A direct stimulating action of Hsp70 on regulatory T cells and its anti-proliferative effects on keratinocytes were confirmed in cell culture experiments. Our observations suggest that Hsp70 may be a promising therapeutic target in psoriasis and potentially other autoimmune dermatoses.

Keywords: psoriasis, regulatory T cells, Treg, Th17, heat shock proteins, Hsp70, immunization

INTRODUCTION

Heat shock proteins (Hsp) are a diverse group of constitutive and stress-induced molecules that are categorized into several families named on the basis of their molecular weight, including Hsp110, Hsp90, Hsp70, Hsp60, Hsp40, and the so-called small Hsp. Hsp act as intracellular chaperones being involved in protein folding and homeostasis but can also be released to extracellular compartments upon stressful conditions or cell death (1).

Given their additional complex immunological roles both inside and outside of cells, multiple studies particularly identified Hsp90 and Hsp70 as important pathophysiological factors and treatment targets of different chronic inflammatory and autoimmune disorders (2–4). Using models of the prototypical autoimmune blistering disease epidermolysis bullosa acquisita, we have previously comprehensively shown that pharmacological Hsp90 inhibition results in attenuation of disease activity by multimodal anti-inflammatory mechanisms (2). It is assumed

that the immunomodulatory effects of Hsp90 inhibition are at least partly mediated by the up-regulation of intracellular Hsp70 (a surrogate marker of Hsp90 blockade) which inhibits the nuclear factor-κB (NF-κB) inflammatory pathway (3). In addition, increased Hsp70 expression achieved independently of Hsp90 inhibition (e.g., by Hsp70 vaccination) has been demonstrated to be associated with down-regulation of inflammatory processes in several preclinical models of autoimmune diseases (4–6). However, some contradictory results suggested that both intra- and extracellular Hsp70 can exert a dual role in autoimmune diseases (i.e., either promoting or silencing immune responses), depending on its origin (i.e., bacterial or self), site of inflammation, type of disease, and possibly other undefined reasons (4).

Evidence suggests involvement of Hsp, including Hsp70, in the development of psoriasis (7), an autoimmune-associated chronic inflammatory skin disease characterized by impaired immunological cell function with altered Th17/regulatory T cell (Treg) balance, autoreactive T cells, and dysregulation of keratinocyte proliferation (8). In this disease, increased expressions of Hsp and immune responses to these proteins have been described (7). Recently, an equivocal role of Hsp70 has been demonstrated in the imiquimod (IMQ)-induced skin inflammation mouse model which has become the most widely used murine model for preclinical studies of psoriasis-like dermatitis (9–11). While one study showed that topical application of Hsp70 led to a reduction of skin lesions and inflammatory markers (10), another study described similar effects using a topical Hsp70 inhibitor (9). Here, we further defined the role of Hsp70 (using murine [m]-, human [h]-, or plant [p]-Hsp70) as a treatment target in the IMQ mouse model and related *in vitro* assays.

MATERIALS AND METHODS

Cloning, Expression, and Purification of Hsp70

Full-length synthetic DNA fragments encoding Hsp70 from *Nicotiana tabacum* (BAM24707.1), *Mus musculus* (NP_034609.2), and *Homo sapiens* (NP_005336.3) have been obtained from Thermo Scientific (GeneArt service). Codon usage was optimized for efficient gene expression in *E. coli* by the GeneOptimizer software. The inserts were synthesized with N-terminal 6x-His-SUMO tag and cloned into the pET151/D-TOPO or pRSET_A_A185 (Thermo Scientific) plasmid. Genetically modified lipopolysaccharide-free *E. coli* ClearColi® BL21(DE3) (Lucigen) strain carrying the respective plasmid was grown in the LB medium supplemented with 1% NaCl, 1 mM IPTG (Sigma), and ampicillin at 18°C overnight. Cells were harvested by centrifugation, resuspended in a lysis buffer, and disrupted by sonication. After centrifugation, the supernatant was loaded on the HIS-Select® Nickel Affinity Gel resin (Sigma) equilibrated with the lysis buffer. To remove unbound proteins and the chaperone-associated substrates, the column was washed with a buffer containing 5 mM ATP, 5 mM $MgCl_2$, 1 M NaCl, and 20 mM Tris-HCl pH=8.0. The Hsp70 containing fractions (eluted with lysis buffer containing 180 mM imidazole) were dialyzed against a dialysis buffer (20 mM Tris-HCl pH=8.0, 250 mM NaCl, 10% glycerol), followed by His-tag cleavage using SUMO protease (Sigma). To remove His-tag from the mixture, the protein sample was loaded on the HIS-Select® Nickel Affinity Gel resin (Sigma) equilibrated with the dialysis buffer. The Hsp70 fraction (99% purity) was filtered (0.22 μm) and stored at −80°C for further analysis. In addition, Hsp70 from *Nicotiana tabacum* leaves has been purified using ATP-agarose, as described previously (12).

Flow Cytometric Immunophenotyping

Single-cell suspensions from spleen of mice were stained with anti-CD4 (clone GK1.5; BioLegend), anti-CD25 (clone 3C7; BioLegend), and anti-FoxP3 (clone MF-14; BioLegend). In the case of intracellular cytokine staining, splenocytes were cultured in RPMI 1640 medium containing 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin in presence of phorbol-12-myristate-13-acetate (PMA) (50 ng/ml; Sigma), ionomycin (1 μg/ml; Sigma), and monensin (BioLegend) for 5 h. Cells were washed, fixed, permeabilized, and stained with anti-IL-17 (clone TC11-18H10.1; BioLegend). Viable single cells were analyzed based on forward and side light scatter properties with a CyFlow Cube 6 flow cytometer (Sysmex) or MACSQuant Analyzer 10 flow cytometer (Miltenyi Biotec).

Detection of Circulating Anti-Hsp70 IgG

Serum levels of IgG against Hsp70 were evaluated by home-made enzyme-linked immunosorbent assay (ELISA), as described previously with minor modifications (13). Briefly, medium-binding 96-well plates were coated with m-Hsp70 at a concentration of 0.5 μg/ml in 0.1 M bicarbonate buffer at 4°C for 18 h. The wells were blocked with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) at room temperature (RT) for 2 h. After being washed with PBS + 0.05% Tween 20, mouse sera diluted 1:100 in PBS + 0.1% BSA were incubated at RT for 1 h. Plates were then incubated with horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Sigma) antibodies diluted 1:5000 in PBS containing 0.1% BSA at RT for 1 h. TMB substrate solution (Sigma) was used to visualize HRP enzymatic reaction, and the reaction was stopped by 0.5 M H_2SO_4. Optical density measurements were performed at 450 nm with an ELISA plate reader.

Mice

Female BALB/c mice aged 6 weeks were purchased from the Tri-City Academic Laboratory Animal Centre - Research and Services Centre (Gdańsk, Poland). Animal experiments were approved by local authorities of the Animal Care and Use Committee (Bydgoszcz, Poland) and performed by certified personnel in the animal facility of the University of Gdańsk, Poland.

Disease Induction and Treatment

To induce psoriasis-like skin inflammation, a 2 x 3 cm area on the mouse back was shaved and depilated on day −2 of the

experiment. Starting on day 0, 50 mg Aldara™ cream, containing 5% IMQ (Meda AB, Sweden) was topically applied to the shaved back skin daily for six consecutive days. Skin inflammation was evaluated daily using a modified version of the Psoriasis Activity and Severity Index (PASI), as described previously (14). Briefly, erythema, infiltration, and desquamation were each scored independently by two blinded persons on a scale from 0 to 4: 0, none; 1, minimal; 2, mild; 3, distinct; and 4 severe. The scores of these individual aspects of dermatitis were summed up to calculate the cumulative score from 0 to 12.

Fourteen days prior to the first Aldara™ application, mice were treated with a single subcutaneous injection of 100 µg recombinant m-Hsp70 or p-Hsp70, PBS, or 100 µg control ovalbumin (OVA) (Sigma) emulsified in 2 mg of adjuvant dimethyl dioctadecyl ammonium bromide (DDA) (Sigma). The use of ClearColi cells warrants that the purified overproduced Hsp70 is free of lipopolysaccharide contaminants.

In a functional assay, one day prior to the 6-day Aldara™ treatment, naive mice were treated with a single intraperitoneal injection of 50 µg mouse anti-Hsp70 IgG_1 mAb (clone BRM-22; Sigma) or 50 µg IgG_1 isotype control (Sigma) in PBS.

Histopathology

For histopathology, skin samples of the back obtained on the final day of the experiments were fixed in 4% (w/v) buffered formalin and embedded in paraffin. 6-µm tissues sections were stained with hematoxylin and eosin (H&E). Dermal leukocyte infiltration and epidermal thickness were scored blindly by an independent researcher on a scale from 0 to 4: 0, none; 1, slight; 2, moderate; 3, marked; and 4, very marked, as described previously (14).

Cell Culture

Splenocytes of naive BALB/c mice were cultured in RPMI 1640 medium containing 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin in the presence of 1 µg/ml immobilized anti-CD3 mAb and 1 µg/ml soluble anti-CD28 mAb in 24-well culture plates at 5% CO_2 and 37°C without (control) or with 20 µg/ml of substrate- and endotoxin-free, non-recombinant *N. tabacum*-derived Hsp70 for 72 h.

HaCaT cells were cultured in DMEM medium (Sigma) at 37°C in 5% CO_2 atmosphere. Cells were seeded on 96-well plates and grown to 80% confluence. Cells were incubated with IMQ (50 µM; Sigma) and cultured in the absence or presence of different concentrations of recombinant h-Hsp70 or p-Hsp70.

Proliferation Assay

Cell proliferation was assayed by ELISA after BrdU (Roche) incorporation at 18 h of IMQ (Abcam) treatment, followed by 6 h of incubation according to manufacturer's protocol.

Cytokine Measurement

IL-8 levels were analyzed in cell culture supernatants by ELISA (BioLegend).

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 5 (San Diego, CA). The Shapiro-Wilk test was used to verify whether the data had normal distribution. Normal and non-normal distributed data was analyzed by Student's t-test and Mann Whitney U test or Kruskal Wallis test, respectively. P values less than 0.05 were considered statistically significant.

RESULTS

Hsp70 Immunization Results in Decreased Disease Severity Associated With Expansion of T Cells in Favor of Regulatory Subtypes in the Imiquimod Mouse Model

The effects of immunization with a highly pure, substrate-, and endotoxin-free recombinant m-Hsp70 and p-Hsp70 were tested in the IMQ-induced skin inflammation mouse model (**Figure 1A**). Immunization of mice with either m-Hsp70 or p-Hsp70 led to the generation of circulating anti-Hsp70 IgG (**Figure 1B**). Clinical disease severity (i.e., infiltration, desquamation, and cumulative PASI) was significantly reduced in p-Hsp70- but not m-Hsp70-treated animals when compared with PBS- and OVA-injected animals (**Figures 1C–G**). Histologically, epidermal hyperplasia was significantly milder in both m-Hsp70- and p-Hsp70-treated mice compared with PBS- and OVA-treated control animals, whereas dermal leukocyte infiltration was not altered by Hsp70 immunization (**Figures 1H–J**).We next investigated whether Hsp70 immunization had an impact on Tregs (i.e., splenic $CD4^+FoxP3^+$ cells and circulating $CD4^+CD25^+$ cells) and pro-inflammatory splenic $CD4^+IL\text{-}17^+$ T cells. Both m-Hsp70- and p-Hsp70-immunized mice had significantly increased frequencies of $CD4^+FoxP3^+$ and pro-inflammatory T cells when compared to PBS-treated control mice (**Figures 2A–C**). However, only immunization with p-Hsp70 was associated with a significant expansion of both types of Tregs and a significant increase of the $CD4^+FoxP3^+$:Th17 ratio (**Figures 2A–D**).

Plant-Derived Hsp70 Induces $CD4^+CD25^+$ and Inhibits Th17 Cells *In Vitro*

Since the above observations concerning p-Hsp70 are based on recombinant protein preparations, we investigated whether non-recombinant Hsp70 directly obtained from *Nicotiana tabacum* leaves had a similar immunomodulatory activity. We found that such Hsp70 preparation led to induction of $CD4^+CD25^+$ cells and reduced the frequency of the Th17 population in anti-CD3/CD28-stimulated spleen cultures (**Figures 3A–C**).

