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

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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

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Immunization with Mycobacterium tuberculosis heat shock protein (hsp) 70 has been shown to protect rats from experimental arthritis. Previously, the protection-inducing capacity was shown to reside in the evolutionary conserved part of the molecule. Now we have studied the nature of the arthritis suppressive capacity of a distinct, antigenically unrelated protein, M. tuberculosis hsp70. Again, a conserved mycobacterial hsp70 sequence was found to be immunogenic and to induce T cells that cross-reacted with the rat homologue sequence. However, in this case parenteral immunization with the peptide containing the critical cross-reactive T cell epitope did not suppress disease. Upon analysis of cytokines produced by these peptide-specific T cells, high IL-10 production was found, as was the case with T cells responsive to whole hsp70 protein. Nasal administration of this peptide was found to lead to inhibition of subsequent adjuvant arthritis induction. The data presented here shows the intrinsic capacity of conserved bacterial hsp to trigger self-hsp cross-reactive T cells with the potential to down-regulate arthritis via IL-10. The Journal of Immunology, 2000, 164: 2711–2717.

Heat shock proteins (hsp)³ are highly immunogenic proteins, with an exceptional degree of evolutionary conservation. They have a function in intracellular protein folding, assembly and transport. Their expression is up-regulated under cellular stress such as occurs during inflammation. By their m.w. they are grouped into four major families: hsp90, hsp70, hsp60, and small hsps. Members of the hsp70 family occur in all known organisms and display the highest level of interspecies conservation. They can be subdivided into endoplasmic reticulum-resident, mitochondrial, and cytosolic proteins. Some of the cytosol-resident hsps of the hsp70 family (like hsp70–1 in the rat) belong to the group of hsps most highly induced by stress.

Their conservation (and the consequent potential for the occurrence of molecular mimicry) made self-hsps into obvious candidate targets for pathologic T cell responses. This was seemingly demonstrated when the Ag of an arthritis-inducing T cell clone in the adjuvant arthritis (AA) model was found to be hsp60 of Mycobacterium tuberculosis (Mt) (1). However, the T cell epitope that was recognized turned out to be from a nonconserved part of the molecule having a molecular mimicry relationship with a cartilage associated non-hsp Ag but not with the corresponding mammalian hsp-sequence (2).

By now, ample evidence has been obtained for a protective role of immunization with hsps in experimental autoimmune diseases (reviewed in Ref. 3). For AA, it has been documented that preimmunization with microbial proteins belonging to different hsp families protect from subsequent arthritis induction: mycobacterial hsp70 (Ref. 4, Prakken et al., submitted)⁵ Mt hsp60 (2, 5, 6), and Mt hsp10 (7). In streptococcal cell wall-induced arthritis, immunization with Mt hsp60 was protective (8). Most importantly, protection with Mt hsp60 was also found in arthritis models induced with nonmicrobial agents like avridine (9), pristane (10, 11), and collagen (12). Other experimental autoimmune diseases were also inhibited by immunization with Mt hsp60: experimental autoimmune encephalomyelitis (13) and diabetes in nonobese diabetic mice (14). However, the mechanism of protection by hsp in autoimmune diseases remained unclear. Recently, we have related the protective effect of Mt hsp60 in experimental arthritis to self-hsp cross-reactive T cells as protection was transferable by these cells (15, 16).

Preimmunization with Mt hsp70 also protected in experimental arthritis, and this was likewise T cell mediated.⁴ In the present study, we analyzed the T cell response to Mt hsp70 to unravel the underlying protective mechanism. T cells specific for conserved Mt hsp70 peptides were tested for cross-reactivity to peptides representing the homologous rat hsp70 sequence. A conserved, cross-reactive T cell epitope of hsp70 was found. T cells raised against Mt hsp70 and this epitope induced strong expression of the anti-inflammatory cytokine IL-10, whereas T cells specific for other conserved protein immunogens did not. The cross-reactive epitope conferred a significant reduction of arthritis. Taken together the

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*Abbreviations used in this paper: hsp, heat shock protein; Mt, Mycobacterium tuberculosis; AA, adjuvant arthritis; PLNC, primed lymphnode cells; DDA, dimethyl dioctadecyl ammonium bromide.

