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

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Objective. Adjuvant arthritis can be induced in Lewis rats by immunization with *Mycobacterium tuberculosis* (Mt). The mycobacterial 65-kd heat-shock protein (Hsp65) is targeted by arthritogenic T cells. However, Hsp65 and the mycobacterial 71-kd heat-shock protein are also recognized by T cells that can downregulate adjuvant-induced arthritis (AIA). We have recently demonstrated that vaccination with human Hsp60 DNA inhibits AIA. The present study was undertaken to analyze the role of the T cell responses to self HSP molecules other than Hsp60 in the control of AIA.

Methods. Lewis rats were immunized with DNA vaccines coding for human Hsp70 or Hsp90 (Hsp70 plasmid [pHsp70] or pHsp90), and AIA was induced. The T cell response to Mt, Hsp60, Hsp70, and Hsp90 (proliferation and cytokine release) was studied, and the T cell response to Hsp60 was mapped with overlapping peptides.

Results. The Hsp70 or Hsp90 DNA vaccines shifted the arthritogenic T cell response from a Th1 to a Th2/3 phenotype and inhibited AIA. We detected immune crosstalk between Hsp70/90 and Hsp60: both the Hsp70 and Hsp90 DNA vaccines induced Hsp60-specific T cell responses. Similarly, DNA vaccination with Hsp60 induced Hsp70-specific T cell immunity. Epitope mapping studies revealed that Hsp60-specific T cells induced by pHsp70 vaccination reacted with known regulatory Hsp60 epitopes.

Conclusion. T cell immunity to Hsp70 and to Hsp90, like Hsp60-specific immunity, can modulate the arthritogenic response in AIA. In addition, our results suggest that the regulatory mechanisms induced by Hsp60, Hsp70, and Hsp90 are reinforced by an immune network that connects their reactivities.

Adjuvant arthritis can be induced in susceptible Lewis rats by immunization with *Mycobacterium tuberculosis* (Mt) (1). T cell reactivity against the mycobacterial 65-kd heat-shock protein (Hsp65) and some of its peptide epitopes is involved both in the pathogenesis and in the regulation of adjuvant-induced arthritis (AIA) (2–5). The regulatory properties of Hsp65 involve the induction of T cells that are cross-reactive with its mammalian counterpart, the 60-kd heat-shock protein (Hsp60) (6–9). Indeed, we and others have recently shown that recombinant vaccinia viruses (10) and DNA vaccines encoding the full-length human Hsp60 (Hsp60 plasmid [pHsp60]) (11) or some of its fragments (12) can boost Hsp60-specific regulatory immunity and inhibit AIA. Moreover, several AIA-regulatory peptides have been identified in Hsp60 (2,7,12,13). Thus, the T cell response to self Hsp60 epitopes can regulate AIA.

Vaccination with the mycobacterial 10-kd or 71-kd heat-shock proteins has also been shown to inhibit the development of AIA (14,15). Mycobacterial Hsp71 T cell epitopes that are cross-reactive with mammalian Hsp70 have been reported to modulate AIA (16,17), but little is known about the regulatory role of T cells that are reactive with self HSP molecules other than Hsp60 in AIA.

In this study, we used DNA vaccines to analyze the role of T cell responses to Hsp70 or Hsp90 in AIA. DNA constructs coding for Hsp70 or Hsp90 could inhibit AIA. We found that vaccination with pHsp70 or pHsp90 modified the immune response to antigens associated with AIA progression and, surprisingly, also influenced the immune response to Hsp60.

MATERIALS AND METHODS

Animals. Female Lewis rats were raised and maintained under pathogen-free conditions in the Animal Breeding Center of the Weizmann Institute of Science. One-month-old rats were used for DNA vaccination experiments. The experiments were performed under the supervision and guidelines of the Weizmann Institute of Science Animal Welfare Committee.

Antigens and adjuvants. Peptides were synthesized as previously described (11). The Hsp65 peptide Mt176–190 used in these studies is EESNTFGLQLELTEG (4), which contains the 180–188 epitope of Hsp65 (5). The panel of overlapping peptides spanning the whole Hsp60 sequence has been described previously (18). Purified recombinant Hsp65 was generously provided by Dr. Ruurd van der Zee (Institute of Infectious Diseases and Immunology, Utrecht, The Netherlands). Recombinant Hsp60 was prepared in our laboratory as described (19). Recombinant human Hsp70 was purchased from Sigma (Rehovot, Israel). Recombinant glutathione S-transferase (GST) and GST-Hsp90 were prepared in our laboratory as described (20). Mt strain H37Ra and Freund's incomplete adjuvant (IFA) were purchased from Difco (Detroit, MI). Tuberculin purified protein derivative (PPD) and mycobacterial Hsp71 were provided by the Statens Seruminstitut (Copenhagen, Denmark). Ovalbumin (OVA) and concanavalin A (Con A) were purchased from Sigma.