Anti-Hsp70 Antibodies Lead to Lowered Disease Activity Associated With Down-Regulation of Pro-Inflammatory T cells in the Imiquimod Mouse Model

To evaluate the role of anti-Hsp70 antibodies in the IMQ mouse model, that are significantly induced upon immunization of

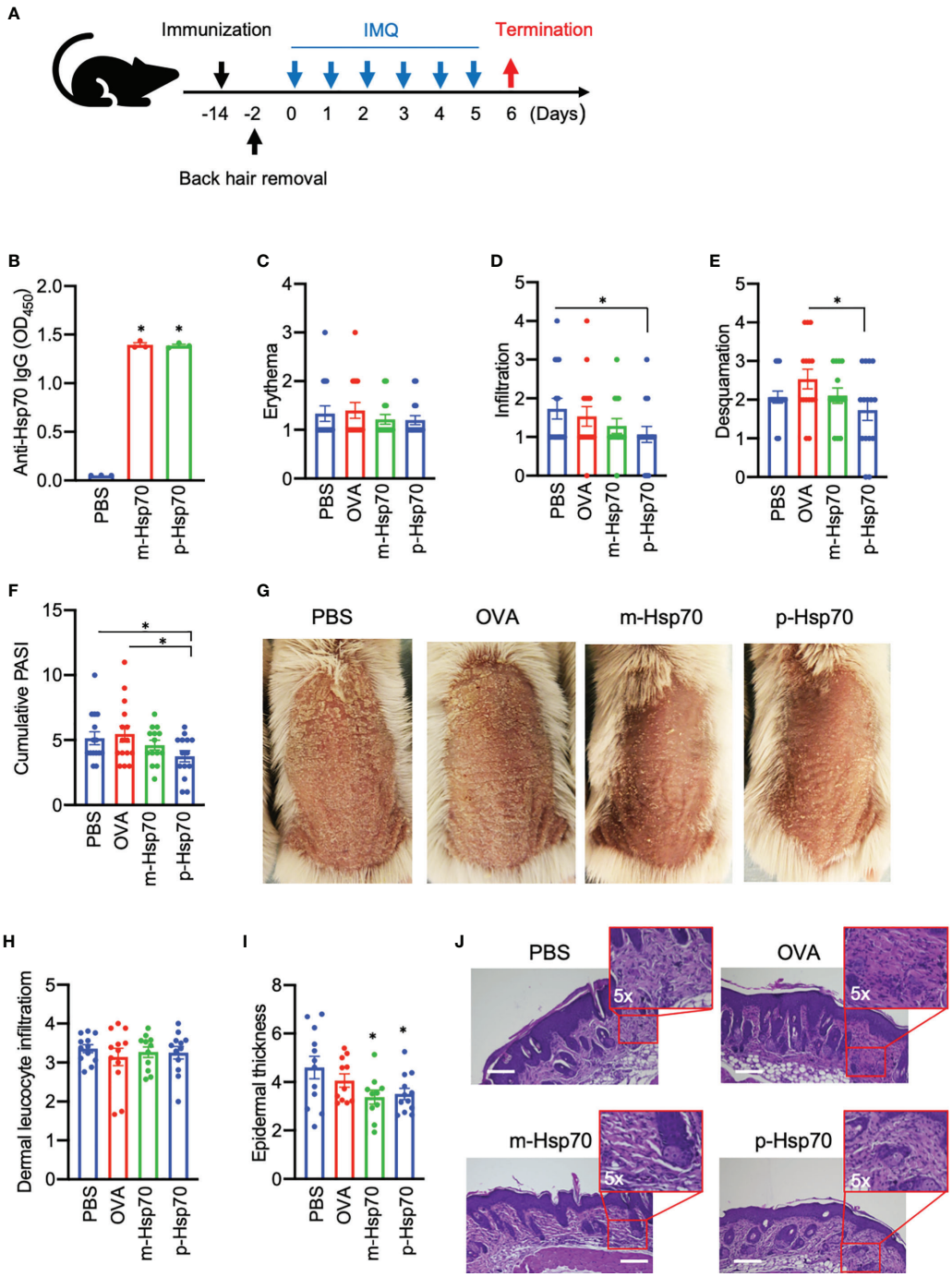

FIGURE 1 | Hsp70 immunization attenuates experimental psoriasis-like dermatitis. **(A)** Schematic illustration of the experimental setup. Mice were treated with a single subcutaneous injection of murine [m]-Hsp70, plant [p]-Hsp70, phosphate-buffered saline (PBS), or ovalbumin (OVA), followed (after two weeks) by daily application of topical imiquimod (IMQ) on shaved backs for 6 consecutive days. **(B)** Anti-Hsp70 IgG serum levels fourteen days after immunization of mice with Hsp70 or PBS injection of controls, as analyzed by enzyme-linked immunosorbent assay (ELISA). Clinical disease severity of PBS-, OVA-, and Hsp70-treated mice shown by scores for **(C)** erythema, **(D)** infiltration, **(E)** and desquamation, as well as **(F)** the resulting cumulative PASI score at the of the experiment. **(G)** Representative clinical presentations. Histological disease severity shown by scores for **(H)** dermal leukocyte infiltration and **(I)** epidermal thickness at the end of the experiment. **(J)** Representative hematoxylin and eosin (H&E) staining of skin biopsies with corresponding higher magnifications of the dermis. Data are representative of three independent experiments and expressed as mean ± SEM of a total of 10–15 mice per group. Dot plots overlaid on bar graphs represent individual data points. *P < 0.05. Bars = 100 μm.

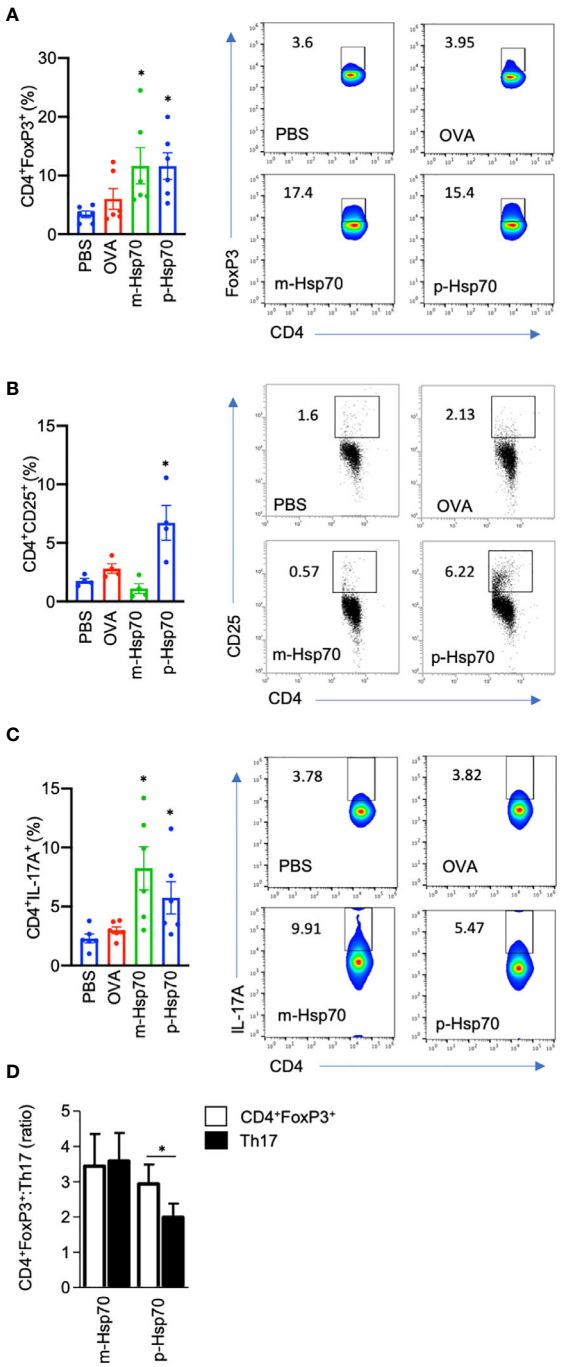

FIGURE 2 | Hsp70 immunization is associated with a predominant increase in regulatory subtypes of T cells. Bar charts show percentages of **(A)** splenic $CD4^+FoxP3^+$ cells, **(B)** blood $CD4^+CD25^+$ cells, **(C)** splenic $CD4^+IL\text{-}17A^+$ cells, as well as **(D)** $CD4^+FoxP3^+$:Th17 ratio at the end of the imiquimod (IMQ)-induced skin inflammation mouse experiment, as analyzed by flow cytometry. The numbers in the gates of the representative results (right) are the percentages of the respective cell populations. To express the ratio, data were normalized to a mean value of the control group [phosphate-buffered saline (PBS)-treated mice]. Data are expressed as mean ± SEM of four to six mice per group. Dot plots overlaid on bar graphs represent individual data points. *$P < 0.05$.

animals with Hsp70, naive mice were injected with murine anti-Hsp70 IgG or IgG isotype control one day prior to the IMQ treatment (**Figure 4A**). We found that anti-Hsp70 IgG-treated mice had a significantly lower PASI scores when compared to control mice (**Figures 4B, C**). Histologically, there was a trend toward a significant ($p=0.09$) decrease in the dermal inflammatory cell infiltrate without influence on epidermal thickness in the anti-Hsp70 IgG-treated mice when compared to controls (**Figures 4D–F**).

Immunophenotyping analysis revealed that anti-Hsp70 treatment had no significant effect on splenic $CD4^+FoxP3^+$ or blood $CD4^+CD25^+$ cell frequencies but was associated with a significantly lower percentage of splenic Th17 cells and a significant increase of the $CD4^+FoxP3^+$:Th17 ratio (**Figures 5A–D**).

Hsp70 Reduces Proliferation and IL-8 Secretion of Imiquimod-Stimulated Human Keratinocyte (HaCaT) Cells

We further explored direct effects of h-Hsp70 and p-Hsp70 on the proliferation of IMQ-stimulated HaCaT cells. We found that both h-Hsp70 and p-Hsp70 significantly inhibited proliferation of activated keratinocytes in a dose-dependent manner (**Figure 6A**). In addition, h-Hsp70 and p-Hsp70 significantly inhibited secretion of IL-8 from activated keratinocytes (**Figure 6B**).

DISCUSSION

Major findings of our study included the observation that immunization with Hsp70, particularly the plant-derived form, protected mice from clinical and histological features of IMQ-induced skin inflammation. Here, we found that p-Hsp70 uniquely induced two regulatory T cell populations ($CD4^+CD25^+$/$CD4^+FoxP3^+$) with a concomitant lesser induction of the pro-inflammatory Th17 cell population *in vivo*, as shown by an increased $CD4^+FoxP3^+$:Th17 ratio. However, our observation that p-Hsp70 actually led to inhibition of Th17 cells in anti-CD3/28-stimulated mouse splenocyte cultures argues against a direct effect of p-Hsp70 on Th17 expansion *in vivo* per se. Concurrent induction of Tregs and Th17 cells in Hsp70-immunized mice can potentially be explained by at least two non-exclusive reasons. First, previous studies in psoriatic patients revealed that the former cell type can differentiate into the latter (15). Second, it is also speculated that autologous extracellular Hsp70 plays a dual role in the cellular immune response which may depend on the type of cells that interact with Hsp70 and the type of the disease (4, 16). Our results are consistent with previously published pre-clinical observations. It has been shown that immunization of animals with bacterial Hsp70 and its highly conserved peptides could be regarded as a potential treatment option for autoimmune arthritis *via* induction of Tregs (4, 17–20). Here, the initially *in vivo* observed beneficial effects of Hsp70 vaccination on Tregs and the epidermal turnover, both known to be impaired in

