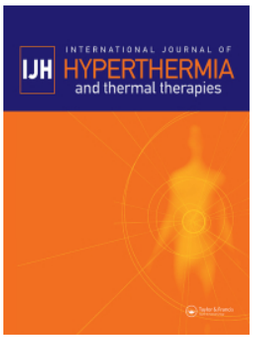

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REVIEW ARTICLE

Heat shock proteins can be targets of regulatory T cells for therapeutic intervention in rheumatoid arthritis

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Abstract

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterised by excessive immune responses resulting in inflammation of the joints. Although current therapies can be successful in dampening inflammation, a long-lived state of tolerance is seldom achieved. Therefore, novel therapies are needed that restore and maintain tolerance in patients with RA. Targeting regulatory T cells (Tregs) is a successful strategy to achieve tolerance, as was shown in studies performed in animal models and in human clinical trials. The antigen-specificity of Tregs is crucial for their effectiveness and allows for very specific targeting of these cells. However, which antigen is suitable for autoimmune diseases such as RA, for which the autoantigens are largely unknown? Heat shock proteins (HSPs) are ubiquitously expressed and can be up-regulated during inflammation. Additionally, HSPs, or HSP-derived peptides are immunogenic and can be recognised by a variety of immune cells, including Tregs. Therefore, this review highlights the potential of HSP-specific Tregs to control inflammatory immune responses. Targeting HSP-specific Tregs in RA can be achieved via the administration of HSPs (derived peptides), thereby controlling inflammatory responses. This makes HSPs attractive candidates for therapeutic intervention in chronic autoimmune diseases, with the ultimate goal of inducing long-lasting tolerance.

Keywords

Antigen-specific regulatory T cells, arthritis, heat shock protein, peptide therapy

History

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Introduction: immune tolerance by regulatory T cells

In order to maintain immunological self-tolerance under homeostasis and control autoreactive or exaggerated immune responses, regulatory T cells (Tregs) have evolved to keep unwanted responses in check [1]. Tregs are specialised suppressor cells that constitute 5–7% of the peripheral CD4+ T cell population. Loss of Treg function leads to multi-organ autoimmune disease [2], while Treg dysfunction can lead to autoimmune diseases such as diabetes and rheumatoid arthritis (RA) [3,4]. CD4+ Tregs are characterised by high expression of the IL-2 receptor alpha chain (CD25) [2] and most Tregs express the transcription factor forkheadbox P3 (FoxP3) [5]. There are several subsets of Tregs, which can be divided into natural Tregs (tTregs) and induced Tregs (pTregs) [6]. tTregs leave the thymus as mature Tregs and predominantly recognise self-antigens, while pTregs differentiated from CD25- precursors in the periphery in response to foreign antigens. It is thought that these subsets complement each other in antigen-specificity [7]: tTregs are specific for self-antigens [8] and circulate in the periphery, while pTregs are specific for foreign antigens (allergens, commensal microbiota, pathogens, alloantigens and altered self-antigens such as tumour antigens and inflammatory antigens) [9] and can be found mainly at mucosal sites.

Both tTregs and pTregs share a variety of mechanisms to suppress the function of target cells upon activation via their T cell receptor (TCR) [10]. Basically, there are four mechanisms of suppression [11]. The first mechanism of suppression is the production of anti-inflammatory cytokines, of which transforming growth factor beta (TGF-β) and interleukin (IL)-10 are the best defined [12]. Recently the inhibitory cytokine IL-35 was discovered and was added to this list [13]. The second mechanism of suppression is the cytolysis of target cells. Although several other immune cells were known to cause cytolysis of target cells, surprisingly this characteristic was also seen in Tregs. Tregs express Granzyme A and B and perforin, which can induce the lysis of CD4+ or CD8+ effector T cells [14]. The third mechanism of suppression is the metabolic disruption of target cells. For instance, via their high expression of CD25, Tregs consume vast amounts of IL-2, thereby depriving other cells of this important growth factor [15]. Finally, the fourth mechanism of suppression is the direct down-modulation of antigen-presenting cells (APC) via inhibitory receptors such as cytotoxic T lymphocyte antigen-4 (CTLA-4) that binds B7 [16], or lymphocyte activation gene 3 (LAG-3) that binds major histocompatibility complex (MHC)-class II [17].