DNA plasmids. The vector containing the human gene *hsp60* (pHsp60) has been described (19). The construct encoding *Mycobacterium leprae* Hsp65 (pHsp65) was kindly provided by Dr. Douglas Lowrie (Medical Research Council, London, UK). Both vectors have been shown to be effective in inhibiting AIA (11,21).

The full-length cDNA of human *hsp70* (GenBank accession no. M11717) and *hsp90α* (GenBank accession no. NM_005348) genes were cloned into the pcDNA3 vector (Invitrogen, Leek, The Netherlands). Briefly, human *hsp70* cDNA in pHLTR-Hsp70 and *hsp90α* cDNA in pGEX-Hsp90 were amplified by polymerase chain reaction (PCR) using specific oligonucleotides containing restriction sites for the enzymes *Bam* HI or *Xba* I. The amplicons and the pcDNA3 vector were purified and digested with *Bam* HI and *Xba* I. The digested PCR products coding for Hsp70 or Hsp90 and the linearized pcDNA3 vector were ligated using a Rapid DNA Ligation Kit, according to the standard protocol recommended by the manufacturer (Roche Diagnostics, Mannheim, Germany). The ligated plasmids were transformed into *Escherichia coli*, and later sequenced to confirm correct insertion of the cDNA (data not shown).

Plasmid DNA was prepared in large scale and injected after pretreatment with cardiotoxin (Sigma), as previously described (11). Briefly, rats were vaccinated by injection into the quadriceps 3 times (on days −40, −26, and −12 relative to AIA induction) with 150 μg of pcDNA3, pHsp60, pHsp65, pHsp70, or pHsp90. Endotoxin levels were checked by *Limulus* amebocyte lysate assay and found always to be below acceptable levels for in vivo use (<0.02 endotoxin units/μg DNA). AIA was induced 12 days after the last injection of DNA. The empty vector pcDNA3 was used as a DNA vaccination control.

AIA induction and assessment. AIA was induced as described (11), by immunizing Lewis rats with 1 mg per rat of heat-killed Mt strain H37Ra suspended in IFA. Each experimental and control group contained at least 8 rats. The day of AIA induction was designated day 0, and disease severity was assessed by direct observation of all 4 limbs in each animal. A relative score of 0–4 was assigned to each limb, based on the degree of joint inflammation, redness, and deformity; thus, the maximum possible score for each individual animal was 16 (11). The mean ± SEM AIA score is shown for each experimental group. The person who scored the disease was blinded to the identity of the groups. Arthritis was also quantified by measuring hind limb diameter with a caliper. Measurements were obtained on the day of the induction of AIA and 26 days later (at the peak of AIA); the results are presented as the mean ± SEM of the difference between the 2 values for all the animals in each group. The experiments were repeated at least 3 times, and yielded similar results.

T cell proliferation. T cell proliferation assays were performed on day 26 after the induction of AIA, when the disease is at its peak, as previously described (11). Briefly, popliteal and inguinal lymph node cells (LNCs) were cultured in quadruplicate in 200-μl round-bottomed microtiter wells (Costar, Cambridge, MA) at 2×10^5 cells per well with or without antigen. The T cell mitogen Con A was used as a positive control for T cell proliferation. Cultures were incubated for 72 hours at 37°C in a humidified atmosphere of 5% CO_2. T cell responses were detected by the incorporation of methyl-^{3}H-thymidine (1 μCi/well; Amersham, Buckinghamshire, UK), which was added to the wells for the last 18 hours. The stimulation index (SI) was computed as the ratio of the mean counts per minute from antigen- or mitogen-containing wells to that from control wells cultured with medium alone. The results of T cell proliferation experiments are shown as the mean ± SEM SI.

Cytokine assays. Supernatants were collected after 72 hours of stimulation with each of the antigens tested. Rat interleukin-10 (IL-10) and interferon-γ (IFNγ) in culture supernatants were quantitated by enzyme-linked immunosorbent assay, using an OptEIA kit (PharMingen, San Diego, CA) as previously described (11). Rat transforming growth factor β1 (TGFβ1) was quantified using the TGFβ1 E_{max} Immuno-Assay System, according to the instructions of the manufacturer (Promega, Madison, WI). Cytokine levels are expressed as pg/ml based on calibration curves constructed using recombinant cytokines as standards. The lower limit of detection for the experiments described in this report was 15 pg/ml for TGFβ1, IL-10, and IFNγ.