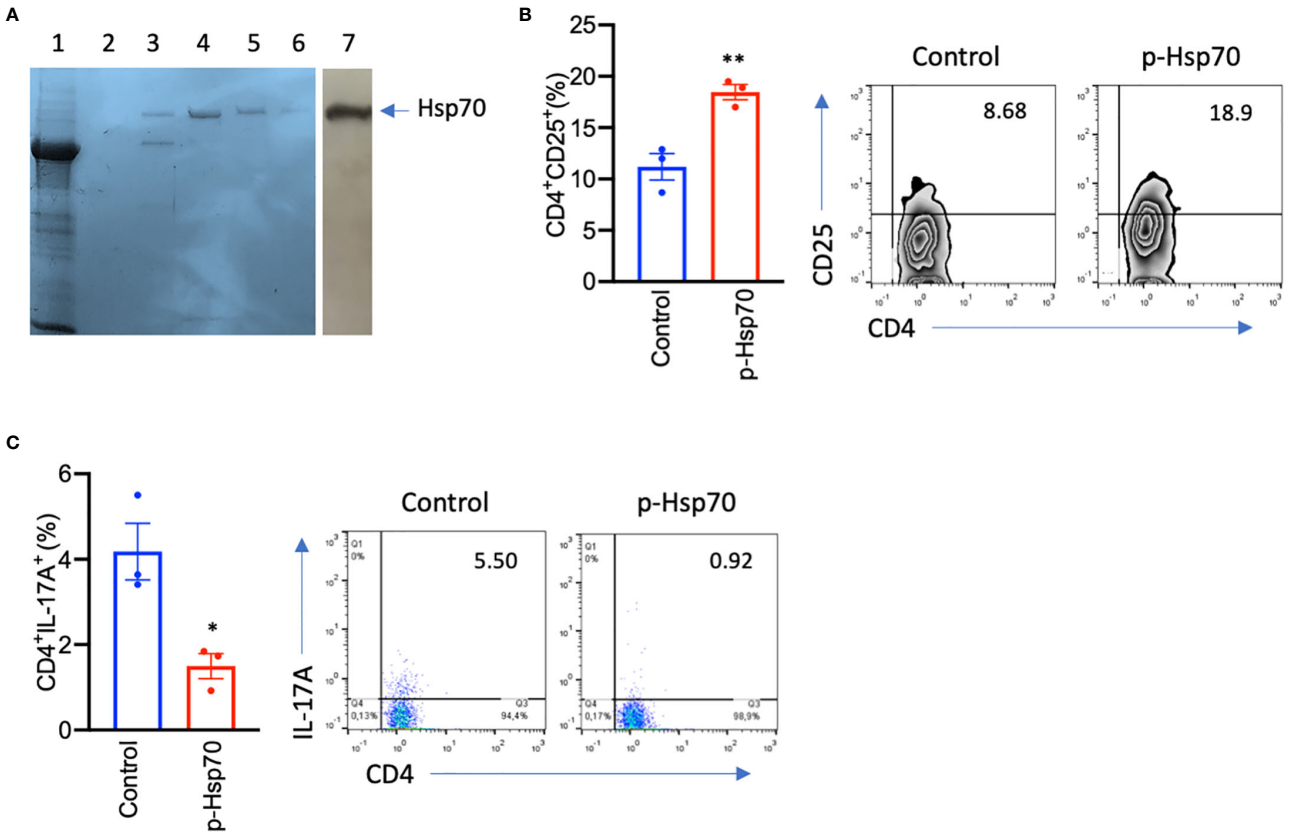

FIGURE 3 | Plant [p]-Hsp70 induces $CD4^+CD25^+$ and inhibits Th17 cells *in vitro*. **(A)** Non-recombinant p-Hsp70 from *Nicotiana tabacum* leaves has been purified using ATP-agarose. Standard Coomassie Brilliant Blue R250 staining method was used, with lane no. 1 representing whole cell lysates, lanes no. 2–6 purification steps, and lane no. 7 immunodetection of [p]-Hsp70 using anti-Hsp70 antibodies. Bar charts show percentages of **(B)** $CD4^+CD25^+$ or **(C)** $CD4^+IL\text{-}17A^+$cells. The numbers in the gates of the representative results (right) are the percentages of the respective cell populations. Data are expressed as mean ± SEM of three mouse donors. Dot plots overlaid on bar graphs represent individual data points. *$P < 0.05$, **$P < 0.01$.

patients with psoriasis (8), were also confirmed in *in vitro* experiments. To prove a direct effect of Hsp70 treatment on Treg expansion and reduction in epidermal thickness, anti-CD3/CD28-stimulated spleen cultures and IMQ-treated HaCaT cells were used, respectively. In fact, a direct stimulating action of Hsp70 on regulatory T cells ($CD4^+CD25^+$) and its anti-proliferative effects on HaCaT cells, along with an inhibitory impact on pro-inflammatory and growth-promoting IL-8, were found in these cell culture studies. These results are partly consistent with our previous study showing that intracellular induction of Hsp70 expression by a Hsp90 inhibitor was associated with suppression of IL-8 production in HaCaT cells (21), one of the key cytokines related to psoriasis pathogenesis (22).

This demonstration extends previous reports of a therapeutic potential of targeting Hsp70 by topical approaches in the IMQ mouse model as well as by Hsp70 vaccination in animal models of different autoimmune diseases such as rheumatoid arthritis and lupus erythematosus (4–6, 9, 10). In fact, there is also some evidence from a randomized controlled trial in which intravenous administration of the endoplasmic reticulum Hsp70 family member binding immunoglobulin protein (BiP) to patients with rheumatoid arthritis was associated with clinical and biological improvements in the disease activity (23). It has been suggested that the promoting effects of extracellular Hsp70 on Tregs are driven by major histocompatibility complex (MHC) class II-T cell receptor interactions, toll-like receptor 2 (TLR2) signaling, and PI3K/AKT-JNK-p38 MAPK pathways, but immunomodulation mediated by induction of tolerogenic dendritic cells has also been described (4). Of note, IMQ induces its psoriasis-like phenotype by activating TLR7/8 (11). Thus, it may be hypothesized that Hsp70 not only interacted with Treg-associated TLR2, but also directly interfered as damage-associated molecular pattern with psoriasis-specific signaling of TLR7/8. In this context, it is also worth mentioning that extracellular Hsp70 is known to interact with TLR2/4 on antigen-presenting cells and activate NF-κB (4). However, these previously described pro-inflammatory

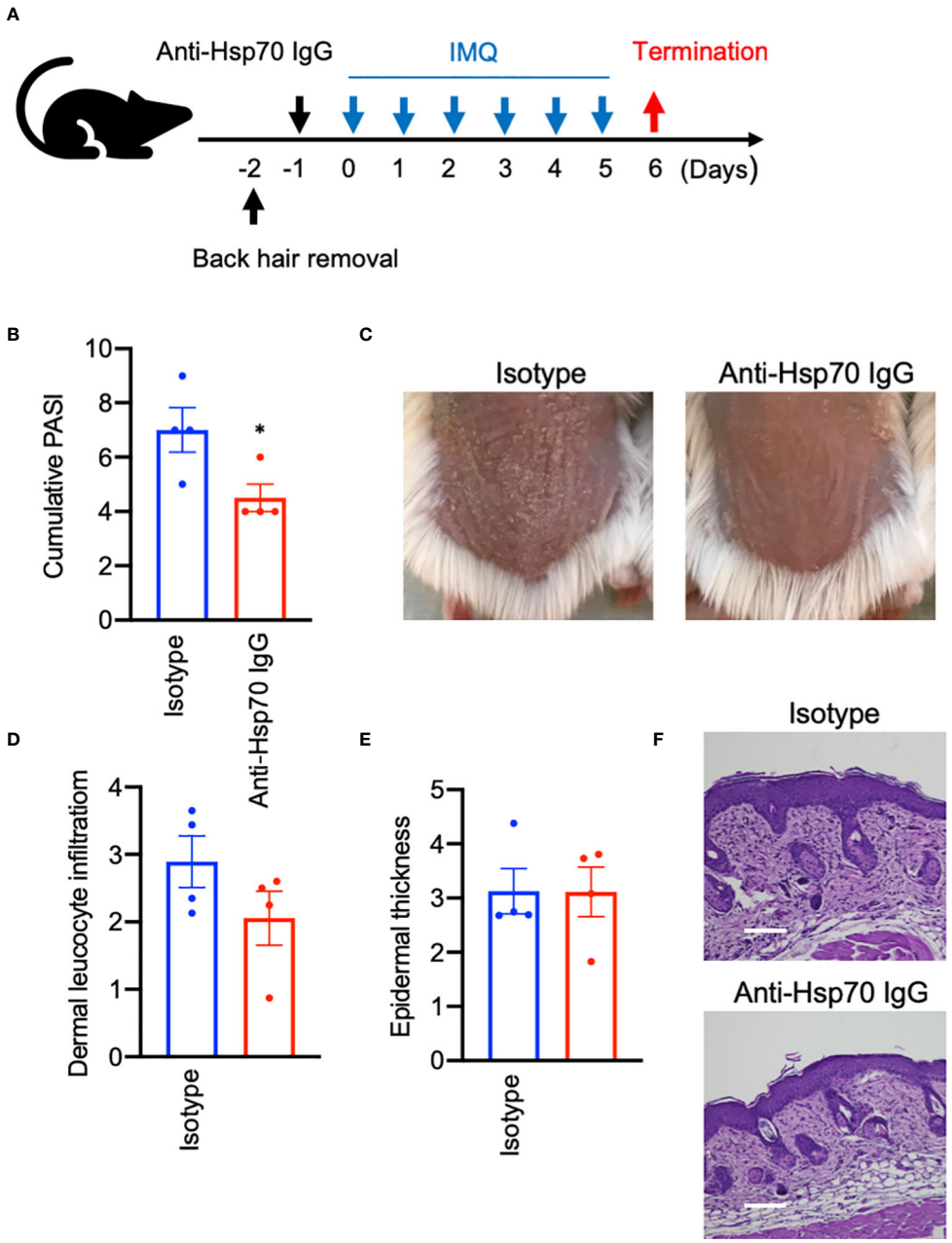

FIGURE 4 | Anti-Hsp70 antibodies ameliorate experimental psoriasis-like dermatitis. **(A)** Schematic illustration of the experimental setup. In a functional assay, naive mice were treated with a single intraperitoneal injection of mouse anti-Hsp70 IgG_1 mAb or isotype control (IgG_1), followed (next day) by daily application of topical imiquimod (IMQ) on shaved backs for six consecutive days. **(B)** Clinical disease severity of isotype- and anti-Hsp70 IgG-treated mice shown by the cumulative PASI score at the end of the experiment. **(C)** Representative clinical presentations. Histological disease severity shown by scores for **(D)** dermal leukocyte infiltration and **(E)** epidermal thickness at the end of the experiment. **(F)** Representative hematoxylin and eosin (H&E) staining of skin biopsies. Data are expressed as mean ± SEM of one experiment using four mice per group. Dot plots overlaid on bar graphs represent individual data points. *P < 0.05. Bars = 100 μm.

properties of exogenous Hsp70 may have potentially resulted from the presence of highly immunogenic bacterial endotoxins (e.g., lipopolysaccharides) in recombinant protein preparations produced in bacterial (e.g., *E. coli*) expression systems (4). In contrast, highly pure, substrate- and endotoxin-free m-Hsp70, h-Hsp70, and p-Hsp70 were used in our study.

Hsp70-derived epitopes can also interact with the B cell receptor, leading to production of anti-Hsp70 antibodies (4).

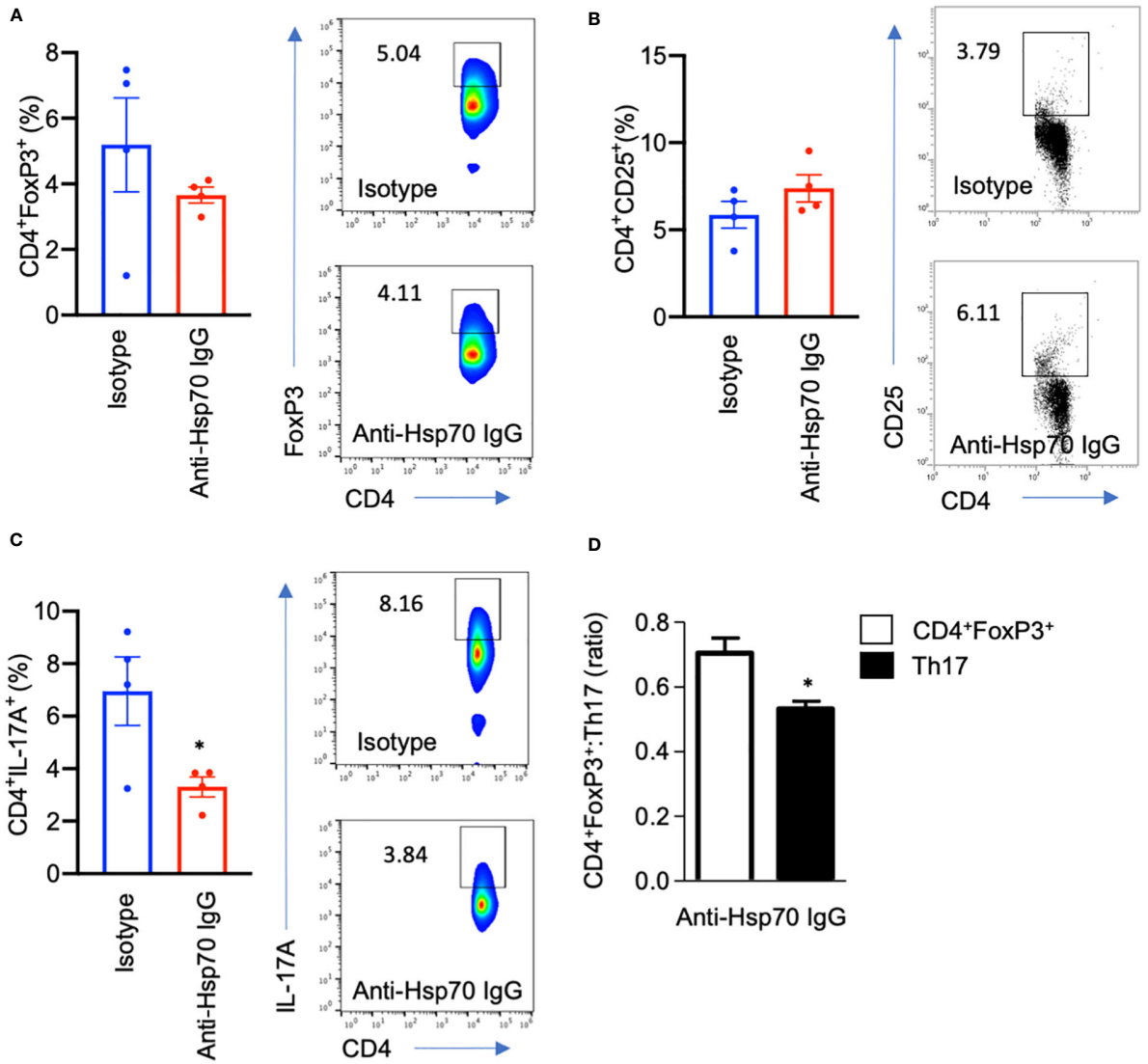

FIGURE 5 | Anti-Hsp70 antibody treatment is associated with a decrease in pro-inflammatory T cells. Bar charts show percentages of **(A)** splenic $CD4^+FoxP3^+$ cells, **(B)** blood $CD4^+CD25^+$ cells, **(C)** splenic $CD4^+IL\text{-}17^+$ cells, as well as **(D)** $CD4^+FoxP3^+$:Th17 ratio at the end of the imiquimod (IMQ)-induced skin inflammation mouse experiment, as analyzed by flow cytometry. The numbers in the gates of the representative results (right) are the percentages of the respective cell populations. To express the ratio, data were normalized to a mean value of the control group (isotype-treated mice). Data are expressed as mean ± SEM of one experiment using four mice per group. Dot plots overlaid on bar graphs represent individual data points. *P < 0.05.

