T-cell regulation in juvenile idiopathic arthritis

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The present approach to control unwanted inflammatory immune responses in juvenile idiopathic arthritis (JIA) – and other autoimmune diseases – is with immunosuppressive drugs that operate by penalizing the whole immune system, even though only a small number of inflammatory cells are responsible for the damage. Besides being aspecific, these treatments fail to induce long-lasting disease remission. As a consequence, long-term treatment with immunosuppressive agents is necessary despite the hazards. Thus, the search for additive and/or alternate strategies needs to continue. Recently, much attention has focussed on the role of regulatory T cells (Tregs) for autoimmunity control. Recent evidence suggests that causal or predisposing factors to chronic inflammatory diseases may be found in a lack of counter-regulation by Tregs. Two types of Tregs, the naturally occurring $CD4^+CD25^+$ Tregs and Tregs with specificity for heat shock proteins (hsps) play a role in determining disease outcome in JIA. Hsps are stress proteins expressed selectively at sites of inflammation. Besides being recognized by Tregs, recent studies have shown that self-hsp60 is able to drive the *in vitro* differentiation from nonregulatory T cells to highly suppressive Tregs. Hence, hsp60 represents an important antigen that can be used potentially for the *in vivo* manipulation of Treg activity, clearing the way for new antigen- and site-specific therapies for JIA. Future studies need to be focussed on the molecular basis of the development of hsp60-induced Tregs and the question of how these cells can be manipulated *in vivo*.

Keywords: adjuvant arthritis, $CD4^+CD25^+$ regulatory cells, heat shock proteins, immunoregulation, immune therapy, juvenile idiopathic arthritis

future medicine

Immunoregulation

Considerable effort is required by the immune system to maintain the balance between activation and regulation. Too much activity may result in chronic inflammation or autoimmunity, while too much regulation may result in chronic infection or tumor growth. Multiple mechanisms exist that contribute to the regulation of immune responses; among them is a dominant form, mediated by so-called regulatory T cells (Tregs).

$CD4^+CD25^+$ Tregs are among the best characterized immunoregulatory subsets shown to actively suppress immune responses [1–3]. These spontaneously occurring T cells can actively and dominantly prevent both the activation and the effector function of autoreactive T cells that escape other mechanisms of tolerance. They are characterized by the constitutive expression of CD25 [4] and the transcription factor FoxP3 [5] and inhibit the activation of autoreactive T cells in an antigen-specific, cell contact-dependent manner [3,6,9]. They develop in the thymus or periphery [8,9] and represent a small but stable fraction (1–3%) of $CD4^+$ T cells in the periphery of every naïve host.

In addition to the role of naturally occurring $CD4^+CD25^+$ Tregs in maintaining the tolerance to self-antigens, there is accumulating evidence for distinct populations of Tregs induced in the periphery after encounters with pathogens and foreign antigens. A variety of *in vitro* and *in vivo* protocols have been used to generate these induced Tregs [10]. In general, they are generated in tolerogenic or anti-inflammatory contexts [11], produce high amounts of inhibitory cytokines, such as interleukin (IL)-10 and/or transforming growth factor (TGF)-β and exhibit suppressive activity that is dependent on these cytokines. These antigen-specific Tregs include Type 1 Tregs (TR1) [12] and T-helper cells (Th) 3 [13,14]. Recently published data showed that TR1 cells, such as $CD4^+CD25^+$ Tregs, are able to suppress autoimmune diseases after adoptive transfer *in vivo* [15].

Autoimmune disease: an immune deficiency?

The reduction in the number of Tregs predisposed to the development of autoimmune disease has been shown extensively in animal models by Sakaguchi and colleagues [16]. These authors showed that the reconstitution of nude mice, through the infusion of CD4 cells depleted in $CD4^+CD25^+$ T cells results in the development of organ-specific autoimmune disease, such as insulitis, gastritis and thyroiditis [4,16],

indicating a direct correlation between the defective number of Tregs and an autoimmune attack. Causal or predisposing factors to autoimmune disease may therefore be found in any genetic abnormality or environmental insult that can tip the balance between Tregs and self-reactive T cells toward the dominance of the latter. The specificity and intensity of the developing autoimmune disease may depend subsequently on the degree of deficiency or dysfunction of Tregs (or the balance between Tregs and self-reactive T cells) and on the host genes, including major histocompatibility complex (MHC) and non-MHC genes [1].