In order to become suppressive, Tregs need to be activated via their TCR in an antigen-specific fashion. However, once

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activated, Tregs can suppress immune responses to other antigens also, which is called 'bystander suppression' [10]. Targeting antigen-specific Tregs with the appropriate antigen can therefore result in dampening of inflammation, even in conditions where the disease inducing antigens are unknown, such as RA. Moreover, Tregs can enable the induction of new suppressor cells by creating an anti-inflammatory environment. This 'infectious tolerance' can be of great benefit for long-term suppression [18], making antigen-specific Tregs ideal targets for therapy in RA.

When tolerance fails: rheumatoid arthritis

Although central tolerance aims to eliminate high affinity autoreactive cells, some potentially dangerous cells do escape deletion. The role of Tregs is to maintain homeostatic tolerance by controlling such cells, however, different circumstances can permit the breach of Treg mediated tolerance, as will be discussed below. In RA, autoreactive immune responses occur in which different immune cells infiltrate the joint, which ultimately leads to bone erosion. The role of Tregs in RA has been extensively studied and has focused on the presence and function of these cells.

One possible explanation for the impaired immunotolerance in RA could be the lack of sufficient Tregs numbers in patients. However, several studies have demonstrated that cell numbers of CD4 + CD25high Tregs in peripheral blood of active RA patients are comparable to healthy individuals [3,19–21]. Moreover, one study has shown that the number of Tregs in peripheral blood of RA patients is even increased as compared to healthy individuals [22]. Additionally, CD4 + CD25high cells have been shown in the synovial fluid (SF) of RA patients and express the proliferation marker Ki-67 [23]. The influx of Tregs into the synovium can be explained by the expression of chemokine receptors such as CXCR4, CCR4 and CCR8 [24] on Tregs, and that the ligands for CCR4 (CCL17 and CCL22) are highly expressed in the synovium [25]. Studies in animal models have shown that Tregs can migrate to the site of inflammation [26,27]. On the other hand, identification by expression of CD25 and Foxp3 is insufficient for the analysis of Tregs, since recently activated T cell effectors (Teff) also up-regulate these markers [28]. Therefore phenotypical analysis of Treg associated markers is not sufficient and the *in vitro* suppressive function of SF CD4 + CD25high cells needs to be tested.

Several studies have looked into the suppressive capacity of SF Tregs. On the one hand, Tregs isolated from the synovial fluid are suppressive *in vitro* [19,22,29–31]. On the other hand, Tregs isolated from the SF of juvenile idiopathic arthritis (JIA) patients are incapable of suppressing responder T cells from the SF [32,33], while in fact these Tregs were capable of suppressing responder T cells from peripheral blood. Others have shown that Tregs from RA patients are unable to suppress pro-inflammatory cytokine production [3,20], and that pro-inflammatory mediators such as tumour necrosis factor alpha (TNFα) can actually impair human Tregs [3,34]. Thus, the pro-inflammatory environment in the inflamed joints leads to Treg dysfunction [23,35]. Together with the unresponsiveness of effector T cells to Treg suppression, this leads to uncontrolled inflammation. Overcoming this imbalance is a major challenge for developing new therapies with long-lasting effect. Interestingly, for the induction of therapeutic tolerance, HSP peptide therapy shows promising results.

Restoring tolerance in RA: peptide therapy with heat shock proteins

Although current treatment for RA is successful in controlling inflammation, patients require life long treatment and can face severe side effects ranging from opportunistic infections to tumour development. Inducing long-lasting tolerance by targeting antigen-specific Tregs via the administration of suitable antigens is considered as the next generation in therapy [36]. For autoimmune diseases such as RA, the disease-inducing antigen(s) are unknown. Therefore, target antigens should be selected on other characteristics. First, those antigens that are immunogenic are suitable candidates, because they can activate antigen-specific Treg efficiently. For instance, it has been shown that heat shock protein (HSP)-derived peptides are recognised by T cells, and that bacterial HSP peptides that cross-react with self-HSP peptides especially induce stronger anti-inflammatory responses than non-conserved epitopes [37].