Although different anti-Hsp autoantibodies were reported to be elevated in the blood of patients suffering from numerous inflammatory and autoimmune diseases, including rheumatoid arthritis, dermatitis herpetiformis, coeliac disease, and psoriasis, their pathological role and value for prediction of the development of autoimmunity is still obscure (7, 13, 24, 25). In our study, Hsp70 immunization of mice led to a robust humoral anti-Hsp70 response, and anti-Hsp70 antibodies were shown to exert clinical activity associated with decreased pro-inflammatory T cell reposes. Similarly, antibodies toward microbial- and self-Hsp60 were found to be effective in protecting and suppressing arthritis and colitis in rodent models (26, 27). The mechanism by which anti-Hsp70 IgG suppress experimental psoriasis and inhibit polarization of the proinflammatory Th17 population has not been completely solved. We can speculate that anti-Hsp70 antibodies may either expose the disease-modifying epitopes of autologous Hsp70 and/or neutralize those epitopes that are responsible for the induction of Th17 cells.

Lastly, the reason for a higher efficacy of plant-derived Hsp70 over the autologous counterpart in the mouse model of our study requires further elucidation. Nevertheless, we think that it is

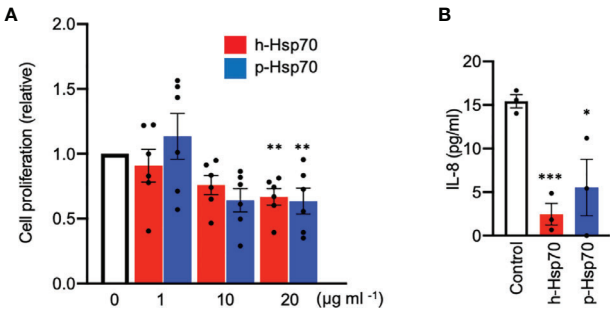

FIGURE 6 | Hsp70 inhibits proliferation and IL-8 secretion of imiquimod (IMQ)-stimulated HaCaT cells *in vitro*. **(A)** Proliferation response of HaCaT cells stimulated by IMQ (50 µmol) in the absence and presence of different concentrations of human [h]-Hsp70 and plant [p]-Hsp70. Cell proliferation was assayed by enzyme-linked immunosorbent assay (ELISA) after BrdU incorporation at 18 h of IMQ treatment, followed by 6 h incubation. Results are mean ± SEM of two independent experiments, each performed in triplicate. $^{**}P < 0.01$. **(B)** Effects of Hsp70 (20 µg/ml) on IL-8 secretion into culture medium from HaCaT cells stimulated by IMQ (50 µmol). Control cells were treated with equal amount of PBS. IL-8 levels in the cell culture supernatants were analyzed by ELISA. Results are mean ± SEM of one experiment performed in triplicate. Dot plots overlaid on bar graphs represent individual data points. $^{*}P < 0.05$, $^{***}P < 0.001$.

associated with disease-modifying regulatory $CD4^+CD25^+$ T cells that are expanded only in mice treated with p-Hsp70. Further studies are needed to specify the disease-modifying epitopes of p-Hsp70 for a potential therapeutic purpose.

CONCLUSION

We demonstrated that targeting Hsp70 exerts beneficial clinical, immunomodulatory, and anti-proliferative effects in the IMQ mouse model and related *in vitro* assays. These data support further investigations of Hsp70-based treatment modalities in psoriasis and other autoimmune dermatoses.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study and experiments were reviewed and approved by the local authorities of the Animal Care and Use Committee (Bydgoszcz, Poland).

AUTHOR CONTRIBUTIONS

ST designed and conceptualized the study. ST, JM, MS, and KB conducted the experiments. ST, JM, MS, KB, and MK analyzed and interpreted the result. ST, KP, ZT, DZ, RL, and MK prepared, revised, and approved the manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Saibil H. Chaperone machines for protein folding, unfolding and disaggregation. *Nat Rev Mol Cell Biol* (2013) 14:630–42. doi: 10.1038/nrm3658
2. Tukaj S, Zillikens D, Kasperkiewicz M. Heat shock protein 90: a pathophysiological factor and novel treatment target in autoimmune bullous skin diseases. *Exp Dermatol* (2015) 24:567–71. doi: 10.1111/exd.12760
3. Tukaj S, Węgrzyn G. Anti-Hsp90 therapy in autoimmune and inflammatory diseases: a review of preclinical studies. *Cell Stress Chaperones* (2016) 21:213–8. doi: 10.1007/s12192-016-0670-z
4. Tukaj S. Heat shock protein 70 as a double agent acting inside and outside the cell: insights into autoimmunity. *Int J Mol Sci* (2020) 21:E5298. doi: 10.3390/ijms21155298
5. Liu A, Ferretti C, Shi F-D, Cohen IR, Quintana FJ, La Cava A. DNA vaccination with Hsp70 protects against systemic lupus erythematosus in (NZB×NZW)F1 mice. *Arthritis Rheumatol* (2020) 72:997–1002. doi: 10.1002/art.41202
6. Quintana FJ, Carmi P, Mor F, Cohen IR. Inhibition of adjuvant-induced arthritis by DNA vaccination with the 70-kd or the 90-kd human heat-shock protein: Immune cross-regulation with the 60-kd heat-shock protein. *Arthritis Rheumatol* (2004) 50:712–20. doi: 10.1002/art.20635
7. Wang WM, Jin HZ. Heat shock proteins and psoriasis. *Eur J Dermatol* (2019) 29:121–5. doi: 10.1684/ejd.2019.3526
8. Greb JE, Goldminz AM, Elder JT, Lebwohl MG, Gladman DD, Wu JJ, et al. Psoriasis. *Nat Rev Dis Primers* (2016) 2:16082. doi: 10.1038/nrdp.2016.82
9. Raghuwanshi N, Yadav TC, Srivastava AK, Raj U, Varadwaj P, Pruthi V. Structure-based drug designing and identification of Woodfordia fruticosa inhibitors targeted against heat shock protein (HSP70-1) as suppressor for Imiquimod-induced psoriasis like skin inflammation in mice model. *Mater Sci Eng C Mater Biol Appl* (2019) 95:57–71. doi: 10.1016/j.msec.2018.10.061
10. Seifarth FG, Lax JE-M, Harvey J, DiCorleto PE, Husni ME, Chandrasekharan UM, et al. Topical heat shock protein 70 prevents imiquimod-induced psoriasis-like inflammation in mice. *Cell Stress Chaperones* (2018) 23:1129–35. doi: 10.1007/s12192-018-0895-0
11. van der Fits L, Mourits S, Voerman JS, Kant M, Boon L, Laman JD, et al. Imiquimod-induced psoriasis-like skin inflammation in mice is mediated via the IL-23/IL-17 axis. *J Immunol* (2009) 182:5836–45.doi: 10.4049/jimmunol.0802999
12. Tukaj S, Tukaj Z. Distinct chemical contaminants induce the synthesis of Hsp70 proteins in green microalgae Desmodesmus subspicatus: Heat

pretreatment increases cadmium resistance. *J Therm Biol* (2010) 35:239–44. doi: 10.1016/j.jtherbio.2010.05.007
13. Tukaj S, Görög A, Kleszczyński K, Zillikens D, Kárpáti S, Kasperkiewicz M. Autoimmunity to heat shock proteins and vitamin D status in patients with celiac disease without associated dermatitis herpetiformis. *J Steroid Biochem Mol Biol* (2017) 173:23–7. doi: 10.1016/j.jsbmb.2016.10.002
14. Sezin T, Zillikens D, Sadik CD. Leukotrienes do not modulate the course of Aldara™-induced psoriasiform dermatitis in mice. *Acta Derm Venereol* (2015) 95:341–2. doi: 10.2340/00015555-1924
15. Bovenschen HJ, van de Kerkhof PC, van Erp PE, Woestenenk R, Joosten I, Koenen HJ. Foxp3+ regulatory T cells of psoriasis patients easily differentiate into IL-17A-producing cells and are found in lesional skin. *J Invest Dermatol* (2011) 131:1853–60. doi: 10.1038/jid.2011.139
16. Tukaj S, Mantej J, Sobala M, Potrykus K, Sitko K. Autologous extracellular Hsp70 exerts a dual role in rheumatoid arthritis. *Cell Stress Chaperones* (2020) 25:1105–10. doi: 10.1007/s12192-020-01114-z
17. van Eden W, van der Zee R, Prakken B. Heat-shock proteins induce T-cell regulation of chronic inflammation. *Nat Rev Immunol* (2005) 5:318–30. doi: 10.1038/nri1593
18. Wieten L, Berlo SE, Ten Brink CB, van Kooten PJ, Singh M, van der Zee R, et al. IL-10 is critically involved in mycobacterial HSP70 induced suppression of proteoglycan-induced arthritis. *PLoS One* (2009) 4:e4186. doi: 10.1371/journal.pone.0004186
19. van Herwijnen MJ, Wieten L, van der Zee R, van Kooten PJ, Wagenaar-Hilbers JP, Hoek A, et al. Regulatory T cells that recognize a ubiquitous stress-inducible self-antigen are long-lived suppressors of autoimmune arthritis. *Proc Natl Acad Sci U S A* (2012) 109:14134–9. doi: 10.1073/pnas.1206803109
20. Wendling U, Paul L, van der Zee R, Prakken B, Singh M, van Eden W. A conserved mycobacterial heat shock protein (hsp) 70 sequence prevents adjuvant arthritis upon nasal administration and induces IL-10-producing T cells that cross-react with the mammalian self-hsp70 homologue. *J Immunol* (2000) 164:2711–7. doi: 10.4049/jimmunol.164.5.2711
21. Tukaj S, Grüner D, Zillikens D, Kasperkiewicz M. Hsp90 blockade modulates bullous pemphigoid IgG-induced IL-8 production by keratinocytes. *Cell Stress Chaperones* (2014) 19:887–94. doi: 10.1007/s12192-014-0513-8
22. Tsai YC, Tsai TF. Anti-interleukin and interleukin therapies for psoriasis: current evidence and clinical usefulness. *Ther Adv Musculoskelet Dis* (2017) 9:277–94. doi: 10.1177/1759720X17735756
23. Kirkham B, Chaabo K, Hall C, Garrood T, Mant T, Allen E, et al. Safety and patient response as indicated by biomarker changes to binding immunoglobulin protein in the phase I/IIA RAGULA clinical trial in rheumatoid arthritis. *Rheumatology (Oxford)* (2016) 55:1993–2000. doi: 10.1093/rheumatology/kew287
24. Kasperkiewicz M, Tukaj S, Gembicki A-J, Silló P, Görög A, Zillikens D, et al. Evidence for a role of autoantibodies to heat shock protein 60, 70, and 90 in patients with dermatitis herpetiformis. *Cell Stress Chaperones* (2014) 19:837–43. doi: 10.1007/s12192-014-0507-6
25. Mantej J, Polasik K, Piotrowska E, Tukaj S. Autoantibodies to heat shock proteins 60, 70, and 90 in patients with rheumatoid arthritis. *Cell Stress Chaperones* (2018) 24:283–7. doi: 10.1007/s12192-018-0951-9
26. Ulmansky R, Cohen CJ, Szafer F, Moallem E, Fridlender ZG, Kashi Y, et al. Resistance to adjuvant arthritis is due to protective antibodies against heat shock protein surface epitopes and the induction of IL-10 secretion. *J Immunol* (2002) 168:6463–9. doi: 10.4049/jimmunol.168.12.6463
27. Ulmansky R, Landstein D, Moallem E, Loeb V, Levin A, Meyuhas R, et al. A humanized monoclonal antibody against heat shock protein 60 suppresses murine arthritis and colitis and skews the cytokine balance toward an anti-inflammatory response. *J Immunol* (2015) 194:5103–9. doi: 10.4049/jimmunol.1500023

