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Inhibition of Adjuvant Arthritis by a DNA Vaccine Encoding Human Heat Shock Protein 60

Francisco J. Quintana, Pnina Carmi, Felix Mor, and Irun R. Cohen

Adjuvant arthritis (AA), inducible in susceptible rats by immunization with heat-killed Mycobacterium tuberculosis (Mt), is characterized by peripheral joint lesions that share many features with human rheumatoid arthritis (RA), although certainly not all features (1). Mycobacterial 65-kDa heat shock protein (HSP65) is a target of pathogenic T cells in AA; a T cell clone (A2b) specific for the epitope contained between amino acids 180 and 188 of HSP65 could adoptively transfer AA (2). The A2b T cell clone was also found to react to an epitope of cartilage proteoglycan, suggesting that targeting of inflammation to the joints might be due to cross-reactivity between P180-188 and a self-component in cartilage (3). However, HSP65 or some of its T cell epitopes can also induce resistance to AA when administered i.p., orally, or injected, expressed in a recombinant vaccinia virus or as a DNA vaccine (4–7). Inhibition of AA by treatment with HSP65 is thought to be mediated by regulatory T cells cross-reactive with the self-60 kDa heat shock protein (HSP60) (8, 9). Indeed, T cell reactivity to self-HSP60 was reported to be associated with a favorable prognosis in human RA; thus, HSP60 autoimmunity is thought to contribute to the control of RA and oligoarticular juvenile chronic arthritis (10, 11). Hence, mycobacterial HSP65 appears to provide epitopes with different immune functions in AA: the cross-reaction of P180-188 with cartilage may be involved in the pathogenic effector mechanism, and a cross-reactivity between HSP65 and self-HSP60 might be involved in regulation of the disease.

In this work we studied the effect on AA of DNA vaccination using vaccines encoding for human HSP60 (pHSP60) or mycobacterial HSP65 (pHSP65). Human HSP60 is 97% identical to rat HSP60 at the amino acid level; thus, pHSP60 is rich in self-epitopes. We found that both DNA vaccines inhibited AA. We then studied both T cell proliferation and cytokine production to several Ags known to be associated with the disease. Our strategy was to document the immune effects of DNA vaccination on the immune reactions to the Ags that develop at the peak of AA.

Materials and Methods

Rats

Female Lewis rats were raised and maintained under pathogen-free conditions in the Animal Breeding Center of this institute. Experiments were conducted under the supervision and guidelines of the Animal Welfare Committee. The rats were 1 mo old at the start of the experiments.

Ags, peptides, and adjuvants

Mt strain H37Ra was obtained from Difco (Detroit, MI). Purified recombinant HSP60 of Mt was kindly provided by Dr. R. van der Zee (Institute of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht, The Netherlands). Recombinant HSP60 was prepared as described (12). The HSP65-derived peptide P176-190 used in this work was EESNT PFLQELTEG (13); this peptide includes the P180-188 epitope. Peptides were synthesized by a standard F-moc procedure. The peptides were purified by reversed-phase HPLC and their compositions were confirmed by amino acid analysis. Con A and OVA were purchased from Sigma (Rehovot, Israel). IFA was purchased from Difco. The purified protein derivative (PPD) of Mt was provided by the Statens Seruminstitut (Copenhagen, Denmark).

DNA plasmids

The vector containing the human HSP60 gene (pHSP60) has been described (14). A construct coding for the 14.5-kDa isoform of murine myelin basic protein (pMBP) was kindly provided by Dr. L. Steinman (Department of Infectious Diseases and Immunology, Weizmann Institute of Science, Rehovot, Israel). IFA was purchased from Difco. The purified protein derivative (PPD) of Mt was provided by the Statens Seruminstitut (Copenhagen, Denmark).