Statistical analysis. The InStat 2.01 program (Graph-Pad Software, San Diego, CA) was used for statistical analysis. Student's *t*-test and the Mann-Whitney U test were performed to identify significant differences between experimental groups.
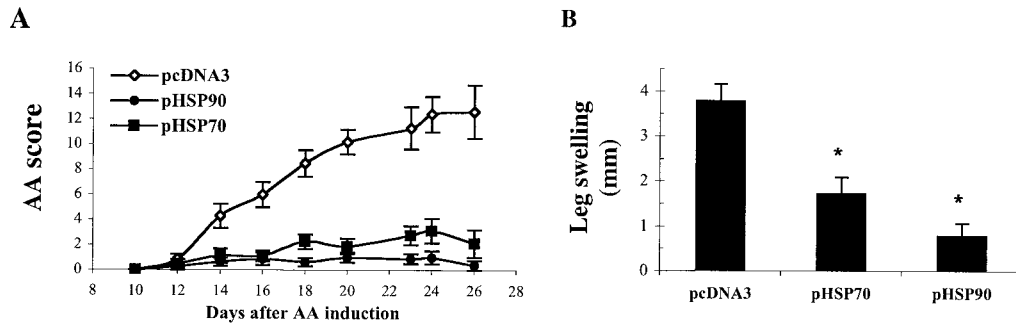
Figure 1. Inhibition of adjuvant-induced arthritis (AA) by vaccination with 70-kd heat-shock protein plasmid (pHsp70) and pHsp90. **A,** Time course of adjuvant-induced arthritis. Lewis rats were vaccinated with pHsp70, pHsp90, or pcDNA3, adjuvant arthritis was induced, and arthritis scores were assessed. Statistically significant differences ($P < 0.05$) were obtained at every determination, from day 14 to day 26, when rats vaccinated with pHsp70 or pHsp90 were compared with pcDNA3-vaccinated rats. **B,** Leg swelling measured on day 26 after adjuvant arthritis induction. Three independent experiments yielded similar results. ∗ = $P < 0.005$ versus pcDNA3-vaccinated rats. Values are the mean ± SEM.

RESULTS

Inhibition of AIA by vaccination with Hsp70 or Hsp90 DNA. The pHsp70 and pHsp90 constructs were functional in an in vitro transcription/translation assay (results not shown). Moreover, immunization of Lewis rats with pHsp70 or pHsp90 led to induction of Hsp70- or Hsp90-specific antibodies, respectively (results not shown), indicating that the constructs were also func-
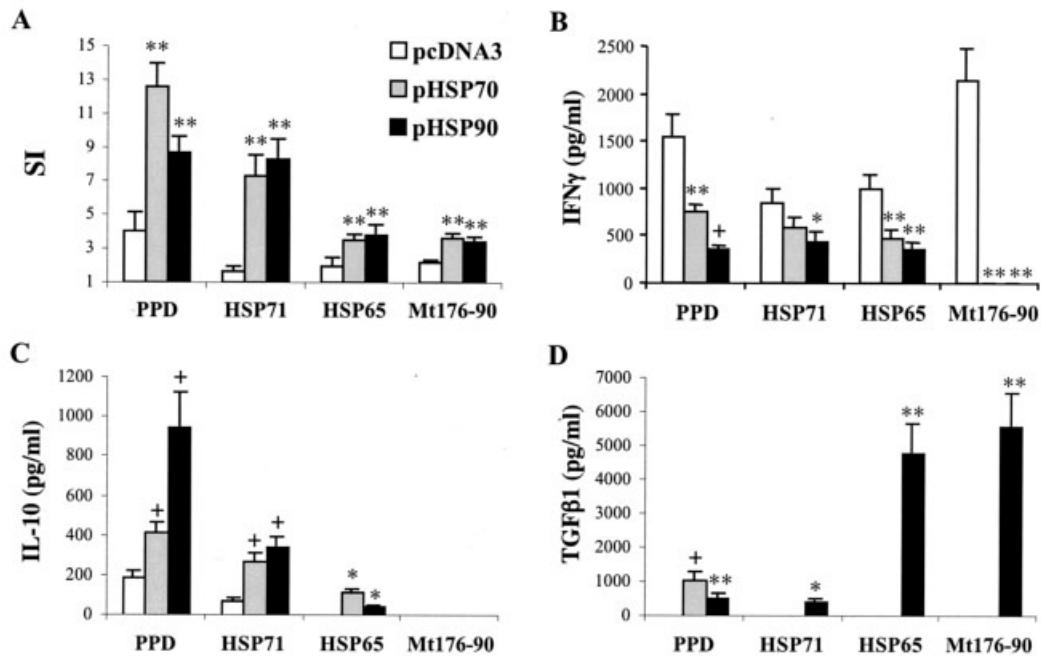