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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RESEARCH ARTICLE

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Evidence for a role of extracellular heat shock protein 70 in epidermolysis bullosa acquisita

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Abstract

Heat shock protein 90 (Hsp90) and Hsp70 are chaperones implicated in different inflammatory disorders, given their property to impact innate and adaptive immune responses. Here, we determined the so far unknown role of extracellular Hsp70 in epidermolysis bullosa acquisita (EBA), an anti-type VII collagen autoantibody-mediated blistering dermatosis. The *in vivo* pathophysiological relevance of extracellular Hsp70 was demonstrated in an anti-type VII collagen antibody transfer-induced EBA mouse model in which elevated blood levels of this chaperone were recorded. We found that Hsp70-treated mice had a more intense clinical disease severity compared to controls that were paralleled by increased levels of cutaneous matrix metalloproteinase 9 and plasma hydrogen peroxide. The latter finding was confirmed in an independent reactive oxygen species release assay using EBA-specific immune complexes combined with recombinant Hsp70. Finally, cell culture experiments using human naive peripheral blood mononuclear cells (PBMC) revealed that extracellular Hsp70 stimulated the secretion of the T cell–derived pro-inflammatory cytokines IL-6 and IL-8. This work extends knowledge about the role of Hsps in autoimmune bullous diseases, suggesting that extracellular Hsp70 represents a pathophysiological factor and potential treatment target in EBA.

KEYWORDS
autoimmune bullous diseases, epidermolysis bullosa acquisita, extracellular Hsp70, heat shock proteins, reactive oxygen species

1 | INTRODUCTION

Highly conserved in evolution heat shock proteins (Hsps) are constitutively expressed molecular chaperones being involved in intracellular polypeptide folding and protein homeostasis.[1] They can be up-regulated by cell stress factors, such as inflammation, and released to extracellular compartments. Within the diverse group of Hsps, Hsp90 and Hsp70 particularly deserved attention in different chronic inflammatory and autoimmune disorders, given their role in impacting innate and adaptive immune responses.[2–4]

Epidermolysis bullosa acquisita (EBA) is a rare autoimmune bullous dermatosis induced by autoantibodies to type VII collagen (COL7) located within the cutaneous basal membrane zone.[5] Experimental model systems have shaped our understanding of EBA pathogenesis.[6,7] In brief, intermediates between short- and long-lived plasma cells produce COL7 autoantibodies. The

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formation of COL7-specific plasma cells depends on the help of CD4 T cells and B cell–helper neutrophils.[8,9] Once in the circulation, the concentration of pathogenic anti-COL7 IgG is controlled by the neonatal Fc receptor, and autoantibodies rapidly bind to their target antigen.[10] Autoantibody binding triggers CD18/ICAM-1-dependent neutrophil extravasation into the target tissue, where neutrophils bind to the tissue-bound immune complexes. This binding is mediated by activating Fc gamma receptors (FcγR).[11,12] FcγR engagement induces a complex signalling cascade within neutrophils,[13–15] leading to the release of reactive oxygen species (ROS) and proteases, which ultimately cause subepidermal blistering.[16,17]

During the last decade, we have unravelled the importance of Hsp90 in EBA pathogenesis.[2] In brief, systemic administration of Hsp90 inhibitors (TCBL-154 and 17-DMAG) suppressed pathogenic anti-COL7 IgG generation and reduced dermal neutrophilic infiltrates in EBA mice as well as inhibited T-cell proliferation in *ex vivo* COL7- or CD3/CD28 mAb re-stimulated EBA lymph node cells.[18] Further *in vitro* observations revealed that 17-DMAG inhibited T lymphocyte proliferation, diminished expansion of major pro-inflammatory T helper 1 (Th1) and Th17 cell populations, and blocked secretion of pro-inflammatory IFN-γ, TNF-α and IL-17 in anti-CD3-activated human PBMC cultures.[19] In addition, COL7-immunized mice treated with Hsp90 inhibitor had reduced total B lymphocytes in spleens and lower levels of pathogenic IgG autoantibodies.[20] The effectiveness of anti-Hsp90 therapy was confirmed when the drug was used topically. 17AAG applied to the skin ameliorated clinical disease severity in experimental EBA that was paralleled with reduced neutrophil infiltration, lowered expression of matrix metalloproteinases (MMPs) and Flightless I, as well as decreased nuclear factor kappa B (NF-κB) activation in the skin.[21]

By contrast, less is known about Hsp70, especially its extracellular form, in autoimmune bullous disorders including EBA. We here aimed to address this knowledge gap and investigated the role of Hsp70 in experimental models of EBA.

2 | MATERIAL AND METHODS

2.1 | Human blood samples

The use of blood samples from healthy volunteers was approved by the Bioethics Committee at Regional Medical Chamber in Gdansk (Poland). Written informed consent has been obtained in accordance with the Declaration of Helsinki.

2.2 | Cloning, expression and purification of Hsp70

Cloning, expression and purification of Hsp70 (NP_005336.3) have been performed as described previously.[22]

2.3 | Mice

Six-week-old BALB/c (♀) mice were obtained from the Tri-City University Animal House—Research Service Centre (Gdansk, Poland). All animal experiments were accepted by the Animal Care & Use Committee in Bydgoszcz (Poland) and conducted by experienced and certified researchers.

2.4 | Purification of the vWFA2 and anti-murine vWFA2 IgG

Mouse von Willebrand factor A-like domain 2 (vWFA2) of the N-terminal non-collagenous domain (NC1) of COL7 was produced as described previously.[23] Rabbit anti-mouse vWFA2 IgG (anti-COL7 IgG) was produced as reported.[24] IgG from immunized rabbit serum was purified as described previously.[24] Activity of IgG was confirmed by immunofluorescence microscopy on mouse skin.

2.5 | Disease induction and treatment

EBA was reproduced in BALB/c mice strain by repetitive subcutaneous (s.c.) injection of anti-vWFA2 IgG as described previously.[24,25] Briefly, 8- to 10-week-old mice got a total of five s.c. injections of 3 mg anti-vWFA2 IgG (anti-COL7 IgG) on every second day. Two weeks prior to the first anti-vWFA2 IgG injection, mice received a single intraperitoneal (i.p.) injection of recombinant Hsp70 (100 μg per mouse) or vehicle (PBS). Disease activity was expressed as the percentage of whole-body surface area that was affected by the typical skin lesions (eg erythema, blisters, erosions, alopecia and crusts) and scored every fourth day (Days 4, 8 and 12), as described previously.[26]

2.6 | Detection of circulating Hsp70

Hsp70 was evaluated in serum/plasma by a HSP70 High Sensitivity ELISA kit (Enzo Life Science), following the manufacturer's instructions.

2.7 | Detection of circulating anti-Hsp70 IgG

Serum levels of anti-Hsp70 IgG were evaluated by an in-house ELISA as described previously.[22]

2.8 | Histopathology

Skin samples of the ears were fixed in Roti®Histofix 4% and embedded in paraffin. Then, skin samples were cut into 6-μm sections

and stained with haematoxylin and eosin (H&E). Dermal neutrophil infiltration was scored semi-quantitatively (blindly) as described previously.[26]

2.9 | Flow cytometry

EDTA-treated blood cells were stained with anti-Ly6G (BioLegend) and anti-CD62L (BioLegend) in CyLyse™ reagents (Sysmex). Viable granulocytes were assayed based on forward scatter (FSC) vs side scatter (SSC) properties with a flow cytometer (CyFlow Cube 6, Sysmex).

2.10 | Immunoblotting

Equal amounts of ear skin lysates (cytoplasmic fractions) were separated in polyacrylamide gels under denaturing conditions (SDS-PAGE) and transferred (semi-dry protein transfer) onto nitrocellulose (0.45 μm) membrane (Bio-Rad). The membrane was incubated in blocking buffer (TBS) containing 3% non-fat milk for 2 h, followed by incubation with primary (rabbit) antibodies to MMP-9 (1:1000; Abcam), Hsp90 (1:1000; Cell Signaling Technology) or β-actin (1:1000; Cell Signaling Technology) at RT for 1.5 hours. HRP-coupled goat anti-rabbit secondary antibodies (1:2000; Sigma-Aldrich) were used. Amersham ECL Plus Western Blotting Detection Reagents (GE Healthcare) was used to visualize the reaction. Relative MMP-9/β-actin protein concentrations were measured by densitometry (Image Lab 6.1.0).

2.11 | H_2O_2 measurement

The concentration of hydrogen peroxide (H_2O_2) in mouse plasma was assayed using the Amplex®UltraRed fluorochrome (Molecular Probes) as described previously with minor modifications.[27] Diluted plasma (1:10) in 0.05 M HEPES (pH 7.0) buffer in presence of Amplex®UltraRed was added to the 96-well plate. The fluorescence at Ex./Em. wavelengths 490/585 nm was monitored for 1 hour using Varioskan Reader (Thermo Fisher Scientific).

2.12 | Reactive oxygen species release assay

Immune complex (IC)-stimulated reactive oxygen species (ROS) release by polymorph nuclear (PMN) cells was studied as described previously with minor modifications.[26] PMN cells were purified from EDTA-treated blood samples from healthy donors by using PolymorphPrep™ (Progen) according to the manufacturer's protocol. The cells were subsequently washed with PBS and resuspended at 2×10^6 cells/ml in RPMI 1640 medium (phenol red-free; Gibco) supplemented with 1% FCS and 25 mM HEPES. Isolated PMN were incubated in absence or presence of Hsp70 at 37°C for 2 h, followed by stimulation with IC (anti-COL7 antibody +COL7 antigen) formed by 2 μg/ml vWFA2 of mouse COL7 and purified rabbit anti-mouse vWFA2 IgG at 37°C. ROS liberation was monitored by luminol (Sigma)-enhanced chemiluminescence. The 96-well plates were analysed for up to 99 repeats with a plate reader (VICTOR™ 3 reader, PerkinElmer Inc.).

2.13 | Peripheral blood mononuclear cell culture

Human peripheral blood mononuclear cells (PBMCs) were isolated from the blood samples (10 ml) of naïve healthy donors by Histopaque 1077 (Sigma) gradient centrifugation and cultured as described previously with minor modifications.[28] PBMCs were resuspended at 1×10^6 cells/ml of the RPMI 1640 medium (Sigma) with 10% FCS (Sigma) and penicillin/streptomycin (Sigma). PBMCs were stimulated with immobilized anti-CD3 monoclonal antibody (mAb) (1 μg/ml; BioLegend) and soluble anti-CD28 mAb (1 μg/ml; BioLegend) in 24-well culture plates at 5% CO_2 and at 37 °C and cultured in presence of Hsp70 for 68 h.

2.14 | Cytokine detection

Cell culture supernatant levels of human IL-6, IL-8, IL-10 and IL-17A were assessed by commercially available ELISA kits (BioLegend).

2.15 | Statistical analyses

Statistical analyses were performed with GraphPad Prism software 5.0. Normally distributed data were analysed by Student's *t* test or ANOVA, and non-normal distributed data were analysed by Mann-Whitney *U* test or Kruskal-Wallis test, respectively.

3 | RESULTS

3.1 | Levels of circulating Hsp70 are increased in mice with EBA

Blood levels of Hsp70 were significantly elevated (2.8-fold mean increase) in the anti-COL7 antibody transfer-induced EBA mouse model as compared to naïve mice (Figure 1).