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3 Abbreviations used in this paper: AA, adjuvant arthritis; HSP65, 65-kDa heat shock protein; HSP60, 60-kDa heat shock protein; pHSP65, DNA vaccine encoding HSP65; pHSP60, DNA vaccine encoding HSP60; Mt, Mycobacterium tuberculosis; RA, rheumatoid arthritis; PPD, purified protein derivative; pMBP, DNA vaccine encoding murine myelin basic protein; DLN, draining lymph node; SI, stimulation index; RT, room temperature.
the values for hind limb diameter taken on days 0 and 26. AA; the results are presented as the mean taken on the day of the induction of AA and 26 days later, at the peak of ed by measuring hind limb diameter with a caliper. Measurements were

A relative score between 0 and 4 was assigned to each limb based on the severity was assessed by direct observation of all four limbs in each animal. Mt suspension. The day of AA induction was designated as day 0. Disease (Amersham Pharmacia Biotech, Little Chalfont, U.K.) added to the wells f o r9 6ha t3 7

(Amersham Pharmacia Biotech, Little Chalfont, U.K.) and has been shown to be effective in controlling AA (7). The empty vector pcDNA3 (14) was used as a DNA vaccination control.

Plasmid DNA was prepared in large scale using the alkaline lysis method of Qiagen Plasmid Mega Prep (Qiagen, Santa Clarina, CA). Plas-mid DNA was ethanol precipitated and resuspended in sterile PBS. Spec-trophotometric analysis revealed 260/280 nm ratios $\leq 1.80$. Purity of DNA preparations was confirmed on a 1% agarose gel. Endotoxin levels were checked by Limulus amebocyte lysate and were found always to be under acceptable levels for in vivo use ($<0.02$ EU/$\mu$g DNA).

Groups of rats were injected i.m. with 200 $\mu$l of 10 mM cardiotonic (Sigma) into the quadriceps using a sterile 27-gauge syringe, witted with a plastic collar to limit needle penetration to 2 mm. The test DNA vaccine (150 $\mu$l of 1 mg/ml) was administered i.m. three times: 5, 19, and 33 days after the cardiotonic. AA was induced 12 days after the last injection of DNA.

Detection of HSP60

Blood samples were collected at day 12 after the last injection of DNA, and serum was prepared as previously described (15) and kept at $-20^\circ$C until used. HSP was quantified by a capture ELISA as previously reported by Rea et al. (16). ELISA plates (Maxisorp; Nunc, Roskilde, Denmark) were coated overnight at 4°C with the murine mAb LK1, specific for human HSP60 (17). Nonspecific binding was blocked by incubation with 1% BSA for 3 h at room temperature (RT), and serum samples or recombinant HSP60 were added and incubated overnight at 4°C. After washing the plates, bound HSP60 was detected using purified anti-human HSP60 rabbit polyclonal Abs (provided by Dr. G. Nussbaum, Department of Immunology, Weizmann Institute of Science, Rehovot, Israel) followed by an anti-rabbit murine mAb conjugated to alkaline phosphatase (Sigma). The plates were washed, alkaline phosphatase substrate (Sigma) was added, and samples were read at 405 nm. HSP60 levels in serum are expressed as micrograms per milliliter based on a calibration curve constructed using recombinant human HSP60 as a standard; the lower limit of detection was 20 ng/ml.

AA induction and assessment

Heat-killed Mt strain H37Ra (Difco) was finely ground using a pestle and mortar, and was suspended to a final concentration of 10 mg/ml in IFA. Test rats were injected at the base of the tail with a total of 100 $\mu$l of the Mt suspension. The day of AA induction was designated as day 0. Disease severity was assessed by direct observation of all four limbs in each animal. A disease score between 0 and 4 was assigned to each limb based on the degree of joint inflammation, redness, and deformity; thus, the maximum possible score for an individual animal was 16. Arthritis was also quantified by measuring hind limb diameter with a caliper. Measurements were taken on the day of the induction of AA and 26 days later, at the peak of AA; the results are presented as the mean $\pm$ SE of the difference between the values for hind limb diameter taken on days 0 and 26.

Joint histology

The rats were killed at day 26, the peak of AA, and their hind paws were removed and fixed in formol saline for 48 h, decalcified in 5% formic acid with formol saline, and stained with H&E (7). Sixteen individual joints were analyzed in each group. The stained sections were scored using a semiquantitative scale (18, 19) that separately measures synovial inflammation and for bone and cartilage erosions (0–4). The results are shown as the mean score $\pm$ SE each for synovial inflammation and for bone and cartilage erosion.