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data presented here suggest that the induction of IL-10 producing T cells with a regulatory phenotype is a characteristic feature of immunization with hsp70.

Materials and Methods

Animals

Male Lewis rats were obtained from the University of Limburg (Maasstricht, The Netherlands). Rats were 6–8 wk old at the start of experiments.

Ags and adjuvants

Heat-killed Mt (strain H37Ra) was obtained from Difco (Detroit, MI). Purified recombinant Mt hsp70 was donated from Gesellschaft für Bio-technologische Forschung (Braunschweig, Germany). The following Ags were obtained from Brunswick/Sigma (Amsterdam, The Netherlands): recombinant GSPDH from Bacillus stearothermophilus (catalog no. G5892), rat GST (G8386), and superoxide dismutase from Escherichia coli (S5639).

Peptides were synthesized as described previously (17). They were prepared by automated simultaneous multiple peptide synthesis. The simultaneous multiple peptide synthesis set-up was developed using a standard autosampler (Gilson 221, Middleton, WI). Briefly, standard F-moc chemistry with in situ PyBop/N-methylmorpholine activation of the amino acids in a 5-fold molar excess with respect to 2 μmol/peptide PAL-PEG-PS resin (Perceptive Biosystems, Cambridge, MA) was employed. Peptides were obtained as C-terminal amides after cleavage with 90–95% TFA/scavenger mixtures. Peptide purity was determined by HPLC.

IFA (Difco) and dimethyl dioctadecyl ammonium bromide (DDA; Eastman Kodak, Rochester, NY) (18) were used as adjuvants. DDA was prepared as a 20 mg/ml suspension in PBS and sonicated/heated to produce a 3% strength of the housekeeping gene (β-actin) were generated. The peptide representing the amino acids 2712–2726 with 100% homology to the Mt hsp70 was used for the generation of cDNA by RT-PCR. PCR was performed with rat cytokine-specific primers (20): IL-2 primers, 5′-GCCGACCCCA CTTCAAGCCCT and 3′-CCACCAAGTGTGCCGTGCTCA; IL-4 primers, 5′-ATGCACCGAGATGTGGTACCCG-3′; and TACCTTGTTGCTAGCG TGGGA; IL-10 primers, 5′-TGCCCTCTAGCTAAGAAGCT and 3′- AAACCTATGTGCTGCTGTA. The signal obtained was measured after gel-electrophoresis with ethidium-bromide using an imaging densitometer (model GS-700; Bio-Rad, Hercules, CA) and standardized to a control PCR product of a housekeeping gene (GPDHPD primers, 5′-AACCAGCTCAT GCCATCCAC and 3′-TCCACACCCGCTGGTCGTA). Data are expressed as percentage of signal-strength for the cytokines in relation to the signal-strength of the housekeeping gene (=100%).

Results

Definition of conserved T cell epitopes in Mt hsp70

For the definition of rat T cell epitopes in Mt hsp70, specific T cell lines were generated by s.c. footpad immunization of Lewis rats with recombinant mycobacterial hsp70 followed by in vitro restimulation of draining PLNC. T cell epitopes of the generated T cell lines were analyzed by peptide scans. Therefore, overlapping 15-mer peptides covering the Mt hsp70 protein (see Fig. 1 for the sequence) were generated. The peptide representing the amino acids 276–290 turned out to be a dominant nonconserved T cell epitope for the Lewis rat (Fig. 2). Subsequently, detailed attention was given to conserved regions as determined by the sequence comparison between rat and bacterial hsp70 sequences. In Fig. 1, a comparison of Mt hsp70 with the highly stress-inducible rat hsp70–1 (GenBank accession no. Q07439) is shown. Due to the high sequence homologies that exist between mammalian members of the hsp70 family, only minor differences are seen when Mt hsp70 is compared with other rat hsp70 molecules such as hsp70-2 (P14659), hsp70-3 (P55063), GR75 (P48721), GR78 (P06761), and hsp70c (P08109) (data not shown). The Mt hsp70 peptides representing conserved regions were tested as potentially cross-reactive T cell epitopes for the Mt hsp70-specific lines. Fig. 2 shows the data obtained for the conserved bacterial hsp70 sequences. Four peptides were found to contain T cell epitopes. These epitopes were found to be present in peptides 111–125, 131–145, 397–411, and 490–504.