Juvenile idiopathic arthritis

Juvenile idiopathic arthritis (JIA) is the most common rheumatic disease of childhood [17]. Local inflammation in the joints results in joint destruction and, overall, an estimated 49% of affected children end up with severe functional limitation because of JIA. The different subtypes of JIA, based on the type of onset of disease, are currently classified according to the International League Against Rheumatism (ILAR) criteria [18] (Table 1). Although the histo-pathological abnormalities found in the three main subtypes seem similar, there is a striking difference in both the severity and outcome of the subtypes. In persistent oligoarticular (pers-OA)-JIA, the disease has a relatively benign course; the disease stays restricted in one to four joints, is often self-remitting and sometimes even self-limiting. In extended (ext)-OA-JIA the disease starts as oligoarticular, but develops into a polyarticular form after 6 months. Ext-OA-, polyarticular- and systemic-JIA are usually nonremitting and crippling diseases requiring aggressive immunosuppressive therapy. Owing to the self-limiting and -remitting character of the inflammatory process, pers-OA-JIA has a unique place among all human autoimmune diseases. The self-limitation of an autoimmune process is often seen in experimental animal models of autoimmune diseases but hardly ever in humans and suggests the active involvement of immunoregulatory processes, such as Tregs.

Current treatment for JIA

The treatment of JIA until now has been mainly focussed on the nonspecific suppression of inflammation by penalizing the whole immune system, even though only a small number of autoreactive T cells (and B cells and macrophages) are responsible for the tissue damage. Although therapeutics, such as corticosteroids, methotrexate and anti-tumor necrosis factor (TNF)-α blockers, have proven to be effective in most patients [19,20], a small group of patients is resistant even to combinations of them. Furthermore, each of these drugs has to be administered for a prolonged time since stopping them will result in full recurrence of the disease. As a consequence, long-term treatment with immunosuppressive agents is necessary, which not only increases the cost of the treatment but also harbors considerable risk for long-term side effects [21,22]. Thus, the need for additive and/or alternate strategies is growing. Ideally, such alternative treatment should, besides being safe and inexpensive, specifically modulate those

Table 1. ILAR classification criteria for juvenile idiopathic arthritis (Durban, South Africa, 1997).

Characteristic	JIA (ILAR)	Clinical features as defined by ILAR classification
Age at onset	< 16 years	
Minimum duration of arthritis	6 weeks	
Subtypes	Oligoarthritis Persistent Extended	Arthritis of 1–4 joints during the first 6 months Affects ≤ 4 joints throughout the disease course Affects ≥ 4 joints after the first 6 months
	Polyarthritis RF negative RF positive	Affects ≥ 5 joints in the first 6 months of disease Test for RF is negative Test for RF is positive on two occasions, at least two months apart
	Systemic arthritis	Arthritis with/preceded by daily fever for at least 2 weeks duration and one/more of: evanescent non-fixed erythematous rash, generalized lymphadenopathy, hepato-spleno-megaly and serositis
	Enthesitis-related arthritis	Sacroiliac tenderness, inflammatory spinal pain and HLA-B27 positive
	Psoriatic arthritis	Dactylitis, nail abnormalities and positive family history

ILAR: International League of Associations for Rheumatology; HLA: Human leukocyte antigen; JIA: Juvenile idiopathic arthritis; RF: Rheumatoid factor.

cohorts of lymphocytes that are responsible leaving others free to handle infectious agents normally, or should enforce the regulatory mechanisms that are intended to keep the autoreactive lymphocytes silenced.