T cell proliferation

T cell proliferation was measured at day 26, the peak of AA. Draining lymph node (DLN) cells (prepared from inguinal and popliteal lymph nodes) were cultured in triplicate in round-bottom microtiter wells (Costar, Cambridge, MA) in 200 $\mu$l of stimulation medium at 2 $\times$ 10^5 cells per well with or without Ag. Stimulation medium was made up of DMEM (Life Technologies, Paisley, U.K.) supplemented with 5 10–5 M 2-ME (Sigma), 2 mM L-glutamine (Biological Industries, Kibbutz Beit Haemek, Israel), 1 mM sodium pyruvate (Sigma), 100 U/ml penicillin (Biological Industries), 100 $\mu$g/ml streptomycin (Biological Industries), 1% v/v non-essential amino acids (Bio Lab, Jerusalem, Israel), and 1% v/v autologous serum. The T cell mitogen Con A was used at a concentration of 1.25 $\mu$g/ml as a positive control for T cell proliferation. Cultures were incubated for 96 h at 37°C in a humidified atmosphere of 7.5% CO₂. T cell responses were detected by the incorporation of [3H]Thymidine (Amersharm Pharmacia Biotech, Little Chalfont, U.K.) added to the wells for the last 18 h. The stimulation index (SI) was computed as the ratio of

the mean cpm of Ag- or mitogen-containing wells to control wells cultured with medium alone. In the case of DLN cells taken from pHSP60- or pHSP65-treated rats, the results are also expressed as the percentage of increase in the proliferation compared with the proliferation seen in DLN cells isolated from pcDNA3-treated rats. The percentage of increase in the proliferation for each Ag was calculated as 100 × (SI in the pHSP60 or pHSP65 group – SI in the pcDNA3 group)/SI in the pcDNA3 group.

Cytokine assays

Supernatants were collected after 72 h of stimulation with test Ags. Rat IL-10 and IFN-γ were quantified in the culture supernatants by ELISA using the OPTEIA kit (BD Pharmingen, San Diego, CA) with some modifi- cations. Briefly, ELISA plates (Maxisorp; Nunc) were coated overnight at 4°C with anti-rat cytokine monoclonal capture Abs. Nonspecific binding
was blocked by incubation with 1% BSA for 1 h at RT; and culture supernatants or recombinant cytokines were added for 2 h at RT. After washing the plates, biotinylated detection Abs were added for 1 h at RT, then extensively washed and incubated with streptavidin conjugated to alkaline phosphatase (Jackson ImmunoResearch Laboratories, West Grove, PA) for 30 min at RT. The plates were washed, alkaline phosphatase substrate (Sigma) was added, and samples were read at 405 nm after 30 min of incubation at RT. Rat TGFβ1 was quantified using the TGFβ1 E_max ImmunoAssay System (Promega, Madison, WI) according to the manufacturer’s instructions. The determinations were made at day 26, the peak of AA. Cytokine levels in supernatants are expressed as picograms per milliliter based on calibration curves constructed using recombinant cytokines as standards. The lower limits of detection for the experiments described in this paper were 15 pg/ml for TGFβ1, IL-10, and IFN-γ. In the case of DLN cells taken from pHSP60- or pHSP65-treated rats, the results are also expressed as the percentage of change in cytokine secretion compared with the secretion detected in DLN isolated from pcDNA3-treated rats. The percentage of change was calculated as 100 × (pg/ml of cytokine in the pHSP60 or pHSP65 group – pg/ml in the pcDNA3 group)/pg/ml in the pcDNA3 group. When no cytokine secretion was detected in DLN cells from pcDNA3-vaccinated rats, 15 pg/ml (the lower detection limit) was used for the calculation of the change in cytokine secretion.

Statistical significance

The InStat 2.01 program was used for statistical analysis. Student’s t test and the Mann-Whitney test were conducted to assay significant differences between the different experimental groups.

Results

DNA vaccination with pHSP60 induces increased levels of serum HSP60

The features of the pHSP65 construct used in these studies have been previously described (7). To determine whether the injection of pHSP60 could lead in vivo to the production of HSP60, eight rats were vaccinated three times (5, 19, and 33 days after the pretreatment with cardiotoxin) with pHSP60 or with the empty vector pcDNA3. A group of naive rats was also included as a control. Twelve days after the last injection, serum samples were collected and HSP60 was quantified using a specific ELISA. Fig. 1 shows that pHSP60-vaccinated rats manifested significantly higher levels of HSP60 in serum (p < 0.0001) when compared with pcDNA3-vaccinated or naive rats. These results are in accordance with previous reports that describe the detection of the Ag encoded by DNA vaccines in the serum of injected animals (20, 21). Furthermore, they demonstrate that vaccination with pHSP60 leads to a significant increase in HSP60 serum levels.