Peptide p111 induces T cells cross-reactive to self-hsp70

For the peptides representing the four major antigenic conserved epitopes (peptides 111–125, 131–145, 397–411, and 490–504), T cell lines were generated by immunization with the peptides and in vitro peptide restimulations. The resulting T cell lines were analyzed for cross-reactivity to the corresponding self-peptides (see Table 1). Peptide p111 (positions 111–125 in Mt hsp70) induced T cells that cross-reacted with the self-hsp epitope (R111; corresponding to rat hsp70-1 aa 139–153), This T cell line proliferated in response to both p111 and R111 to a similar level (Fig. 3A). Most importantly, it was also possible to establish a T cell line by immunization with self-peptide R111, which cross-reacted with the microbial peptide (see Fig. 3B). T cells recognizing Mt hsp70 p111 were RT1.B1 restricted (Fig. 4), as responses were inhibited...
FIGURE 1. Sequence alignment of rat and Mt hsp70. Sequence comparison of rat hsp70-1 (GenBank accession no. Q07439, a stress-inducible member of the hsp70 family) with Mt hsp70 (accession no. S29698). Identical amino acids in both sequences are interconnected by lines, and conserved substitutions are interconnected by dots. Conserved regions are underlined. T cell epitopes of Mt hsp70-specific T cell lines are shaded. The epitope-inducing T cells cross-reactive with the mammalian homologue are shaded dark.

FIGURE 2. Conserved T cell epitopes of Mt hsp70. Proliferation of an Mt hsp70-specific T cell line in response to synthetic peptides representing conserved parts of the protein. The line was previously submitted to four cycles of in vitro restimulation with Mt hsp70. Background proliferation without added Ag was 169 cpm. Peptides were added at 20 μg/ml and Mt hsp70 and Mt at 10 μg/ml; Con A was added at 2.5 μg/ml. Additionally, the response to peptide 276–290 is shown, which was found to be the dominant non-conserved epitope of Mt hsp70 in the rat.
in the presence of B\textsuperscript{1}-specific Abs (OX6) and not D\textsuperscript{1}-specific Abs (OX17). The response of T cells to peptide R111 was similarly inhibited by anti-B\textsuperscript{1} Abs.

No disease inhibitory effect was induced by cross-reactive epitopes upon parenteral immunization

The induction of cross-reactive responses nominated peptide p111 as a candidate T cell epitope responsible for the in vivo protective effect of Mt hsp70 preimmunization in experimental arthritis. Therefore, p111 in PBS together with the adjuvant DDA was s.c. injected in the footpad of Lewis rats followed by induction of AA 10 days later. Disease was first observed at day 10 in the p111 group and at day 8 in the PBS-only control group. Rats immunized with p111 showed a mean maximum disease score of 11.8, whereas the PBS control rats had a mean maximum score of 12.4. Thus, except for a weak delay in onset, no significant influence on disease course by parenteral p111 immunization could be observed.

Mt hsp70 and a conserved Mt hsp70 peptide induce IL-10 production in responding T cell populations

Subsequently, the cytokine profile of the T cells responding to Mt hsp70 and epitopes thereof were analyzed. As control proteins other conserved bacterial proteins with mammalian homologues were chosen (G3PDH and superoxide dismutase) as well as a rat self-protein (GST). Earlier, these proteins had been demonstrated to be immunogenic but not to induce arthritis protection when used

| Table I. Sequence comparison of antigenic conserved Mt hsp70 peptides and their corresponding rat hsp70-1 peptides\textsuperscript{a} |
|---------------------------------|-----------------|-----------------|
| **Sequence** | **Proliferative Response** | **Proliferative Response** |
| | **Mt hsp70 Line** | **Peptide Line** |
| p111 | Mt ITDAVTTTPAYFMDA | + | + |
| p131 | Mt KDAGOIAGLNVLRI | + | + |
| p397 | Mt SETFTADNQPSVQ | + | + |
| p490\textsuperscript{b} | Mt IKDAAHAEEDPRR | + | + |

\textsuperscript{a} Recognition by a Mt hsp70-specific T cell line and by the relevant mycobacterial peptide-specific T cell line is indicated. The presumed RT1.B\textsuperscript{1} binding core (22) is underlined; identical amino acids are depicted as dashes. Sequences originate from GenBank database: Mt hsp70, accession no. S29698; rat hsp70-1, accession no. Q07439.