3.2 | Treatment with recombinant Hsp70 results in increased disease severity in mice with EBA

To evaluate the role of extracellular Hsp70 in EBA, naive mice were injected intraperitoneally with full-length Hsp70 fourteen days prior to the first anti-COL7 IgG injection. We observed that Hsp70-immunized mice had a significantly higher clinical disease severity when compared to control mice (Figure 2A), which was associated with significantly elevated serum levels of anti-Hsp70 IgG (Figure 2B). Both dermal

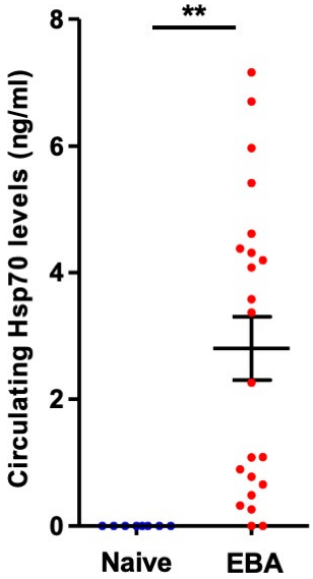


FIGURE 1 Levels of circulating Hsp70 are increased in experimental EBA. BALB/c mice were injected subcutaneously with anti-COL7 IgG on Days 0, 2, 4, 6 and 8. Levels of Hsp70 in the blood of naive mice and mice with experimental EBA were measured by enzyme-linked immunosorbent assay (ELISA) at Day 12. Data are presented as mean (±SEM) of 8–22 mice per group. Dot plots represent individual data points. $^{**}p < 0.01$

inflammatory cell infiltrate (Figure 2C) and neutrophil activity indicated by CD62L expression on circulating Ly6G-positive cells (Figure 2D) were not significantly altered by Hsp70 treatment. On the contrary, plasma content of H_2O_2 (Figure 2E) and skin expression of MMP-9 (Figure 2F,H) were significantly elevated in the Hsp70-injected mice compared to vehicle-injected mice. There were no significant effects of the treatment on the amount of cutaneous Hsp90 (Figure 2G, H).

3.3 | Hsp70 stimulates the release of ROS from human neutrophils

We further evaluated whether the higher plasma content of H_2O_2 in Hsp70-treated EBA mice was due to direct effects of this protein on human blood neutrophils. The presence of Hsp70 significantly triggered ROS release from EBA immune complex-stimulated neutrophils in a dose-dependent way (Figure 3A,B).

3.4 | Hsp70 stimulates the release of pro-inflammatory cytokines from human T cells

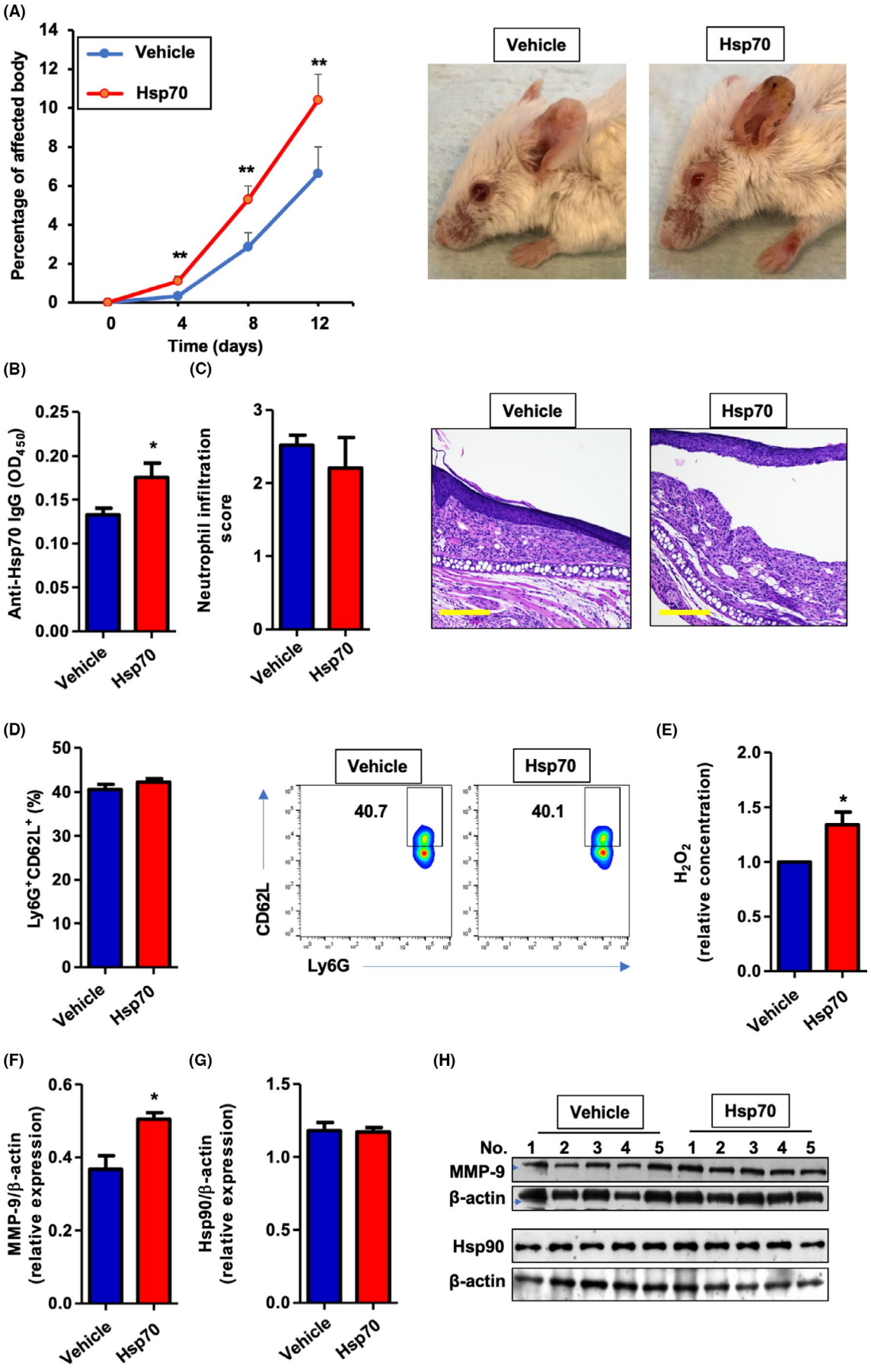
We further measured immunomodulatory effects of Hsp70 on selected pro-/anti-inflammatory cytokines of anti-CD3/28-stimulated T cells in human naïve PBMC cultures. While Hsp70 had no significant effects on IL-10 and IL-17A secretion, IL-6 and IL-8 secretion was significantly increased by this chaperone in a dose-dependent fashion (Figure 4).

4 | DISCUSSION

In this study, using different experimental approaches with human and murine material, we could show that extracellular Hsp70 might be involved in the pathophysiology of EBA. We found that the content of circulating Hsp70 was significantly elevated in mice with experimental EBA compared to naive mice. In this well-established anti-COL7 antibody transfer model, anti-COL7 antibodies require neutrophils which directly cause skin injury by MMP- and ROS-mediated degradation of cutaneous basement membrane zone components.[12] In fact, Hsp70-treated mice showed a more intense clinical disease severity of EBA compared to the control group which additionally demonstrated increased levels of both cutaneous MMP-9 and plasma hydrogen peroxide. The latter finding was confirmed in an independent ROS release assay using EBA-specific immune complexes combined with recombinant Hsp70. It seems that the increase of disease activity in the Hsp70-treated EBA mice was independent of Hsp90 expression since its cutaneous levels were unchanged compared to vehicle-treated EBA mice. Finally, further cell culture experiments revealed that extracellular Hsp70 stimulated the secretion of the T cell–derived IL-6 and IL-8. Both CD4 T cells and neutrophils are involved in autoantibody production in EBA,[8,9] and these cells are known to be activated by IL-6 and IL-8, respectively.[29]

The results of our study are somewhat opposite to the clinical and immunological outcomes we recently observed in a similar experimental setting with Hsp70 immunization (ie silencing vs. enhancing the pro-inflammatory immune response), but in a different mouse model of skin inflammation (ie psoriasis).[22] This can potentially be explained by the assumption that Hsp70 plays ambiguous role outside the cell that may depend on the type of immune cells

FIGURE 2 Hsp70 treatment leads to a higher disease severity in mice with EBA. (A) Naive BALB/c mice were injected intraperitoneally with recombinant Hsp70 or vehicle (PBS) fourteen days prior to the first of five subcutaneous anti-COL7 IgG injections (Days 0, 2, 4, 6 and 8). Clinical scores were higher in Hsp70- compared with PBS-treated animals. Representative clinical pictures of vehicle- and Hsp70-injected mice at the end of the observation period (Day 12) are presented (right). (B) Anti-Hsp70 IgG serum levels were significantly elevated in Hsp70- compared to vehicle-treated mice at the end of the observation period (Day 12). (C) Dermal neutrophil infiltration in H&E-stained tissue sections revealed no significant differences between vehicle- and Hsp70-injected mice at the end of the observation period (Day 12). Scale bars = 200 μm. (D) Flow cytometric analysis showed that Hsp70 treatment had no effect on peripheral blood neutrophil activity compared to the vehicle-treated group, as indicated by an unchanged expression of CD62L on Ly6G-positive cells at the end of the observation period (Day 12). (E) Fluorescent assay-based plasma hydrogen peroxide (H_2O_2) content was significantly elevated in Hsp70-treated mice when compared to vehicle-treated animals at the end of the observation period (Day 12). (F-H) Skin content of matrix metalloproteinase 9 (MMP-9) and Hsp90 in vehicle- and Hsp70-treated mice at the end of the observation period (Day 12) analysed in ear skin lysates by Western blotting and expressed relative to the β-actin level. Data are presented as mean (±SEM) of 5 mice per group $^{*}p < 0.05$, $^{**}p < 0.01$


(A)
Percentage of affected body
Vehicle
Hsp70
Time (days)
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(B)
Anti-Hsp70 IgG (OD450)
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Hsp70
(C)
Neutrophil infiltration score
Vehicle
Hsp70
(D)
Ly6G+CD62L+ (%)
Vehicle
Hsp70
CD62L
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40.7
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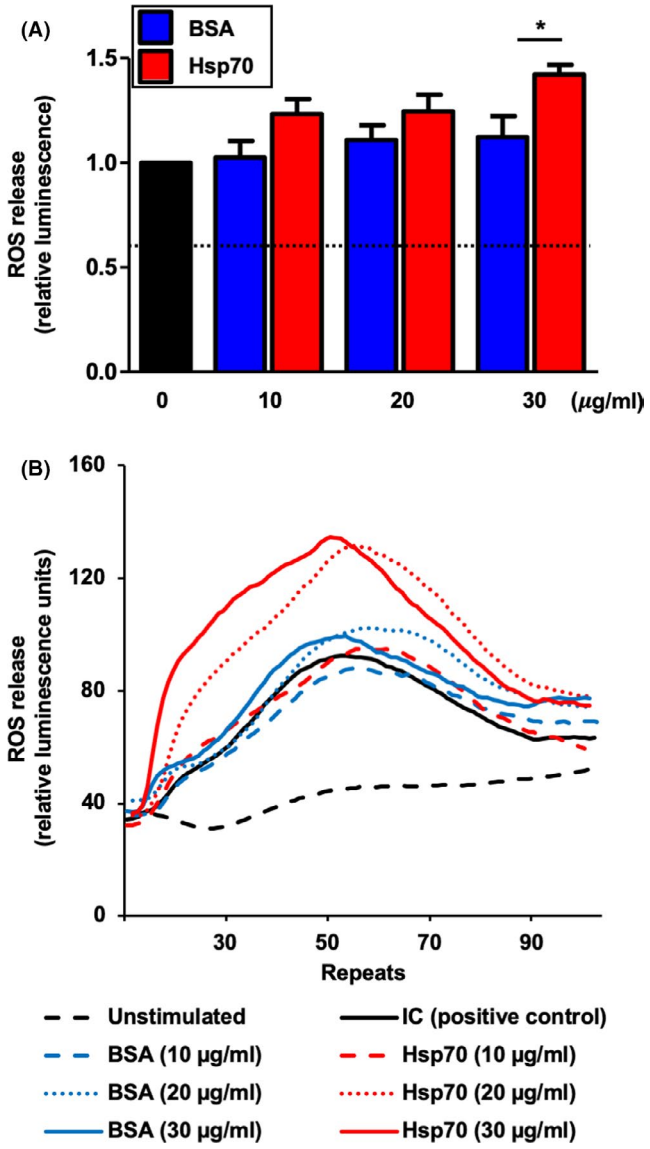

FIGURE 3 Hsp70 promotes the release of reactive oxygen species from EBA-specific immune complex-stimulated human neutrophils. (A) *Ex vivo* extracellular reactive oxygen species (ROS) release (normalized) from human PMNs stimulated by EBA-specific IC (anti-COL7 IgG +COL7) was analysed by luminol-enhanced chemiluminescence. ROS release was significantly triggered by recombinant Hsp70 when compared to bovine serum albumin (BSA)-treated control cells in a dose-dependent fashion. Dotted lines represent cells unstimulated by IC. Data are presented as mean (±SEM) of 3–4 healthy blood donors of three independent experiments. (B) Corresponding representative example of the time course of ROS secretion. $^{*}p < 0.05$