Figure 2. Arthritogenic responses after DNA vaccination. Lewis rats were vaccinated with 70-kd heat-shock protein plasmid (pHsp70), pHsp90, or pcDNA3, and adjuvant arthritis was induced. Twenty-six days later, lymph node cells were prepared, stimulated with purified protein derivative (PPD), Hsp71, Hsp65, or *Mycobacterium tuberculosis* 176–190 (Mt176–190), and antigen-induced proliferation (**A**) and cytokine release (**B–D**) were studied. Three independent experiments yielded similar results. Values are the mean and SEM. ∗ = $P < 0.05$; ∗∗ = $P < 0.01$; + = $P < 0.005$, versus the pcDNA3-treated group. SI = stimulation index; IFNγ = interferon-γ; IL-10 = interleukin-10; TGFβ1 = transforming growth factor β1.
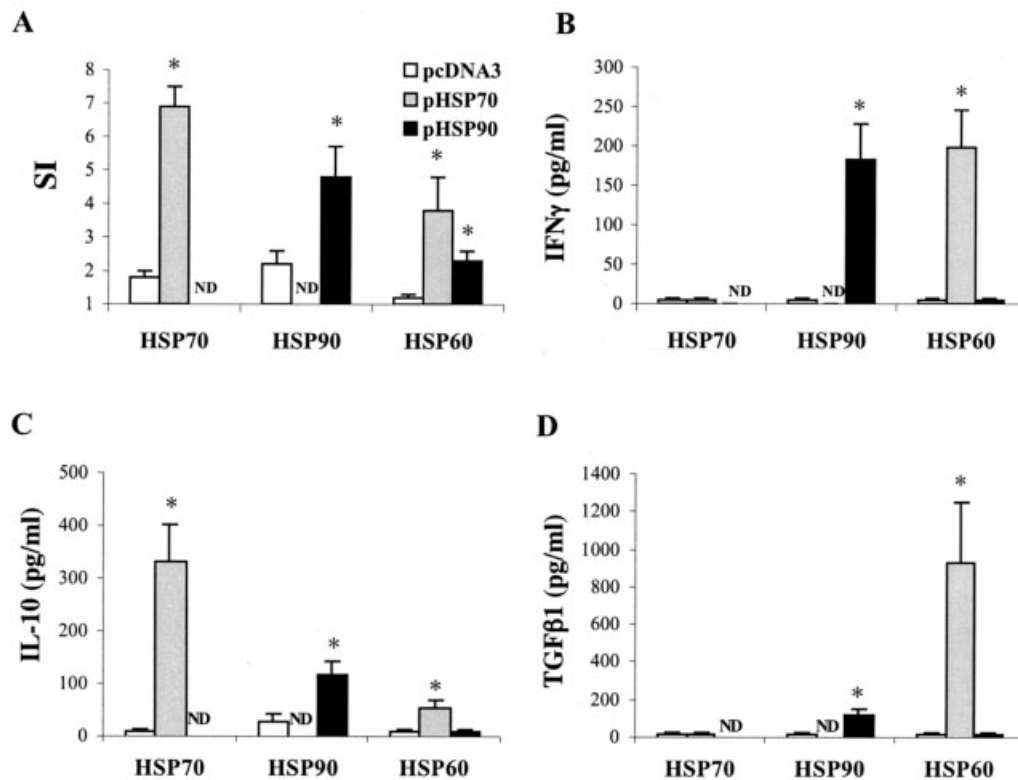
Figure 3. Mammalian HSP–specific responses after DNA vaccination. Lewis rats were vaccinated with pHsp70, pHsp90, or pcDNA3, and adjuvant arthritis was induced. Twenty-six days later, lymph node cells were prepared, stimulated with Hsp70, Hsp90, or Hsp60, and antigen-induced proliferation (**A**) and cytokine release (**B–D**) were studied. Three independent experiments yielded similar results. Values are the mean and SEM. ∗ = $P < 0.05$ versus the pcDNA3-treated group. ND = not done (see Figure 2 for other definitions).

tional in vivo. We therefore investigated the effects on AIA of vaccination with pHsp70 or pHsp90 DNA. Administration of the empty control vector pcDNA3 (Figure 1A) or of a vector encoding myelin basic protein (data not shown) did not inhibit the development of AIA, as previously reported (11). But vaccination with pHsp70 or pHsp90 induced a significantly milder arthritis, as assessed by clinical score (Figure 1A) and ankle swelling (Figure 1B). The mean ± SEM maximum clinical score was 14.7 ± 0.9 in the pcDNA3-treated rats, compared with 4.5 ± 1.1 in the pHsp70-treated rats and 1.6 ±1.2 in the pHsp90-treated rats ($P < 0.001$ for both experimental groups compared with the pcDNA3 group). Thus, DNA vaccination with vectors encoding mammalian Hsp70 or Hsp90 could inhibit AIA.