\textsuperscript{b} For p490, the comparison was done with the corresponding rat GR78 (P06761) peptide as this hsp70 family member shows the highest degree of homology in this particular region.

FIGURE 3. A highly conserved epitope of Mt hsp70 induces T cells, which strongly cross-react with the corresponding peptide of rat self-hsp70. A, T cells specific for Mt hsp70 p111 cross-react with the corresponding self-hsp epitope. A T cell line generated from a rat immunized with p111 is stimulated by the homologous rat peptide (Rat p111). Con A is used at 2.5 \( \mu \)g/ml, and Mt hsp70 is used at 10 \( \mu \)g/ml. Peptide concentration is 20 (●) and 5 \( \mu \)g/ml (□). Mt hsp70 peptide p490 was used as a nonstimulatory control. The background proliferation in the test was 332 cpm. SI, Stimulation index. B, T cells specific for Rat p111 cross-react with the corresponding Mt hsp70 p111 epitope. PLNC from a rat immunized with Rat p111 cross-react with the Mt hsp70 p111 peptide. The background proliferation was 6856 cpm.

FIGURE 4. Mt hsp70 p111 T cell line is RT1.B\textsuperscript{1} restricted. Recognition of p111 and R111 is RT1.B\textsuperscript{1} restricted. Abs specific for the rat MHC class II isotypes RT1.B\textsuperscript{1} (OX6) and D\textsuperscript{1} (OX17) were used to block p111- and R111-specific proliferation of a p111-specific T cell line, respectively. Peptides were added at 1 or 5 \( \mu \)g/ml (indicated between brackets), and Abs were added at 40 \( \mu \)g/ml. The background value in the test was 61 cpm.
for preimmunization. Cytokine mRNA levels detected by RT-PCR in PLNC from rats immunized with the different Ags are shown in Fig. 5. IL-2 was produced to a similar degree in response to the Ags, correlating with similar levels of proliferation found (not shown). Overall, very low expression of IL-4 was detectable. Analysis of IL-10 expression showed a divergent pattern: high levels of IL-10 for Mt hsp70 and low levels or absence of IL-10 mRNA for the other proteins (Fig. 5). The p111-specific T cell line also induced IL-10 mRNA upon stimulation with the peptide and, most importantly, also with the homologous self-peptide R111. Because we were interested specifically in the cytokine levels induced by antigenic stimulation of the T cells, we could not differentiate between mRNA derived from residual APC and from the T cells. However, in the absence of T cells or with T cells with an irrelevant specificity, no induction of IL-10 mRNA was found.

**Figure 5.** Mt hsp70-specific T cells induce IL-10. Ag-specific cytokine expression of PLNC 14 days after immunization with conserved microbial proteins. The cDNA from Ag-stimulated PLNC of conserved protein-immunized rats was analyzed by RT-PCR with rat-cytokine-specific primers. The following Ags were used: Mt hsp70, peptides p111 and R111, the bacterial proteins G3PDH and superoxide dismutase (SOD) and rat GST. Data are expressed as percentage of signal-strength related the signal of a housekeeping gene. A T cell line specific for the conserved epitope p111 of Mt Hsp70, having been restimulated in vitro with p111 for 4 cycles, was tested with p111 and R111 as Ags.

**Figure 6.** Nasal administration of conserved Mt hsp70 peptide p111 down-modulates AA. Intranasal treatment with Mt hsp70 p111 in PBS (▲, n = 5) suppresses the development of subsequently induced arthritis as compared with PBS-only controls (○, n = 5).