Identification of Tregs in JIA: naturally occurring $CD4^+CD25^+$ Tregs

The synovial lymphocyte infiltrate in JIA consists predominantly of Th1 type cells [23] and, like rheumatoid arthritis (RA), is therefore believed to be the result of a polarization towards a persistent proinflammatory Th1 response. Despite this strong Type 1 phenotype in JIA, variable immune components that favor downregulation of inflammation can be present, especially in early OA disease. In particular, the presence of a Th2 component early in the disease process of JIA, in terms of Type 2 cytokines (IL-4 and -10) and the expression of chemokine receptors (CCR4) [24], is suggested to function in an anti-inflammatory capacity and to correlate with a favorable prognosis [24–26].

In addition to these Th2 components, $CD4^+CD25^+$ Tregs play a role in the determination of the disease course in JIA. Ext-OA-JIA patients have significantly reduced peripheral blood $FoxP3^+CD4^+CD25^+$ Treg frequencies when compared with healthy controls, as well as significantly reduced peripheral blood and synovial fluid frequencies when compared with pers-OA JIA patients [27]. Thus, the number of $CD4^+CD25^+$ Tregs in peripheral blood and synovial fluid of JIA patients correlates with the clinical course of the disease, suggesting that these cells play a role in determining disease phenotype.

The above mentioned study, as well as other studies that try to identify $CD4^+CD25^+$ regulatory cells in inflamed tissue, was complicated by the fact that CD25 is not a reliable marker for identifying regulatory cells, since it is also expressed on activated effector cells. However, it has been shown that human $CD4^+CD25^+$ Tregs reside mainly in the $CD25^{bright}$ population, therefore this problem could be partly addressed by differentiating between $CD25^{bright}$- and $CD25^{intermediate}$-expressing $CD4^+$ T cells and determining the mRNA FoxP3 levels in both populations.

Interestingly however, Ruprecht and colleagues showed recently that CD27 can be used in conjunction with CD25 expression to discriminate Tregs in inflamed tissues that express high amounts of FoxP3 and are endowed with potent suppressive activity, from $FoxP3^-$ effector T cells devoid of suppressor activity [28]. Using CD27 for the identification of Tregs in the joints of JIA patients, Ruprecht confirmed the correlation between synovial fluid $CD4^+CD25^+$ Treg frequencies and clinical course of the disease. This was achieved by showing that patients with polyarticular JIA have a higher proportion of synovial fluid $CD27^-$ activated/effector cells and lower proportion of synovial fluid $CD27^+$ Tregs compared with patients with OA disease [28].

Identification of Tregs in JIA: human hsp60-specific Tregs

Evidence is now accumulating that, besides $CD4^+CD25^+$Tregs, T cells with specificity for heat-shock proteins (hsps) may serve an immunoregulatory function and play a role in the downregulation of pathogenic, inflammatory processes [29] (Box 1). Most evidence for a protective role for hsp60-specific T cells came from the experimental animal model adjuvant arthritis (AA). In this model, preimmunization with mycobacterial hsp60 protected animals from the development of arthritis. This regulation was mediated by Tregs cross reactive with the self-60 kDa hsp (hsp60) and capable of downregulating inflammation [31,32].

JIA shares many features with the experimental animal model AA. Besides a clinical and histopathological resemblance, evidence is growing that hsps also play a central role in the immunoregulation of JIA. Cells from the synovial lining of patients with JIA show an increased expression of endogenously produced hsp60 [33]. This endogenous hsp60 is a target for the immune system, as shown clearly by the detection of immunoglobulin (Ig) G antibodies and T-cell reactivity to both human and mycobacterial hsp60 in serum and synovial fluid from patients with JIA [34]. Interestingly, cross-sectional patient studies revealed that most prominent T-cell responses to self-hsp60 were

Box 1. Heat-shock proteins (hsps).