Arthritogenic immune response in vaccinated rats. *T cell proliferation in response to Mt antigens.* The inhibition of AIA by DNA vaccination (11,12) or by other means (22) has been associated with increased proliferation in response to Mt antigens. We studied the LNC proliferative responses to a panel of relevant mycobacterial and mammalian antigens, 26 days after the induction of AIA, in rats vaccinated with control pcDNA3, pHsp70, or pHsp90. Figure 2A shows that the LNCs of the rats protected by pHsp70 or pHsp90 vaccination showed stronger proliferative responses to PPD, mycobacterial Hsp71, mycobacterial Hsp65, and peptide Mt176–90 (antigens known to be targeted in or associated with AIA [5,14,23]) than did the control rats. None of the experimental groups showed significant T cell responses to OVA, and they did not differ in their responses to Con A (data not shown). Thus, as we have found following pHsp60 vaccination (11,12), inhibition of AIA by vaccination with pHsp70 or pHsp90 was associated with increased T cell proliferation against a variety of mycobacterial antigens associated with AIA.

Cytokine secretion in response to Mt antigens. The induction of AIA has been reported to up-regulate antigen-specific IFNγ secretion, while immunomodulation of AIA has been associated with the down-

Table 1. Lymph node cell proliferative response to overlapping peptides of 60-kd heat-shock protein (Hsp60)*

Antigen (amino acids)	Sequence	Stimulation index	
		pHsp60 DNA vaccine	pHsp70 DNA vaccine
Hu3 (31–50)	KFGADARALMLQGVDLLADA	4.5 ± 0.8	–
Hu19 (271–290)	LVIIAEDVDGEALSTLVLNR	–	3.6 ± 0.3
Hu24 (346–365)	GEVIVTKDDAMLLKGKGDKA	–	4.3 ± 0.6
Hu25 (361–380)	KGDKAQIEKRIQEIIEQLDV	–	3.5 ± 0.4
Hu27 (391–410)	NERLAKLSDGVAVLKVGGTS	–	3.2 ± 0.4
Hu28 (406–425)	VGGTSDVEVNEKKDRVTADL	–	3.2 ± 0.3
Hu30 (436–455)	IVLGGGCALLRCIPALDSLT	–	3.1 ± 0.5
Hu32 (466–485)	EIIKRTLKIPAMTIAKNAGV	–	3.4 ± 0.6
Hu33 (481–500)	KNAGVEGSLIVEKIMQSSSE	–	4.1 ± 0.4
Hu34 (496–515)	QSSSEVGYDAMAGDFVNMVE	–	3.4 ± 0.3

* Lewis rats were vaccinated with Hsp60 plasmid (pHsp60), pHsp70, or pcDNA3, and lymph node cells were prepared on day 26 after induction of adjuvant arthritis. Values are the mean ± SEM stimulation index from quadruplicate cultures, in the presence of 25 μg/ml of each peptide. No significant proliferative response to any of the peptides was observed in pcDNA3-vaccinated animals.

regulation of IFNγ secretion and up-regulation of Th2/3-like cytokines in response to relevant Mt antigens (11,16). We studied the effects of vaccination with pHsp70 or pHsp90 DNA on the profile of cytokines secreted 26 days after induction of AIA.

LNCs from the rats vaccinated with pHsp70 or pHsp90 secreted significantly lower amounts of IFNγ upon stimulation with mycobacterial PPD, Hsp71, Hsp65, or its T cell epitope Mt176–90 than did arthritic control rats vaccinated with pcDNA3 (Figure 2B). In contrast, those LNCs secreted IL-10 in response to stimulation with PPD, Hsp65, or Hsp71 (Figure 2C). Stimulation with PPD, Hsp71, Hsp65, or MT176–90 led to secretion of significant amounts of TGFβ1 from LNCs of pHsp90-vaccinated rats, particularly in response to Hsp65 and its peptide Mt176–90 (Figure 2D). LNCs from pHsp70-vaccinated rats secreted TGFβ1 only upon activation with PPD (Figure 2D). We did not detect significant secretion of IFNγ, IL-10, or TGFβ1 upon stimulation with OVA, and the different experimental groups did not differ in their cytokine responses to Con A (data not shown).

In summary, inhibition of AIA by pHsp70 or pHsp90 vaccination was associated with a decrease in secretion of IFNγ and an increase in IL-10 and/or TGF1 secretion by T cells in response to Mt antigens.

Immune responses to mammalian HSP. We have previously reported that the inhibition of AIA by vaccination with Hsp60 DNA involves activation of T cells that are reactive with Hsp60 (11,12). We therefore studied the effect of vaccination with pHsp70 or pHsp90 on T cell responses to Hsp70, Hsp90, and Hsp60.

Both DNA vaccines, pHsp70 and pHsp90, in-

duced antigen-specific proliferative responses: pHsp70-vaccinated rats manifested T cell responses to Hsp70 (Figure 3A) and not to OVA (data not shown), and pHsp90-vaccinated rats manifested T cell responses to GST-Hsp90 (Figure 3A) and not to the control protein GST (data not shown). Unexpectedly, vaccination with pHsp70 or pHsp90 DNA induced modest but significant T cell proliferative responses to Hsp60 (Figure 3A).