HSPs are major components of bacteria, and immune responses against HSPs can have a protective effect in infectious diseases [38]. For instance, protective HSP-specific T cell responses have been found after bacterial infections with *Listeria* [39], *Mycobacterium* [40] and *Wolbachia* [41]. However, in these cases, the HSP responses included both effector and regulatory responses (see Table I for Treg-associated responses found after infection). This indicates that both HSP-specific effector T cells and Tregs are induced or activated during infections. It has been shown that pTregs directed against pathogenic peptides can suppress pathogen-induced immunopathology [9], giving a possible explanation for the induction of HSP-specific Tregs during infection. Interestingly, although the HSP-specific Tregs induced after *Listeria* infection made rats more susceptible for infection [39], these cross-reactive antigen-specific T cells were capable of suppressing adjuvant arthritis in rats upon transfer [42]. This shows that T cells raised against bacterial HSPs that are cross-reactive with self- HSPs are capable of suppressing experimental arthritis. Therefore, bacterial HSP peptides that have human homologues are particularly suited for selection as potential candidate antigens. Several animal models of experimental arthritis that are induced by non-bacterial antigens (e.g. pristane, collagen type II, proteoglycan) show spontaneous responses against HSPs, indicating their immunogenicity and relevance, especially in inflammation [27,43,44]. Second, candidate antigens for peptide therapy should be expressed at the site of inflammation; in the case of RA, that would be the synovial tissue. The local expression of target antigens will allow for the activation of antigen-specific Treg only there where suppression of the immune system is needed, reducing non-specific immunosuppression. HSPs are ubiquitously expressed and can be found at sites of inflammation, for instance HSPs are expressed in inflamed synovial tissue of RA patients [45–47]. Third, next to the local expression, target antigens need to be specific for

Table I. Treg-associated makers expressed on T cells responsive to HSP. All markers depicted were identified on CD4+ HSP-responsive T cells that were functionally tested for suppressive capacity *in vitro* (e.g. suppression assay), or *in vivo* (adoptive transfer).

Molecule	Function	Hsp	Model (of disease)
CD25	IL-2 receptor alpha chain	Hsp40	JIA [75], none [76], lung cancer [68,77]
		Hsp60	AA [50,78], AD [79], atherosclerosis [69], DTH [58], filariasis [41], infection [58], none [66,80–82]
		Hsp70	PGIA [27], none [83]
		Hsp90	EAE [67], cancer [84]
CD27	TNF receptor	Hsp60	None [56]
		Hsp70	None [83]
CD28	Co-stimulatory molecule	Hsp60	AA [85]
CD30	TNF receptor	Hsp60	None [56]
CD45RA	On naive cells	Hsp60	None [80]
CD45RO	On memory cells	Hsp60	None [80]
CD69	Activation marker	Hsp40	JIA [75]
CD86	Co-stimulatory molecule	HS APC	AA [62]
CTLA-4	Co-stimulatory molecule	Hsp40	JIA [75]
		Hsp60	None [56]
		Hsp70	None [83]
FoxP3	Transcription factor	Hsp40	RA [49,74], none [76]
		Hsp60	AA [50,78], AD [79], atherosclerosis [69], none [56,66,80]
		Hsp70	PGIA [27]
		Hsp90	Cancer [84]
GITR	Co-stimulatory molecule	Hsp60	None [56]
IL-4	Th2 cytokine	HS APC	AA [62]
		Hsp70	AA [63], CIA [43]
IL-5	Th2 cytokine	Hsp70	CIA [43]
IL-10	Anti-inflammatory cytokine	HS APC	AA [62]
		Hsp40	JIA [75]
		Hsp60	AA [86], atherosclerosis [69], DTH [58], infection [58], none [56,80]
		Hsp70	AA [63], CIA [43], listeriosis [39], PGIA [27]
IL-13	Th2 cytokine	Hsp60	None [80]
LAG-3	Inhibitory molecule	Hsp70	PGIA [27]
Nrp-1	Inhibitory molecule	Hsp70	PGIA [27]
TGF-β	Anti-inflammatory cytokine	Hsp60	AA [85–87], DTH [58], infection [58]
		Hsp70	Listeriosis [39]

AA, adjuvant arthritis (rat); AD, atopic dermatitis (human); CIA, collagen-induced arthritis (mouse); CTLA-4, cytotoxic T-lymphocyte antigen 4; DTH, delayed-type hypersensitivity; EAE, experimental autoimmune encephalomyelitis; FoxP3, forkhead box P3; GITR, glucocorticoid-induced TNFR family-related gene; Hsp, Heat shock protein; HS APC, heat-shocked antigen presenting cells; IL, interleukin; JIA, juvenile idiopathic arthritis (human); LAG-3, lymphocyte-activation gene 3; None, studies performed without inducing disease; Nrp-1, Neuropilin-1; PGIA, proteoglycan-induced arthritis (mouse); RA, rheumatoid arthritis (human); TGF-β, transforming growth factor beta.

inflammation itself (i.e. up-regulated as a consequence of inflammation). In this manner Treg activation only occurs in the presence of inflammation. In this case, Treg activation will no longer occur once tolerance has been established, which will reduce continued immunosuppression when not needed. For HSP it is known that cells up-regulate HSP under conditions of stress [48], for which inflammation also qualifies, thereby enhancing their expression. In this case, stress (including inflammation)-inducible HSP is the best candidate antigen, because it is a 'bystander' antigen that is expressed at sites of inflammation and it is immunogenic in the sense that it is recognised by the immune system. Peptide therapy with bacterial HSP peptides can induce or expand cross-reactive HSP-specific Tregs that are activated locally at the site of inflammation [27]. It needs to be determined whether the resolution of the inflammation leads to Treg inactivation, leading to a tailor-made immunosuppression with potentially very few side-effects.