Ubiquitous and abundant proteins are essential for cellular viability [30] and are present in cells of almost all living organisms. They are induced when a cell undergoes various types of environmental stresses, such as heat, cold and oxygen deprivation. Hsps are highly conserved during evolution, which has resulted in extensive amino acid sequence identities between mammalian and microbial hsps.

found in patients with human leukocyte antigen (HLA) B27-negative OA JIA, the subtype of JIA with the best prognosis. In a subsequent prospective follow-up study in newly diagnosed patients with JIA, T-cell reactivity to self-hsp60 occurred very early in the course of OA-JIA and patients lacking early responsiveness to self-hsp60 developed a much more severe form of JIA with a polyarticular course. Phenotypic analysis of the T cells responding to self-hsp60 revealed a clear regulatory phenotype. After activation with human hsp60, T cells from patients with remitting JIA express CD25 and CD30 and are capable of producing regulatory cytokines such as IL-4, IL-10 and TGF-β [35,36]. Furthermore, recent experiments using a newly developed technique to capture HLA class II-restricted peptide-specific T cells [37,38] revealed the expression of FoxP3 mRNA in self-hsp60 epitope-specific T-cells isolated from JIA patients [39].

In addition, recent data related to the peripheral induction of Tregs in JIA show that the stimulation of human $CD4^+CD25^-$ T cells with human hsp60 or hsp60-derived peptides induces a clear population of $CD4^+CD25^{bright}$ T cells. Besides high levels of CD25, these newly formed $CD4^+CD25^+$ T cells showed high expression levels of the regulatory cell markers glucocorticoid-induced TNF receptor (GITR), cytotoxic T-lymphocyte-associated protein-4 (CTLA-4) and CD30 and high levels of FoxP3 mRNA expression (de Kleer and colleagues [Unpublished Data]).

The importance of knowing the antigen specificity of Tregs

The finding that, in JIA, the frequency of $CD4^+CD25^+$ Tregs and hsp60-specific Tregs correlates with the clinical course of the disease emphasizes earlier findings in animal models that the outcome of an immune response depends on the balance between Tregs and effector T cells. Future studies, therefore, need to be focussed on finding ways to enhance the local proliferation of Tregs without abrogating their regulatory capacity. Both $CD4^+CD25^+$ Tregs and antigen-specific Tregs (TR1 and Th3-type Tregs) require recognition of the antigen through their T-cell reactivity (TCR) to become activated and retain their suppressive activity. Subsequently, the Tregs are able to not only suppress responses to their own antigen but also to other antigens in the same tissue. This process is called linked suppression [40,41]. In several animal models, it has indeed now been shown that the induction of Tregs by autoantigens can suppress disease, even if the primary initiating autoantigens are unknown and if inflammation is progressive. Thus, the *in vitro* or *in vivo* establishment of Treg cell lines to predefined antigens presented at the site of inflammation might be useful in harnessing such linked suppression and cause a local, rather than systemic, control of inflammation. By contrast, a random and thus polyclonal stimulation of Tregs may result in unwanted suppression of immune responses, leading to the induction of tumors and chronic infections.

Not much is known regarding the antigen specificity of naturally occurring $CD4^+CD25^+$ Tregs. $CD4^+CD25^+$ Tregs have a polyclonal TCR repertoire and could conceivably recognize a wide spectrum of antigens [42]. It is believed generally that the repertoire of $CD4^+CD25^+$ Tregs is biased towards self-antigens. With the finding that $CD4^+$ T cells specific for human hsp60-derived peptides express CD25 and FoxP3 mRNA, human hsp60 is the first naturally occurring antigen identified that is recognized by $CD4^+CD25^+$ Tregs.