**Disease inhibitory effect of the cross-reactive epitope by nasal administration**

As the IL-10 data suggested the possibility that Mt hsp70 and, more particularly, its conserved p111 peptide generated regulatory T cells that induce IL-10, we chose to optimize the induction of regulatory T cell activity by administering the peptides intranasally. Rats were given p111 in PBS intranasally four times over a period of 2 wk before arthritis was induced. Control groups of rats received PBS alone or received a control peptide. P111-treated rats developed a significantly reduced disease severity compared with control rats. In the experiment shown (Fig. 6), the mean maximum score for p111-treated rats was 4.4 (SEM, 0.9) and 8.6 (SEM, 1.5) for the PBS control group (p < 0.05; Mann-Whitney U test). An immunogenic, nonconserved Mt hsp70 peptide (p276–290) did not suppress disease upon nasal administration (mean maximum score 7.4 (SEM, 1.6)) as we found before with other irrelevant peptides (21). In a second experiment, similar results were obtained (data not shown).

**Discussion**

In earlier studies, we have reproduced the arthritis protective effect of microbial hsp60 immunization using a selected conserved hsp60 sequence that induced a mammalian self-hsp cross-reactive T cell response (15, 16). In the present study, we have triggered self-hsp cross-reactive T cells using a conserved sequence of another hsp molecule (antigenically unrelated to Mt hsp60): mycobacterial hsp70. Furthermore, by exposing rats to this conserved sequence by nasal peptide administration, a significant reduction of arthritis was observed. Therefore, it is likely that the earlier-described arthritis protection by Mt hsp70 was provoked by a conserved T cell epitope inducing T cells cross-reactive with self-hsp70. Based on these combined observations, it can be concluded that the arthritis inhibitory effects of conserved bacterial hsps, as shown in various experimental models, may well be a consequence of the induction of self-reactive T cell responses directed to mammalian self-hsp homologues of bacterial hsps. The induction of IL-10 appears to be a prominent feature of these self-hsp cross-reactive T cells, strongly suggesting that microbial hsp protects against arthritis by inducing self-hsp-reactive T cells that produce cytokines such as IL-10 that have an inhibiting effect in inflammation.

In the present study, the immune response to Mt hsp70 in the Lewis rat was analyzed by screening T cell responses directed to a selected set of hsp70 peptides. First of all, the T cell response was dominated by a nonconserved T cell epitope involving a part of Mt hsp70 that has a low degree of sequence homology with mammalian hsp70 (aa 276–290). Within conserved parts of the molecule, multiple subdominant T cell epitopes were found, even in areas of the molecule where five or more amino acids of a potential MHC class II binding nonamer were identical with self-hsp70 homologues (Table I). However, it seems that T cell recognition was mainly determined by nonconserved residues within such conserved regions as most Mt hsp70 peptide-specific T cell lines failed to recognize their mammalian homologue peptides as measured by proliferation. Nonetheless, upon direct testing with the rat homologues, one T cell epitope of Mt hsp70 (p111) was identified where the specific T cells strongly cross-reacted with the self-peptide. In the case of p111, eight of nine amino acids from the RTI1.B1-binding core epitope (as predicted by a recently published RTI1.B1 binding motif (22)) were identical within the bacterial and rat sequence. The facts that bacterial and self-peptide were recognized to an identical extent (Fig. 3A) and in the context of the same restriction element (RTI1.B1, Fig. 4) and that in the reciprocal situation the T cell line generated against the self-hsp70 peptide was also stimulated by the mycobacterial peptide (Fig. 3B) indicate that the same T cells were indeed triggered by both peptides. Thus, self-hsp70-reactive T cells can be activated and expanded by immunization with Mt hsp70. As it has been demonstrated that the synthesis of hsp, such as hsp60, is up-regulated in
arthritic joints (23) and as this also was shown more recently for hsp70 (24), it is likely that MHC presentation of self-hsp peptides is also enhanced in arthritic joints. Therefore, migrating p111-specific T cells may encounter their Ag in joints (or the respective draining lymph node) of arthritic rats where they can recognize the rat homologue peptide on “stressed” APC or MHC II-positive activated T cells (which also have an increased expression of hsp70 (25)). After activation in the joint (or local lymph node), the self-hsp-reactive T cells could exert their predicted regulatory activity. This could well be reflected by the course of arthritis seen in hsp70 peptide pretreated rats (Fig. 6), where an initial joint inflammation started to develop (local up-regulation of hsps) and then rapidly subsided (attraction and effect of regulatory T cells).