We also studied the effect of pHsp70 and pHsp90 vaccination on the cytokine responses to Hsp70, Hsp90, and Hsp60. Figures 3B–D show that vaccination with pHsp90 induced T cell secretion of IFNγ, IL-10, and TGFβ1 following stimulation with Hsp90. LNCs from rats vaccinated with pHsp70 secreted IL-10, but not IFNγ or TGFβ1, upon stimulation with Hsp70. Strikingly, LNCs from pHsp70-vaccinated rats secreted IFNγ, TGFβ1, and to a lesser degree, IL-10 upon activation with Hsp60.

Thus, vaccination with pHsp90 or pHsp70 induced T cell responses against the HSP encoded by the DNA, but pHsp70 also activated Hsp60-specific T cell immunity. We therefore designed studies to investigate whether pHsp70 vaccination induces T cell responses to the same Hsp60 epitopes as does pHsp60 vaccination.

Differential recognition of Hsp60 T cell epitopes in pHsp70- and pHsp60-vaccinated rats. We previously reported that the suppression of AIA by pHsp60 DNA vaccination was associated with T cell reactivity to a single Hsp60 peptide epitope, Hu3 (12). Since vaccination with pHsp70 induced strong T cell responses to Hsp60 (Figure 3), we studied the proliferation of LNCs in response to a panel of overlapping peptides spanning
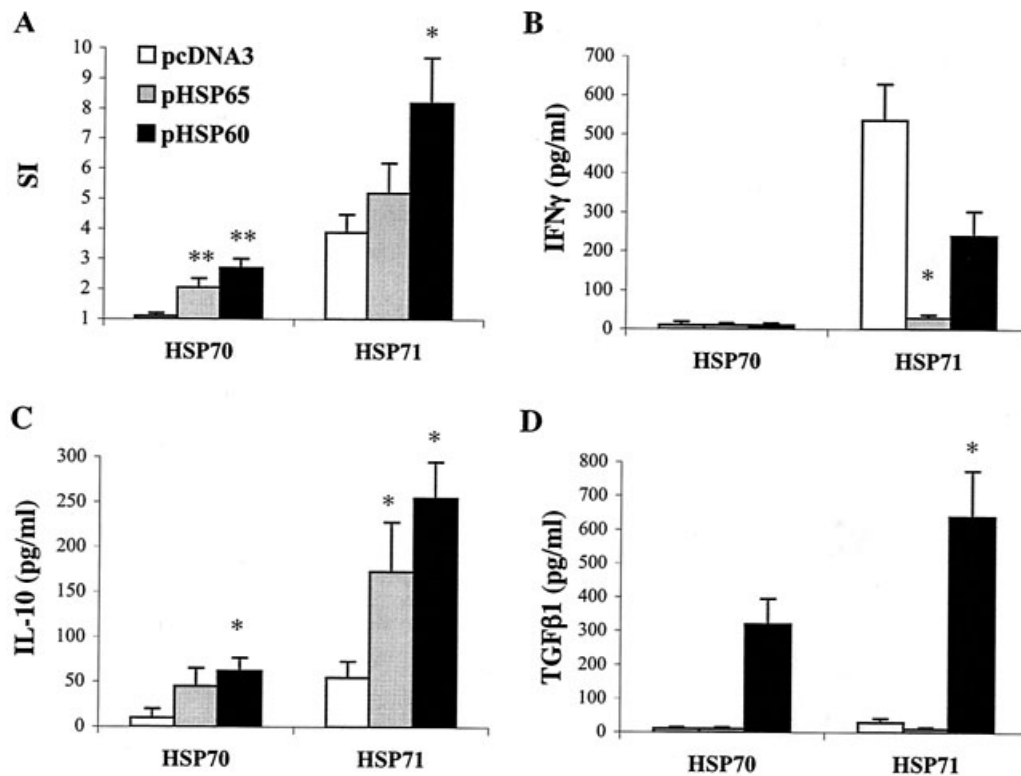
Figure 4. T cell responses to Hsp70 and Hsp71 after pHsp60 or pHsp65 vaccination. Lewis rats were vaccinated with pHsp60, pHsp65, or pcDNA3, and adjuvant arthritis was induced. Twenty-six days later, lymph node cells were prepared, stimulated with Hsp70 or Hsp71, and antigen-induced proliferation (**A**) and cytokine release (**B–D**) were studied. Three independent experiments yielded similar results. Values are the mean and SEM. ∗ = $P < 0.05$; ∗∗ = $P < 0.01$, versus the pcDNA3-treated group. See Figure 2 for definitions.

the human Hsp60 sequence (18). Control LNCs were prepared from rats vaccinated with pcDNA3 (negative control) or pHsp60 (positive control). AIA was induced in the rats by challenge with Mt, and the responses were assayed on day 26. Table 1 shows that LNCs from the pHsp60-vaccinated rats responded only to peptide Hu3 (amino acids 31–50); LNCs from pHsp70- or pcDNA-vaccinated rats did not respond to Hu3. LNCs from pHsp70-vaccinated rats responded to several other Hsp60 peptides, i.e., Hu19 (amino acids 271–290), Hu24 (amino acids 346–365), Hu25 (amino acids 361–380), Hu27 (amino acids 391–410), Hu28 (amino acids 406–425), Hu30 (amino acids 436–455), Hu32 (amino acids 466–485), Hu33 (amino acids 481–500), and Hu34 (amino acids 496–515).