Autologous stem cell transplantation for JIA: an effective though a risky way to restore the balance

For a small group of treatment-resistant JIA patients (5–10% of children with the systemic and polyarticular onset forms) autologous stem cell transplantation (ASCT) has emerged as an alternate treatment option. ASCT induces complete, drug-free remission in as many as 50% of the treated JIA patients (maximum follow-up is 7 years) [43]. Recently, it became clear that the basis for this success lies in the fact that ASCT restores the regulatory network [39]. ASCT completely restored the frequency of $CD4^+CD25^+$ Tregs in the transplanted patients to levels comparable with healthy controls, and ASCT restored the regulatory capacity of hsp60-specific Tregs. While before ASCT, hsp60-peptide-specific T cells expressed a low mRNA IL-10:interferon-γ ratio and a low *GATA3*:*Tbet* ratio, after ASCT, both ratios were reversed. These data showed that ASCT is an effective way to restore the balance between regulation and (auto)immunity and confirmed the hypothesis that this balance is needed to gain tolerance. Although successful, there is one major drawback to this procedure: ASCT is an invasive procedure with a significant mortality risk of 10–15%. Thus,

ASCT is an important new treatment for a small group of severely ill, treatment-resistant JIA patients, but does not eliminate the need to find less invasive and more specific ways to restore the immune balance.