It is possible that peptide therapy as a mono-therapy in ongoing inflammation may need additional support in order to be effective. Therefore, combination therapy with low doses of anti-inflammatory drugs such as disease-modifying anti-rheumatic drugs (DMARDs) or prednisone together with HSP might be effective. This approach has already been tested and proved effective in a phase II clinical trial [49]. In addition, combination therapy with low-dose anti-TNFα together with Hsp40 was able to suppress adjuvant arthritis in rats [50], indicating that combining current therapies in RA with the administration of HSP can dampen inflammation.

In the case of infection during autoimmunity, the regulation by HSP-specific Tregs can be difficult to predict. As mentioned, pathogen-specific Tregs can be formed, but their suppressive role is only apparent at the end stage of infection, when the pathogen is neutralised and the immune system needs to be calmed down. Therefore, it is expected that infection will not suppress autoimmunity immediately, and it is not sure if it will suppress autoimmunity at all. Currently, there is no literature about HSP-specific Tregs induced during infection in an autoimmunity model.

Thus, HSP peptide therapy has the potential to activate T cells. However, for successful induction of tolerance these T cells should be able to effectively suppress other immune cells involved in the inflammatory process. The suppressive capacity of HSP-specific T cells has been shown and several Treg-associated markers have been identified on these suppressive HSP-specific Tregs, which will be discussed next.

Heat shock protein specific Tregs

HSP-responsive T cells were previously identified in animal models for RA [51,52], and in peripheral blood of humans [53,54]. Although many effector functions of HSP-specific T cells were identified, such as antigen-dependent proliferation and interferon gamma (IFN-γ) production [54,55], more recently also Treg-associated markers have been identified on HSP-responsive T cells. Apart from CD25 and FoxP3, which substantially contribute to the suppressive function of Tregs, other markers or characteristics have been identified to be associated with HSP-responsive Tregs after stimulation with HSP (Table I). Some of the markers or characteristics identified were actually needed for HSP-directed suppression. For instance, the lack of CD25 or FoxP3 on HSP-induced T cells resulted in disease development as compared to control CD4 + CD25− cells [27]. Additionally, CD30, glucocorticoid-induced TNFR-related protein (GITR), or LAG-3 was required by HSP-specific Tregs to be suppressive in the models tested [27,56]. In transfer experiments performed in a mouse model for experimental arthritis, LAG-3 was necessary for suppression as CD4 + CD25 + LAG-3 + Tregs were suppressive, while CD4 + CD25 + LAG-3− Tregs were not able to suppress disease development [27]. Additionally, as little as only 4,000 LAG-3+ cells were required to achieve disease suppression, indicating that HSP-specific Tregs can be suppressive at low numbers. The underlying mechanism could be fast proliferation of the transferred HSP-specific Tregs (in this case specific against Hsp70 peptide B29), due to the small clonal size which allows for the efficient generation of memory cells [57]. This was demonstrated by the increased presence of HSP-specific Tregs as compared to control Tregs, as well as an increased expression of Ki-67 on the transferred HSP-specific Tregs. Apart from cell surface molecules, secreted anti-inflammatory cytokines such as IL-4, IL-10 and TGF-β have been identified as effector molecules of HSP-specific Tregs. These cytokines were required for suppression, as was shown in knockout mice, or by blocking the function by neutralising antibodies [43,58], proving that some specific Treg characteristics are required for the suppressive function of HSP-specific Tregs.

Given the number of Treg markers expressed by HSP-responsive T cells, the mechanism by which HSP-specific Tregs can suppress inflammation seems rather broad. For instance, CD25 is involved in the metabolic disruption pathway [15], whereas CD30 [59], GITR [60] and LAG-3 [17] are used for cell–cell interactions which can lead to the inhibition of the target cell. The inhibitory cytokines IL-10 and TGF-β are secreted to suppress target cells, and it has been shown that IL-10 is crucial for Hsp70-mediated suppression of PGIA [61]. Also, the Th2 cytokine IL-4 has been found to be secreted by HSP-specific Tregs [43,62,63], which was responsible for the suppression of collagen-induced arthritis as a result of immune deviation from Th1 to Th2.