In summary, these results show that LNCs from rats vaccinated with pHsp70 recognize different Hsp60 T cell epitopes than do LNCs from rats vaccinated with Hsp60 itself. Vaccination with pHsp70 activates T cells that are reactive with Hsp60, but the T cells respond to

peptides other than peptide Hu3, which is the epitope to which T cells react after pHsp60 vaccination.

DNA vaccination with Hsp60 or Hsp65 boosts immune responses to both mammalian and mycobacterial Hsp70. To further investigate immune crosstalk between Hsp60 and Hsp70, we studied T cell reactivity to mammalian Hsp70 and mycobacterial Hsp71 26 days after induction of AIA in rats that had been vaccinated with pHsp60, pHsp65, or pcDNA3. We previously found that DNA vaccination with pHsp60 does in fact activate T cell reactivity to Hsp60, as evidenced by both proliferation and cytokine secretion (11). Here, however, we found that LNCs from pHsp60- or pHsp65-vaccinated rats manifested increased proliferative responses to Hsp70 and Hsp71 (Figure 4A); the rats did not show significant T cell responses to OVA, and the groups did not differ in their responses to Con A (data not shown). Moreover, LNCs from pHsp60- or pHsp65-vaccinated rats secreted significantly less IFNγ upon stimulation with Hsp71 (Figure 4B), while they secreted detectable

amounts of IL-10 upon stimulation with Hsp70 and significantly higher levels of IL-10 upon activation with Hsp71, compared with LNCs from pcDNA3-vaccinated rats (Figure 4C). Only LNCs from pHsp60-vaccinated rats secreted significant amounts of TGFβ1 upon stimulation with Hsp70 or Hsp71 (Figure 4D). Thus, vaccination with pHsp60 or pHsp65 DNA activated Th2/3 T cell responses to Hsp71 and to Hsp70, and the crosstalk between Hsp60 and Hsp70 was mutual.

DISCUSSION

This report describes the effects of vaccination with Hsp90 or Hsp70 DNA on AIA. Vaccination with pHsp70 or pHsp90 significantly inhibited AIA (Figure 1) and led to increased T cell proliferative responses to PPD, Hsp71, Hsp65, and Mt176–190, accompanied by increased secretion of IL-10 and/or TGFβ1 and decreased secretion of IFNγ (Figure 2). The T cell responses to Mt176–190 would seem to determine the pathogenicity of T cells involved in the disease. Indeed, a T cell clone specific for Mt176–190, which contains the 180–188 epitope of Hsp65, cross-reacts with collagen and can adoptively transfer AIA (24,25). We and others have reported that inhibition of AIA by vaccination with Hsp65 or Hsp60 leads to down-regulation of Th1 together with up-regulation of Th2/3 T cell responses to relevant Mt antigens (11,12,16). Furthermore, IL-10 and TGFβ1 are immunomodulatory cytokines associated with the arrest of experimental arthritis (7,16,26–29). It is therefore conceivable that the cytokine modulations that are part of the T cell responses to key Mt antigens induced by vaccination with pHsp70 or pHsp90 reflect control of the pathogenic reaction that drives AIA. This cytokine modulation can account for the inhibition of AIA by the DNA vaccines.

We have recently reported that DNA vaccination with the whole Hsp60 (11) or with its N-terminus region (12), or peptide vaccination with the Hu3 peptide of Hsp60 (12), can shift the Mt-specific T cell cytokine response toward Th2/3 and inhibit AIA. Inhibition of AIA by vaccination with mycobacterial Hsp65 DNA (11) or with Hsp65-derived peptides (3,6) also induces Hsp60-specific T cells (11). Hsp60-specific responses in rheumatoid arthritis (30,31) and juvenile chronic arthritis (32–34) are associated with a better prognosis and milder arthritis symptoms. Taken together, these observations suggest that Hsp60-specific T cells have a regulatory role in arthritis in both humans and rats (8,9).

Hsp70 seems to be similar to Hsp60 in controlling arthritis. Vaccination with mycobacterial Hsp71 (14,16,28) or some of its T cell epitopes (16,17) has been

reported to inhibit AIA. Remarkably, T cells specific for the regulatory T cell epitopes within mycobacterial Hsp71 have been demonstrated to cross-react with mammalian Hsp70 (16,17) and secrete the regulatory cytokines IL-10 and TGFβ1 (35). Herein we show that vaccination with pHsp70 itself also inhibits AIA and induces T cells that target Hsp70 and secrete IL-10 (Figure 3). Thus, our results support the notion that Hsp70-specific responses have a regulatory role in AIA. Study of T cell responses to Hsp70 in RA and juvenile chronic arthritis appears to be warranted.