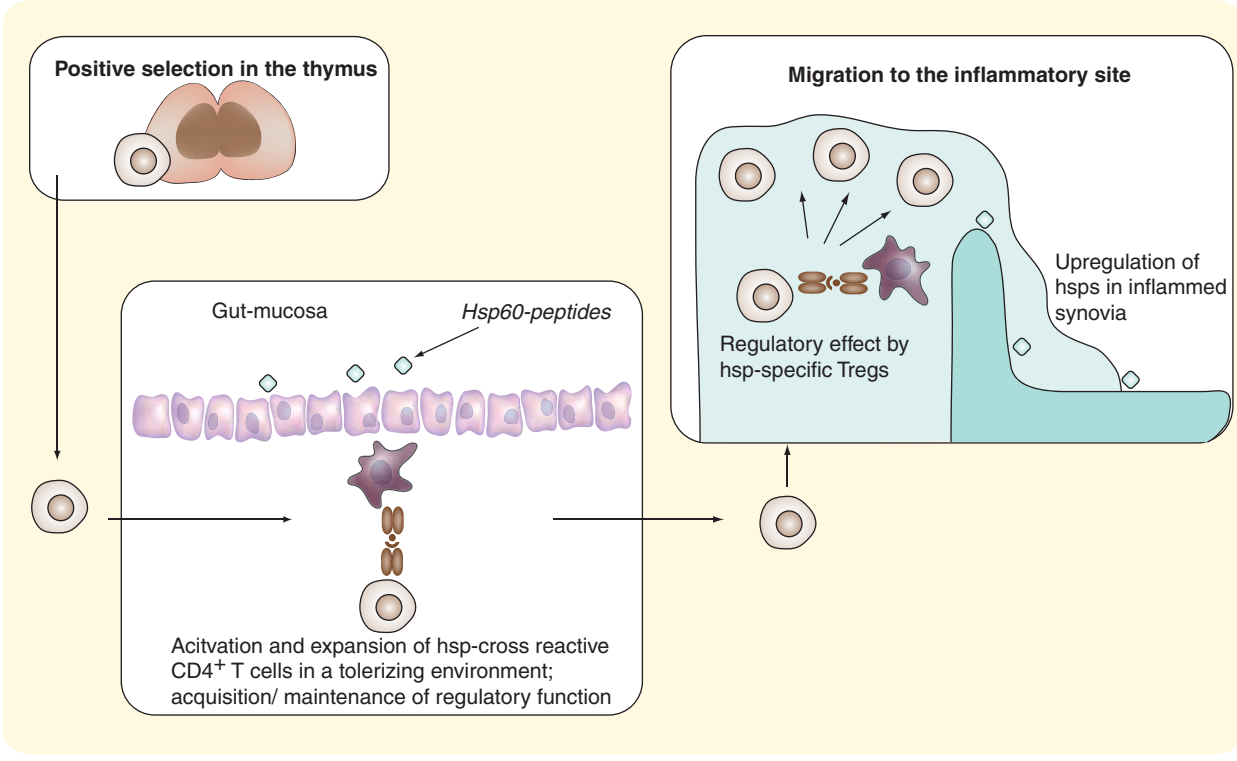
Conclusion

The quality and quantity of Tregs present in the peripheral blood and the synovial fluid of JIA patients clearly correlate with the clinical course of disease. The *in vivo* expansion of antigen- and site-specific Tregs seems therefore to be an extremely attractive therapeutic strategy. Hsp60 represents an antigen that can be used potentially for the *in vivo* manipulation of Tregs in JIA (Figure 1). It is a protein expressed selectively at the inflammatory site, recognized by Tregs and able to induce the expansion of Tregs. Epitopes of human hsp60 administered repeatedly via the mucosa or in combination with anti-TNF-α therapy seem promising ways to achieve this feat. Hopefully, in the near future this form of immunoregulation will complement and even substitute for the currently used conventional antigen nonspecific immunosuppression with all of its hazards.

Future perspective: hsp60 as a tool to control the balance

Hsp60 is expressed selectively at the inflammatory site and is recognized by Tregs and able to induce their expansion. Thus, hsp60 represents an important antigen that can potentially be used for the *in vivo* manipulation of the balance between regulatory and effector T-cell function in JIA. A major hurdle in the application of Tregs is the development of methods to manipulate and regulate the quality of TR1 and/or $CD4^+CD25^+$ Treg suppressor function *in vivo*. Although there is now a great interest in exploiting *in vitro* expanded Treg cell lines as therapeutic agents, this does not seem a very realistic approach. Besides being unattractive for commercial development, it would require a

Figure 1. Induction and expansion of self-hsp60 specific Tregs by mucosal administration of self-hsp60 derived peptides.



Hsp60-specific cells are positively selected in the thymus and circulate in the periphery. Mucosal administration of hsp60-derived peptides cause hsp60-specific cells to become activated and to expand. These hsp60-specific cells recognize selectively their antigen at the site of inflammation and infect neighboring T cells with suppressive activity (linked suppression), resulting in local downregulation of inflammation.
Hsp: Heat-shock protein; Treg: Regulatory T cell.

major commitment of transplant centers to cellular manipulations and to the storage of preexpanded T-cell lines. A more realistic, and presumably less risky, way to harness the therapeutic use of Tregs is by promoting their antigen-specific expansion *in vivo*. The following methods are currently under investigation.

In vivo *expansion of Tregs by the induction of mucosal tolerance*

Antigens elicit qualitatively distinct immune responses based on their portal of entry [44]. Systemically introduced antigens, whether by injection or injury, lead to local infiltration by inflammatory cells and specific immunoglobulin production. By contrast, antigens introduced at mucosal surfaces (such as the gastrointestinal and respiratory tracts) elicit tolerance to those antigens systemically. In AA [45,46], as well as in other animal models of autoimmune diseases [47–49], mucosal tolerance has proven to be effective in suppressing the disease. Mucosal tolerance, induced by repeated exposure to relatively low doses of antigen, is known to be mediated by the active suppression of immune responses by Tregs, such as $CD4^+CD25^+$ Tregs [50,51] and TR1/Th3 cells [52,53]. That the same mechanisms may apply in humans is suggested by the results of a Phase I clinical trial, in which patients with early RA were treated orally with a peptide of the hsp dnaJ (dnaJP1). This treatment induced an intriguing change from proinflammatory to Treg function [54].

As discussed, each of the described Treg subsets need to be activated in an antigen-specific manner, but are able to subsequently suppress immune responses in the immediate surrounding area in an antigen nonspecific manner (linked suppression). Expansion and/or

Executive summary

Immunoregulation

- Regulatory T cells (Tregs) play a dominant and active role in the maintenance of self-tolerance.
- The main subsets of Tregs are the naturally occurring $CD4^+CD25^+$ Tregs, characterized by the expression of CD25, CD27 and FoxP3 mRNA, and Type 1 Tregs (TR1)/Th3 cells, characterized by the production of the regulatory cytokines interleukin (IL)-10 and T cell growth factor (TGF)-β.