Thus, up-regulated (stress response) self-hsp at sites of inflammation could attract and/or activate self-hsp-reactive T cells. It is possible that they display a regulatory phenotype influencing neighboring harmful autoreactive T cells, either directly or indirectly through cytokines. This would be in line with our findings because the T cells generated by immunization with M t hsp70 were strong inducers of IL-10 (Fig. 5), as was noted earlier for mycobacterial hsp70 in a Listeria infection model in rats (26). Most importantly, in our case, IL-10 was also induced by the self-reactive p111-specific T cells (see Fig. 5) when stimulated with the self-peptide. The most likely source of the IL-10 mRNA were the Ag-specific stimulated T cells. Formally, the APC present for Ag-specific stimulation could not be excluded as a source, but IL-10 mRNA was not induced by hsp70 Ags in the absence of T cells or with irrelevant T cells.

The mechanism through which (self) hsp70-specific T cells developed an intrinsic capacity for IL-10 production may consist of a combination of several factors. First, presentation of self-hsp Ags is strongly associated/increased with cellular stress, a phenomenon which in itself promotes the production of IL-10, as has been shown in various cell types (27). Second, the generation of T cells in the dominant presence of IL-10 leads to T cells of a regulatory, IL-10-producing phenotype (28) or at least of an IL-10-producing Th2 phenotype (29). Third, for cross-reactive epitopes of bacterial hsps, it seems significant that they are taken up and presented in the gut mucosa where IL-10 (and TGF-β) biased T cells are generated due to the local cytokine environment. (30). Therefore, stress-induced hsps may expand and propagate T cells that have already adopted a propensity to produce IL-10.

A study by Katsikis et al. (31) implicated a regulatory role for IL-10 in rheumatoid arthritis. Blocking of IL-10 in synovial membrane cultures from rheumatoid arthritis patients markedly increased the production of proinflammatory cytokines. Since then, a number of studies have demonstrated the beneficial effect of IL-10 in experimental collagen-induced arthritis (32, 33).

The actual regulatory mechanism of IL-10 could be inhibition of Ag presentation capacities of local APCs. This regulatory mechanism has been described for synovial macrophages (34). Alternatively (or perhaps additionally), the working mechanism of IL-10 in the suppression of arthritis might be related either to inhibition of IL-12 production by macrophages, thereby inhibiting a destructive Th1 immune response (35–37), or to direct induction of anergy in T cells present in the joint (38). Most importantly, IL-10 production has recently been described (28) as being the most prominent characteristic of a subset of regulatory T cells (Tr1 cells) generated in the presence of IL-10. In this study, similar to the data we have obtained for our hsp70-specific T cells (Fig. 5), the T cells did not display a regular Th2 phenotype as they did not produce IL-4.

IL-10 production as a mediator of arthritis suppression seems compatible with the disease inhibitory effect of intranasal treatment with p111 (Fig. 6) and the failed protection after parenteral immunization with the peptide. The mucosal environment may well influence developing immune responses by promoting the generation of IL-10-producing T cells (28). In the case of the peptide p111, this apparently leads to successful inhibition of disease (Fig. 6). It is possible that, in the case of the total hsp70 protein, the presence of multiple conserved T cell epitopes obviates the need for the more dominantly IL-10-promoting, intranasal routing to obtain an arthritis-reducing effect. In contrast, the fast loading of MHC class II molecules possible by the intranasal route as opposed to the slow release of peptide from adjuvant will influence the momentarily available dose and thus may influence T cell triggering. However, subsequent experiments, in vitro and in vivo, to inhibit the actions of IL10 using specific Abs will be required to clarify its role in the disease suppressive mechanism exerted by the hsp cross-reactive T cells.

Altogether, the present data have substantiated previous evidence that the arthritis suppressing quality of bacterial hsp immunization resides in conserved sequences of these molecules that have the potential of triggering self-hsp-reactive T cells. The regulatory phenotype (inherent or developing) of such T cells, characterized by IL-10 production, may be responsible for the mechanism through which such cells mediate bystander suppression targeted to sites of inflammation with up-regulated self-hsp. The triggering of such T cells in vivo through administration of conserved hsp peptides under IL-10-promoting conditions, such as nasal administration, may be a possible strategy for the development of novel immunotherapeutic interventions.

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References