BiP (GenBank accession no. X87949) is a member of the Hsp70 family of proteins usually localized in the endoplasmic reticulum; however, different stimuli associated with stress lead to its up-regulation and relocalization to the nucleus and the cell surface (for review, see ref. 36). BiP has been recently identified as an autoantigen targeted by T cells and B cells of rheumatoid arthritis patients (37,38). It is also targeted in several experimental models of arthritis including AIA (38), and AIA can be prevented by administration of BiP (38). BiP has 64% homology with the Hsp70 used in our studies (GenBank accession no. M11717).

The Hsp70 used in the present investigation is dominantly localized in the cytoplasm, and its expression is induced by cellular stress factors. We found that, unlike BiP, Hsp70 is not targeted spontaneously in the course of AIA (Figure 3). Thus, the immune responses to BiP and Hsp70 differ during the natural course of AIA, possibly due to alternative pathways of antigen processing leading to the presentation of different epitopes of BiP or Hsp70 depending on the state of cell stress. This hypothesis should be tested experimentally. It should be noted that BiP and the Hsp70 we used are both inducible members of the Hsp70 family; it would be interesting, therefore, to study the immunomodulatory effects of noninducible forms of Hsp70.

Little has been reported regarding the role of autoimmunity to Hsp90 in human or experimental arthritis. Hsp90-specific antibodies have been found in rheumatoid arthritis patients (39); however, the T cell response to Hsp90 has not yet been studied. The results reported here suggest that the immune response to Hsp90 and its mycobacterial homolog, HPTG, should be investigated in human arthritis.

Surprisingly, immunization with pHsp70 or pHsp90 induced varying degrees of T cell response to Hsp60 (Figure 3). Hsp60, Hsp70, and Hsp90 share no sequence homology and are not immunologically cross-reactive (data not shown). One possible explanation for the induction of Hsp60-specific T cell responses by pHsp70 or pHsp90 is self-vaccination with endogenous

self Hsp60 induced and/or released as a result of the DNA vaccinations. Proteins encoded by DNA vaccines are detectable in the blood after vaccination (40,41), and HSP molecules are ligands for innate receptors (42). Thus, it is conceivable that Hsp60, Hsp70, and Hsp90 mutually up-regulate their expression via an innate receptor-mediated mechanism that finally leads to self-vaccination with the newly synthesized endogenous HSP molecules. This hypothesis is supported by the detection, in preliminary experiments, of increased levels of circulating Hsp60 in pHsp70-vaccinated rats (data not shown). However, this and other possible explanations for HSP immune crosstalk need further investigation.

Although vaccination with pHsp90 triggered only a mild T cell proliferative response and no detectable cytokine responses to Hsp60, vaccination with pHsp70 induced strong reactivity with Hsp60. However, the T cell epitopes targeted by the T cells of pHsp60- or pHsp70-vaccinated rats were different. Vaccination with pHsp60 induced a response to the Hu3 peptide alone, while pHsp70 vaccination induced responses to several Hsp60 peptides, but not to Hu3 (Table 1).

One might wonder how induction of Hsp60-specific T cell responses triggered by pHsp70 or pHsp90 vaccination contributes to the control of AIA. Actually, several of the Hsp60 peptides recognized in pHsp70-vaccinated rats contain AIA-regulatory T cell epitopes. Peptide Hu19 (271–290) partially overlaps with the peptide 283–297 described by Paul et al (position relative to Hsp60) (7). Those investigators demonstrated that T cells reactive with this region of Hsp60 could inhibit AIA. In addition, 3 other Hsp60 peptides targeted by LNCs from pHsp70-treated rats (Hu30 [436–455], Hu32 [466–485], and Hu34 [496–515]) overlap with a set of C-terminus Hsp60 peptides described by Moudgil et al (441–458, 469–483, and 491–507 [positions relative to Hsp60]) (2,13). Vaccination with these Hsp60 peptides, or transfer of peptide-specific T cells, also inhibited AIA. Thus, the regulatory properties of Hsp70 and Hsp90 in AIA might be reinforced by the induction of an immune response directed to regulatory Hsp60 epitopes.

Although we still do not know how Hsp60, Hsp70, and Hsp90 autoimmunity might control AIA, our results confirm the regulatory nature of immune responses directed to self HSP molecules. Indeed, a peptide derived from human Hsp60 has been shown to arrest autoimmune diabetes in NOD mice (43) and recent-onset type 1 diabetes mellitus in humans (44). Controlled autoreactivity is needed for the proper functioning of the immune system and body homeostasis

(45,46). Hence, therapies aimed at activating built-in regulatory networks might serve as effective tools for the management of autoimmune disease.

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