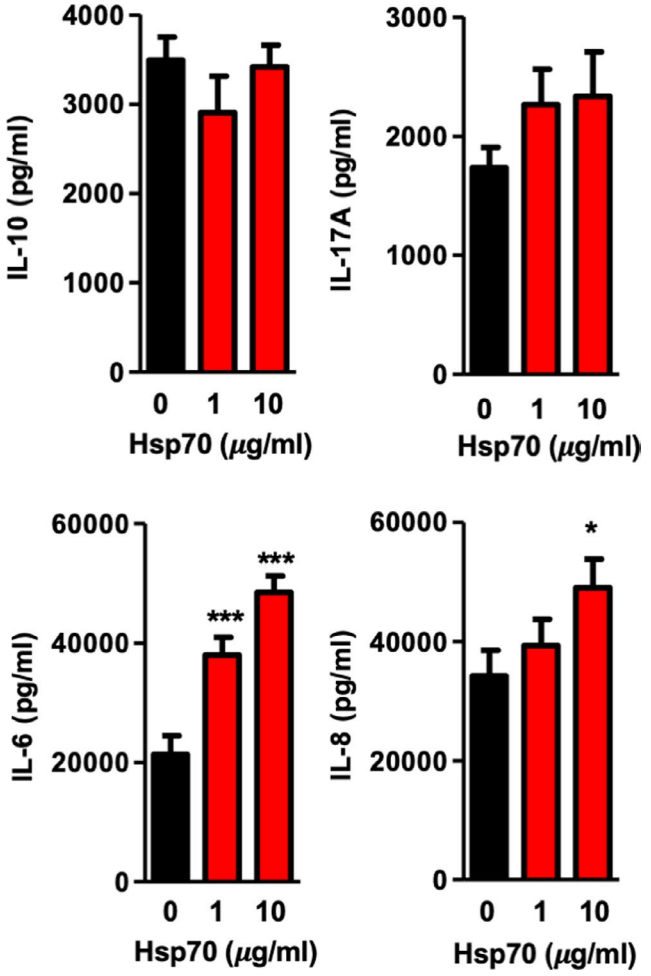

FIGURE 4 Hsp70 stimulates the secretion of T cell–derived pro-inflammatory cytokines. Evaluation of the effects of recombinant Hsp70 on IL-6, IL-8, IL-10 and IL-17A secretion into culture medium by anti-CD3/28-stimulated T cells in human PBMC cultures by ELISA. Data are presented as mean (±SEM) of 3–4 healthy blood donors of three independent experiments. $^{*}p < 0.05$, $^{***}p < 0.001$

and receptors that interact with this chaperone, the type of the disorder, and possibly other yet undefined reasons.[4] On the one hand, extracellular Hsp70 can promote regulatory T cells via the major histocompatibility complex class II molecule and toll-like receptor (TLR).[2] On the other hand, extracellular Hsp70 can interact with TLR-2/4 and activate the inflammatory signalling pathway (eg NF-κB) in innate immune cells including monocytes/macrophages, dendritic cells and granulocytes.[4] In our study, highly pure and LPS-free recombinant Hsp70 was used, arguing against non-specific pro-inflammatory properties of Hsp70 due to protein production-related LPS contamination as described previously.[4]

In contrast to the role of Hsp70 outside the cell, data on intracellular Hsp70 have been mostly consistent throughout studies, with evidence indicating that intracellular Hsp70 can inhibit NF-κB activity.[4] It is worth noting that pharmacological inhibition of Hsp90, which proofed effective in several models and assays of EBA, is associated with the activation of HSF-1 driving the intracellular expression of Hsp70.[2] In fact, intracellular Hsp70 has been generally regarded as a surrogate marker of Hsp90 blockade and found to be up-regulated in our previous EBA studies using Hsp90 inhibitors.[2,21]

In conclusion, this work extends knowledge about the role of Hsps in autoimmune bullous diseases, suggesting that, in addition to Hsp90, extracellular Hsp70 represents a pathophysiological factor and potential treatment target in EBA. Our results further highlight the dichotomous nature of extracellular Hsp70 in different inflammatory disorders.

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CONFLICT OF INTEREST

The authors state no conflict of interest.

AUTHOR CONTRIBUTIONS

ST involved in study design, conceptualization and supervision. ST, JM, KS, MB and KB involved in analysis. ST and MK involved in original draft preparation. ST, JM, KS, MB, DZ, RJL, KB and MK involved in data interpretation and critical revision of the manuscript.

DATA AVAILABILITY STATEMENT

The crude data that support the findings of this study are available from the corresponding author upon reasonable request.

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REFERENCES

1. Saibil H. Chaperone machines for protein folding, unfolding and disaggregation. *Nat Rev Mol Cell Biol*. 2013;14:630-642.
2. Tukaj S, Zillikens D, Kasperkiewicz M. Heat shock protein 90: a pathophysiological factor and novel treatment target in autoimmune bullous skin diseases. *Exp Dermatol*. 2015;24:567-571.
3. Tukaj S, Węgrzyn G. Anti-Hsp90 therapy in autoimmune and inflammatory diseases: a review of preclinical studies. *Cell Stress Chaperones*. 2016;21:213-218.
4. Tukaj S. Heat shock protein 70 as a double agent acting inside and outside the cell: insights into autoimmunity. *Int J Mol Sci*. 2020;21:5298.
5. Goletz S, Zillikens D, Schmidt E. Structural proteins of the dermal-epidermal junction targeted by autoantibodies in pemphigoid diseases. *Exp Dermatol*. 2017;26:1154-1162.
6. Koga H, Prost-Squarcioni C, Iwata H, Jonkman MF, Ludwig RJ, Bieber K. Epidermolysis Bullosa Acquisita: The 2019 Update. *Front Med (Lausanne)*. 2019;5:362.
7. Ludwig RJ, Vanhoorelbeke K, Leypoldt F, et al. Mechanisms of autoantibody-induced pathology. *Front Immunol*. 2017;8:603.
8. Iwata H, Bieber K, Tiburzy B, et al. B cells, dendritic cells, and macrophages are required to induce an autoreactive CD4 helper T cell response in experimental epidermolysis bullosa acquisita. *J Immunol*. 2013;191:2978-2988.
9. Samavedam UK, Iwata H, Müller S, et al. GM-CSF modulates autoantibody production and skin blistering in experimental epidermolysis bullosa acquisita. *J Immunol*. 2014;192:559-571.
10. Hundt JE, Iwata H, Pieper M, et al. Visualization of autoantibodies and neutrophils in vivo identifies novel checkpoints in autoantibody-induced tissue injury. *Sci Rep*. 2020;10:4509.
11. Kasperkiewicz M, Nimmerjahn F, Wende S, et al. Genetic identification and functional validation of FcγRIV as key molecule in autoantibody-induced tissue injury. *J Pathol*. 2012;228:8-19.
12. Kovacs B, Tillmann J, Freund LC, et al. Fcγ receptor IIB controls skin inflammation in an active model of epidermolysis bullosa acquisita. *Front Immunol*. 2020;10:3012.
13. Ludwig RJ. Signalling and targeted therapy of inflammatory cells in epidermolysis bullosa acquisita. *Exp Dermatol*. 2017;26:1179-1186.
14. Koga H, Kasprick A, López R, et al. Therapeutic effect of a novel phosphatidylinositol-3-kinase δ inhibitor in experimental epidermolysis bullosa acquisita. *Front Immunol*. 2018;9:1558.
15. Samavedam UK, Mitschker N, Kasprick A, et al. Whole-genome expression profiling in skin reveals SYK as a key regulator of inflammation in experimental epidermolysis bullosa acquisita. *Front Immunol*. 2018;9:249.
16. Hiroyasu S, Zeglinski MR, Zhao H, et al. Granzyme B inhibition reduces disease severity in autoimmune blistering diseases. *Nat Commun*. 2021;12:302.
17. Chiriac MT, Roesler J, Sindrilaru A, Scharffetter-Kochanek K, Zillikens D, Sitaru C. NADPH oxidase is required for neutrophil-dependent autoantibody-induced tissue damage. *J Pathol*. 2007;212:56-65.
18. Kasperkiewicz M, Müller R, Manz R, et al. Heat-shock protein 90 inhibition in autoimmunity to type VII collagen: evidence that nonmalignant plasma cells are not therapeutic targets. *Blood*. 2011;117:6135-6142.
19. Tukaj S, Zillikens D, Kasperkiewicz M. Inhibitory effects of heat shock protein 90 blockade on proinflammatory human Th1 and Th17 cell subpopulations. *J Inflamm (Lond)*. 2014;11:10.
20. Tukaj S, Tiburzy B, Manz R, et al. Immunomodulatory effects of heat shock protein 90 inhibition on humoral immune responses. *Exp Dermatol*. 2014;23:585-590.
21. Tukaj S, Bieber K, Kleszczyński K, et al. Topically applied Hsp90 blocker 17AAG inhibits autoantibody-mediated blister-inducing cutaneous inflammation. *J Invest Dermatol*. 2017;137:341-349.
22. Tukaj S, Mantej J, Sobala M, et al. Therapeutic implications of targeting heat shock protein 70 by immunization or antibodies in experimental skin inflammation. *Front Immunol*. 2021;12:614320.
23. Leineweber S, Schönig S, Seeger K. Insight into interactions of the von-Willebrand-factor-A-like domain 2 with the FNIII-like domain 9 of collagen VII by NMR and SPR. *FEBS Lett*. 2011;585:1748-1752.
24. Iwata H, Witte M, Samavedam UK, et al. Radiosensitive hematopoietic cells determine the extent of skin inflammation in experimental epidermolysis bullosa acquisita. *J Immunol*. 2015;195:1945-1954.
25. Kasprick A, Bieber K, Ludwig RJ. Drug Discovery for Pemphigoid Diseases. *Curr Protoc Pharmacol*. 2019;84:e55.
26. Tukaj S, Bieber K, Witte M, et al. Calcitriol treatment ameliorates inflammation and blistering in mouse models of epidermolysis bullosa acquisita. *J Invest Dermatol*. 2018;138:301-309.
27. Pokora W, Aksmann A, Baścik-Remisiewicz A, et al. Changes in nitric oxide/hydrogen peroxide content and cell cycle progression: study with synchronized cultures of green alga *Chlamydomonas reinhardtii*. *J Plant Physiol*. 2017;208:84-93.
28. Tukaj S, Mantej J, Sobala M, Potrykus K, Sitko K. Autologous extracellular Hsp70 exerts a dual role in rheumatoid arthritis. *Cell Stress Chaperones*. 2020;25:1105-1110.
29. Kany S, Vollrath JT, Relja B. Cytokines in Inflammatory Disease. *Int J Mol Sci*. 2019;20:6008.

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Oświadczenie

Oświadczam, że mój wkład w powstanie niniejszej pracy:

Mantej J, Polasik K, Piotrowska E, Tukaj S. (2018) Autoantibodies to heat shock proteins 60, 70, and 90 in patients with rheumatoid arthritis. *Cell Stress Chaperones.* 24:283-287. doi:10.1007/s12192-018-0951-9

polegał na optymalizacji i przeprowadzeniu eksperymentów obejmujących wykonanie testów immunoenzymatycznych (ELISA) w materiale ludzkim. Dodatkowo, mój udział w tworzeniu pracy polegał na współudziale w planowaniu, analizie i interpretacji wyników.

Jagoda Mantej

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Mantej J, Polasik K, Piotrowska E, Tukaj S. (2018) Autoantibodies to heat shock proteins 60, 70, and 90 in patients with rheumatoid arthritis. *Cell Stress Chaperones.* 24:283-287. doi:10.1007/s12192-018-0951-9

polegał na optymalizacji i przeprowadzeniu eksperymentów obejmujących wykonanie testów immunoenzymatycznych (ELISA) w materiale ludzkim.

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polegał na pomocy w redakcji manuskryptu oraz interpretacji wyników.

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polegał na kierowaniu projektem, tworzeniu koncepcji publikacji, zaplanowaniu doświadczeń, koordynacji pracy laboratoryjnej i udziale w analizie oraz interpretacji wyników. Dodatkowo, mój udział w tworzeniu pracy polegał na przygotowaniu ostatecznej wersji manuskryptu i odpowiedzi na pytania oraz uwagi recenzentów i redaktora czasopisma.

Stefan Tukaj

Mantej J*, Bednarek M*, Sitko K, Świętoń M, Tukaj S. (2021) Autoantibodies to heat shock protein 60, 70, and 90 are not altered in the anti-SARS-CoV-2 IgG-seropositive humans without or with mild symptoms. Cell Stress Chaperones Jul;26(4):735-740.

