“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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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.

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