As mentioned previously, tTregs and pTregs are two separate subsets that rise from different sites (thymus or periphery) and recognise different sources of antigens (self or foreign). Which subset of Tregs would be HSP-specific? Since HSP epitopes can share great homology between bacteria and humans, we hypothesise that tTregs (mostly directed against self-antigens) might be able to cross-recognise bacterial HSP epitopes at mucosal sites, including the gut. Additionally, pTregs formed after encountering bacterial HSP in mucosal tissue can be cross-reactive to self- HSP due to the homology of the sequence. Commensals induce the up-regulation of Hsp70 in gut endothelial cells [64,65]. In this manner, the HSP from mucosal bacteria would allow for the maintenance of both HSP-specific tTregs and pTregs. There is actually one study that shows the induction of Tregs from naïve cells in response to Hsp60 derived from gut micro flora [66]. Additionally, we have unpublished data that shows that HSP-specific Tregs induced *in vivo* after immunisation with bacterial Hsp70 peptide B29 can suppress proteoglycan-induced arthritis in recipients after adoptive transfer. These studies indicate that induction of HSP-specific Tregs in response to bacterial HSP can occur.

Thus, HSP-responsive T cells can express or produce molecules that enable them to suppress inflammation and the source of Tregs could be the pTreg subset. Adoptive transfer therapy using these HSP-specific Tregs in animal models for inflammatory diseases has shown the feasibility of using HSP-specific Tregs for therapy, and could be a new therapeutic approach in the induction of tolerance for patients with RA.

Adoptive transfer therapy with HSP-specific Tregs

Various studies in animal models have shown the potential of Tregs to suppress inflammation, including RA [27,58,67–69]. Especially antigen-specific Tregs are suited for the control of inflammation since antigen-specific Tregs are functionally superior over the polyclonal Treg population [70,71]. Clinical trials with adoptive transfer therapy of autologous polyclonal Tregs are currently studied for graft-versus-host disease, type 1 diabetes and kidney transplantation rejection [72]. These involve the isolation and expansion of Tregs and re-infusion into the patient. Therapeutic approaches using Tregs to suppress inflammation currently involve the *in vivo* induction of polyclonal Tregs (for instance via the administration of anti-CD3 [73], or the induction of antigen-specific Tregs via peptide therapy [74]. Additionally, *ex vivo* expansion or induction of polyclonal Tregs which are then reintroduced into the patient is an option. The rationale behind this approach is to increase the total number of Tregs and thereby to tip the balance from inflammation to tolerance. Both protocols require the isolation of ultrapure T cells (e.g. CD4 + CD25– cells for induction of Tregs, or CD4 + CD25high cells for expansion of Tregs), and sufficient controls to determine Treg function (FoxP3 expression and *in vitro* suppressive capacity) under good manufacturing practice (GMP) conditions. Due to the lack of well defined and abundantly expressed autoantigens with specificity for RA, antigen-specific Tregs have not yet been introduced into the clinic. Nonetheless, antigen-specificity of the generated Tregs can be evaluated after stimulation with antigens through FACS analysis for Ki-67, neuropillin-1 (Nrp)-1 and IL-10 as previously described by us for adoptive transfer therapy for the mouse [27]. The outcome of current clinical trials with Treg transfers mentioned earlier will determine the future for

the implementation of antigen-specific Treg as a source for T cell therapy. Given the superior suppressive capacity of antigen-specific Tregs over polyclonal Tregs, and the existence of HSP-specific Tregs that can suppress chronic inflammation, the introduction of HSP-specific Treg through HSP vaccination or possibly hyperthermia may lead to innovative therapeutic strategies, including adoptive transfer of HSP-specific Treg.

Conclusion

Autoimmune diseases such as RA are characterised by uncontrolled inflammation directed against self tissues. Although current therapies can be successful in suppressing inflammation, RA patients can suffer severe side effects and new therapies are needed that induce long-term tolerance. In this respect, peptide therapy with HSP-derived peptides that specifically activate antigen-specific Tregs shows promising results. Identifying suitable epitopes that can be recognised by suppressive T cells is therefore needed, as well as expansion protocols for HSP-specific Tregs that can be used for adoptive transfer therapy.

Declaration of interest

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