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polegał na optymalizacji i przeprowadzeniu eksperymentów obejmujących nadprodukcję i oczyszczanie rekombinowanego białka Hsp70, rekrutacji pacjentów oraz wykonanie testów immunoenzymatycznych (ELISA). Dodatkowo, mój udział w tworzeniu pracy polegał na udziale w planowaniu, analizie i interpretacji wyników, a także uzyskaniu finansowania na badania.

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Mantej J*, Bednarek M*, Sitko K, Świętoń M, Tukaj S. (2021) Autoantibodies to heat shock protein 60, 70, and 90 are not altered in the anti-SARS-CoV-2 IgG-seropositive humans without or with mild symptoms. *Cell Stress Chaperones.* 26:735-740. doi: 10.1007/s12192-021-01215-3

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polegał na rekrutacji pacjentów oraz optymalizacji i przeprowadzeniu większości eksperymentów obejmujących doświadczenia z użyciem ludzkich surowic, a także analizie i interpretacji uzyskanych wyników.

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polegał na rekrutacji pacjentów, wykonaniu testów immunoenzymatycznych (ELISA), analizie, interpretacji wyników, redakcji manuskryptu oraz udziale w wizualizacji uzyskanych wyników.

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polegał na pomocy w przeprowadzeniu eksperymentów obejmujących doświadczenia z użyciem surowic ludzkich.

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polegał na współkierowaniu projektem, tworzeniu koncepcji publikacji, zaplanowaniu doświadczeń, koordynacji pracy laboratoryjnej oraz udziale w analizie i interpretacji wyników. Dodatkowo, mój udział w tworzeniu pracy polegał na przygotowaniu ostatecznej wersji manuskryptu i odpowiedzi na pytania oraz uwagi recenzentów i redaktora czasopisma.

Tukaj S*, **Mantej J*,** Sobala M, Potrykus K, Tukaj Z, Zillikens D, Ludwig R.J, Bieber K, Kasperkiewicz M. (2021) Therapeutic Implications of Targeting Heat Shock Protein 70 by Immunization or Antibodies in Experimental Skin Inflammation.
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polegał na kierowaniu projektem, tworzeniu koncepcji publikacji, zaplanowaniu doświadczeń, koordynacji pracy laboratoryjnej i udziale w analizie oraz interpretacji wyników. Dodatkowo, mój udział w tworzeniu pracy polegał na przygotowaniu ostatecznej wersji manuskryptu i odpowiedzi na pytania oraz uwagi recenzentów i redaktora czasopisma.

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polegał na optymalizacji i przeprowadzeniu eksperymentów obejmujących nadprodukcję i oczyszczanie rekombinowanych białek Hsp70, wykonanie testów immunoenzymatycznych (ELISA), a także indukcji, terapii i ewaluacji łuszczycy w zwierzęcym modelu doświadczalnym, prowadzeniu hodowli ludzkich linii keratynocytów oraz przygotowaniu materiału do analiz cytometrycznych. Dodatkowo, mój udział w tworzeniu pracy polegał na udziale w planowaniu, analizie i interpretacji wyników.

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Polegał na zaprojektowaniu i optymalizacji metody oczyszczania rekombinowanych białek Hsp70.

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Tukaj S, Mantej J, Sitko K, Bednarek M, Zillikens D, Ludwig RJ, Bieber K, Kasperkiewicz M. Evidence for a role of extracellular heat shock protein 70 in epidermolysis bullosa acquisita. Exp Dermatol. 2021. doi: 10.1111/exd.14495. Epub ahead of print.

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Tukaj S, Mantej J, Sitko K, Bednarek M, Zillikens D, Ludwig RJ, Bieber K, Kasperkiewicz M. Evidence for a role of extracellular heat shock protein 70 in epidermolysis bullosa acquisita. Exp Dermatol. 2021. doi:
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Tukaj S, Mantej J, Sitko K, Bednarek M, Zillikens D, Ludwig RJ, Bieber K, Kasperkiewicz M. Evidence for a role of extracellular heat shock protein 70 in epidermolysis bullosa acquisita. Exp Dermatol. 2021. doi: 10.1111/exd.14495. Epub ahead of print.

I declare that my contribution to the above publications involved in the histological analysis.

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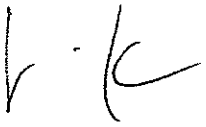
Sincerely,



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Tukaj S*, **Mantej J*,** Sitko K, Bednarek M, Zillikens D, Ludwig R J, Bieber K, Kasperkiewicz M. (2022) Evidence for a role of extracellular heat shock protein 70 in epidermolysis bullosa acquisita. Exp Dermatol. Apr;31(4):528-534.

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polegał na kierowaniu projektem, tworzeniu koncepcji publikacji, zaplanowaniu doświadczeń, koordynacji pracy laboratoryjnej i udziale w analizie oraz interpretacji wyników. Dodatkowo, mój udział w tworzeniu pracy polegał na przygotowaniu ostatecznej wersji manuskryptu i odpowiedzi na pytania oraz uwagi recenzentów i redaktora czasopisma.

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Tukaj S*, Mantej J*, Sitko K, Bednarek M, Zillikens D, Ludwig R J, Bieber K, Kasperkiewicz M. (2021) Evidence for a role of extracellular heat shock protein 70 in epidermolysis bullosa acquisita. *Exp Dermatol.* Nov 6. doi: 10.1111/exd.14495

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polegał na optymalizacji i przeprowadzeniu eksperymentów obejmujących nadprodukcję i oczyszczanie rekombinowanych białek Hsp70, wykonanie testów immunoenzymatycznych (ELISA), a także indukcji, terapii i ewaluacji mysiego modelu nabytego oddzielenia się naskórka (EBA), prowadzeniu hodowli ludzkich komórek jednojądrzastych krwi (PBMC), analizie dot. wydzielania reaktywnych form tlenu (ROS), immunodetekcji, i przygotowaniu materiału do analiz cytometrycznych. Dodatkowo, mój udział w tworzeniu pracy polegał na udziale w planowaniu, analizie i interpretacji wyników.

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Oświadczam, że mój wkład w powstanie niniejszej pracy:

Tukaj S*, Mantej J*, Sitko K, Bednarek M, Zillikens D, Ludwig R J, Bieber K, Kasperkiewicz M. (2021) Evidence for a role of extracellular heat shock protein 70 in epidermolysis bullosa acquisita. *Exp Dermatol.* Nov 6. doi: 10.1111/exd.14495

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polegał na uczestnictwie w części doświadczeń dotyczących hodowli ludzkich komórek jednojądrzastych krwi (PBMC), analizie dot. wydzielania reaktywnych form tlenu (ROS), i immunodetekcji, a także analizie, interpretacji uzyskanych wyników oraz przygotowaniu manuskryptu.

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I declare that my contribution to the above publications involved in the interpretation of data and a critical revision of the manuscripts.

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I declare that my contribution to the above publications involved in the histological analysis.

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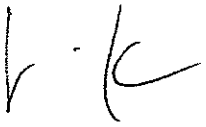
To whom it may concern,

I declare that my contribution to the above publications included the interpretation of data and help in manuscript preparation.

Sincerely,



Prof. Dr. Michael Kasperkiewicz

DOROBEK NAUKOWY

Artykuły w czasopismach i rozdziały w monografiach

Cykl publikacji opublikowanych w czasopiśmie z listy ministerialnej (A) wchodzący w skład dorobku będącego podstawą wszczęcia przewodu doktorskiego:

1. **Mantej J**, Polasik K, Piotrowska E, Tukaj S. (2019) Autoantibodies to heat shock proteins 60, 70, and 90 in patients with rheumatoid arthritis. Cell Stress Chaperones. Jan;24(1):283-287. (**IF = 3,667; MNiSzW = 70).**

2. **Mantej J***, Bednarek M*, Sitko K, Świętoń M, Tukaj S. (2021) Autoantibodies to heat shock protein 60, 70, and 90 are not altered in the anti-SARS-CoV-2 IgG-seropositive humans without or with mild symptoms. Cell Stress Chaperones. Jul;26(4):735-740. (**IF = 3,667; MNiSzW = 70**).

3. Tukaj S*, **Mantej J***, Sobala M, Potrykus K, Tukaj Z, Zillikens D, Ludwig RJ, Bieber K, Kasperkiewicz M. (2021) Therapeutic Implications of Targeting Heat Shock Protein 70 by Immunization or Antibodies in Experimental Skin Inflammation. Front Immunol. Feb 23;12:614320. (**IF = 7,561; MNiSzW = 140**).

4. Tukaj S*, **Mantej J***, Sitko K, Bednarek M, Zillikens D, Ludwig RJ, Bieber K, Kasperkiewicz M. (2022) Evidence for a role of extracellular heat shock protein 70 in epidermolysis bullosa acquisita. Exp Dermatol. Apr;31(4):528-534. (**IF = 3,960; MNiSzW = 100**).

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Pozostały dorobek:

5. Tukaj S, **Mantej J**, Sitko K, Zillikens D, Ludwig RJ, Bieber K, Kasperkiewicz M. (2022) Pathological Relevance of Anti-Hsp70 IgG Autoantibodies in Epidermolysis Bullosa Acquisita. Front. Immunol., 20 April. doi.org/10.3389/fimmu.2022.877958

6. Gaffke L, Szczudło Z, Podlacha M, Cyske Z, Rintz E, **Mantej J**, Krzelowska K, Węgrzyn G, Pierzynowska K. (2022) Impaired ion homeostasis as a possible associate factor in mucopolysaccharidosis pathogenesis: transcriptomic, cellular and animal studies. Metab Brain Dis. Feb;37(2):299-310.

7. Sitko K, Bednarek M, **Mantej J**, Trzeciak M, Tukaj S. (2021) Circulating heat shock protein 90 (Hsp90) and autoantibodies to Hsp90 are increased in patients with atopic dermatitis. Cell Stress Chaperones. Nov;26(6):1001-1007.

8. Pierzynowska K, Cyske Z, Gaffke L, Rintz E, **Mantej J**, Podlacha M, Wiśniewska K, Żabińska M, Sochocka M, Lorenc P, Bielańska P, Giecewicz I, Węgrzyn G. (2021) Potencjał autofagii indukowanej przez genisteinę w leczeniu chorób

neurodegeneracyjnych [Potential of genistein-induced autophagy in the treatment of neurodegenerative diseases]. Postepy Biochem. Apr 26;67(2):117-129.

9. Juchniewicz P, Piotrowska E, Kloska A, Podlacha M, **Mantej J**, Węgrzyn G, Tukaj S, Jakóbkiewicz-Banecka J. (2021) Dosage Compensation in Females with X-Linked Metabolic Disorders. Int J Mol Sci. Apr 26;22(9):4514.

10. Tukaj S, **Mantej J**, Sobala M, Potrykus K, Sitko K. (2020) Autologous extracellular Hsp70 exerts a dual role in rheumatoid arthritis. Cell Stress Chaperones Nov;25(6):1105-1110.

11. Pierzynowska K, Podlacha M, Gaffke L, Majkutewicz I, **Mantej J**, Węgrzyn A, Osiadły M, Myślińska D, Węgrzyn G. (2019) Autophagy-dependent mechanism of genistein-mediated elimination of behavioral and biochemical defects in the rat model of sporadic Alzheimer's disease. Neuropharmacology Apr;148:332-346.

12. Węgrzyn G, Pierzynowska K, Podlacha M, Brokowska J, Gaffke L, **Mantej J,** Cyske Z, Rintz E, Osiadły M, Bartkowski M, Puchalski M, Grabski M, Pierzynowski M, Pankanin D, Piotrowska E, Tukaj S. (2018) Molekularne mechanizmy działania genisteiny w świetle terapii chorób genetycznych i immunologicznych [Molecular mechanisms of genistein action in the light of therapies for genetic and immunological diseases]. Postepy Biochem. Dec 29;64(4):262-276.

13. Pierzynowska K, Gaffke L, Cyske Z, Puchalski M, Rintz E, Bartkowski M, Osiadły M, Pierzynowski M, **Mantej J,** Piotrowska E, Węgrzyn G. (2018) Autophagy stimulation as a promising approach in treatment of neurodegenerative diseases. Metab Brain Dis. Aug;33(4):989-1008.

14. Pierzynowska K, Gaffke L, Hać A, **Mantej J**, Niedziałek N, Brokowska J, Węgrzyn G. (2018) Correction of Huntington's Disease Phenotype by Genistein-Induced Autophagy in the Cellular Model. Neuromolecular Med. Mar;20(1):112-123.

Rozdziały w monografiach:

1. Rintz E, Gaffke L, Pierzynowska K, Podlacha M, **Mantej J,** Bednarek M, Cyske Z, Bałuch M, Bielańska P, Bilak A, Guzowski J, Wegrzyn G. (2020) Genistein – a natural antioxidant and its use in treatment of various diseases, 16, Bentham Science.