Autoimmune disease: an immunodeficiency

- A reduction in the number of Tregs predisposed the development of autoimmune disease in animal models, indicating a direct relation between the defective number of Tregs and autoimmune attack.
- Boosting the activity and/or induction of Tregs may offer a promising treatment strategy for autoimmune diseases.

Juvenile idiopathic arthritis

- Due to its self-limiting and remitting character, persistent oligoarticular juvenile idiopathic arthritis (pers-OA-JIA) takes a unique place among all human autoimmune diseases, suggesting the involvement of immunoregulatory processes.
- Treatment of JIA is focused on nonspecific suppression of inflammation and fails to induce a long-lasting remission of disease.

$CD4^+CD25^+$ regulatory T cells in juvenile idiopathic arthiritis

- In JIA, the frequency of $CD4^+CD25^+$ Tregs in peripheral blood and synovial fluid correlates with the clinical course of the disease.

Regulatory T cells specific for heat-shock proteins in arthritis models and JIA

- In adjuvant arthritis, preimmunization with heat-shock proteins (hsps) protects animals from arthritis. This protection results from the induction of self-hsp60 cross-reactive T cells capable of downregulating inflammation.
- In JIA, early responsiveness to self-hsp60 is associated with a remitting, oligoarticular course of the disease (pers-OA JIA).
- T cells from patients with pers-OA JIA responding to self-hsp60 express the regulatory markers CD25, CD30 and FoxP3 mRNA and are capable of producing the regulatory cytokines IL-4, IL-10,and TGF-β.
- Activation with human hsp60 or hsp60-derived peptides drives the differentiation of CD25 and FoxP3 mRNA-expressing regulatory cells from $CD4^+CD25^-$ nonregulatory T cells.
- Hsp60-induced $CD4^+CD25^+$ Tregs are able to suppress *in vitro* immune responses.

Future perspectives: hsp60 as a tool to control the immune balance

- Owing to their selective expression at sites of inflammation and their ability to induce the expansion of Tregs, hsps may be instrumental in restoring the immunological balance and may thus contribute to a long-lasting disease remission in JIA.
- Epitopes of human hsp60 administered repeatedly via the mucosa or in combination with anti-TNF-α seem promising ways to achieve this.

reactivation of hsp60-specific Tregs through the mucosal administration of peptides derived from hsp60, can be a potentially powerful therapeutic tool for restoring the immune balance and thus controlling autoimmune/inflammatory conditions. Potential peptides that can be used for this purpose were identified recently by using a novel computer algorithm for the analysis of potential pan-D related binding epitopes (provided by Sette A, Epimmune, La Jolla, CA, USA). Testing of the selected peptides revealed clear T-cell responses, as measured by T-cell proliferation and antigen-specific cytokine production, in the vast majority (60–90%) of patients with JIA [38].

Combining anti-TNF-α treatment & antigen specific therapy

The induction of mucosal tolerance with hsp-derived peptides has proven to be effective in the AA model; not only in the prevention but also in the treatment of ongoing arthritis [46]. The question is whether, in the more complex human situation, self-hsp60-specific T cells will be sufficient to downregulate ongoing disease, especially once massive inflammation and joint destruction has taken place. When this is not the case, a combination therapy of anti-TNF-α therapy followed by the mucosal administration of hsp-derived peptides might be the solution. While long-term side effects and limited efficacy in certain groups of patients set bounds to the use of anti-TNF-α therapy, it does induce impressive immunological changes. Besides the induction of a 'ceasefire', by a general suppression of proinflammatory pathways, an increase in the frequency of $CD4^+CD25^+$ Tregs was reported after treatment with infliximab [55]. It is indeed possible that hsp-specific Tregs, induced by mucosal administration during this 'ceasefire', have more regulatory impact than when induced during ongoing inflammation. The newly induced hsp60 Tregs might even be powerful enough to lead to sustained remission of the disease.

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