DNA vaccination: human pHSP60 is more effective than is mycobacterial pHSP65

We investigated the effects on AA of vaccination with DNA encoding human pHSP60 compared with mycobacterial HSP65. We used a construct encoding the full-length human HSP60 (pHSP60) and a construct encoding the full-length HSP65 of M. leprae

FIGURE 3. Histological examination of AA joints following DNA vaccination. A semi-quantitative histological analysis was performed on joints (16 per group) obtained from DNA-vaccinated rats 26 days after the induction of AA. A, Mean histological scores ± SE for synovial inflammation (maximal score, 4). B, Mean histological scores ± SE for bone and cartilage erosion (maximal score, 4). *, p < 0.0001 compared with the control groups; **, p < 0.05 compared with the control groups; +, p = 0.005 compared with the pHSP65 group.

FIGURE 4. Effect of DNA vaccination on T cell proliferation in AA. A, T cell responses in pcDNA3-vaccinated rats. Rats were vaccinated with the empty vector pcDNA3, and their DLN T cell responses were measured on day 26 after induction of AA. The simulating Ags, at 1, 5, or 25 μg/ml, were HSP60, HSP65, PPD, and P178-190 peptide of HSP65. The proliferative responses are expressed as the mean SI ± SEM of quadruplicate cultures. B, Changes in T cell responses in pHSP60- and pHSP65-vaccinated rats. Rats were vaccinated with pHSP60 or pHSP65, and on day 26 after induction of AA the T cell responses of their DLN cells were studied. The results are presented as the mean of three independent experiments and are expressed as the percentage of increase in proliferation relative to the responses of control vaccinated rats for the same Ag (see A). Only the results corresponding to the optimum concentration of the Ag are shown. The SE was <15% of the mean between different experiments. *, p < 0.005 compared with the pcDNA3 group; **, p < 0.04 compared with the pcDNA3 group; +, p < 0.05 compared with the pHSP65 group.
Table I. Proliferation and cytokine secretion by DLN cells of DNA-treated rats upon in vitro Ag stimulation

<table>
<thead>
<tr>
<th>Treatment</th>
<th>pcDNA3</th>
<th>pHSP60</th>
<th>pHSP65</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ag</td>
<td>SI</td>
<td>IFN-γ</td>
<td>IL-10</td>
</tr>
<tr>
<td>None</td>
<td>1</td>
<td>BD</td>
<td>BD</td>
</tr>
<tr>
<td>HSP60</td>
<td>1.5 ± 0.5</td>
<td>62 ± 13</td>
<td>BD</td>
</tr>
<tr>
<td>HSP65</td>
<td>3.7 ± 0.7</td>
<td>1440 ± 87</td>
<td>BD</td>
</tr>
<tr>
<td>PPD</td>
<td>9.6 ± 3.2</td>
<td>2084 ± 127</td>
<td>128 ± 31</td>
</tr>
<tr>
<td>P178-190</td>
<td>2.9 ± 0.5</td>
<td>2152 ± 91</td>
<td>BD</td>
</tr>
</tbody>
</table>

* Rats (eight per group) were vaccinated with DNA preparations and AA was induced. The DLN cells were pooled on day 26, the peak of AA, and proliferation and cytokine secretion were measured. Similar results were obtained in each of three independent experiments. Only the results corresponding to the optimal concentration of the Ag are shown.

Proliferation is shown as SI. Cytokine secretion into the culture medium is shown as picograms per milliliter. The sensitivity for IFN-γ, IL-10, and TGFβ1 was 15 pg/ml BD. Below detection.

(pHSP65), shown in the past to modulate AA (7). Two control constructs were used: an empty vector (pcDNA3) and pMBP. Fig. 2A shows that vaccination with pcDNA3 or pMBP did not have any effect on AA. In contrast, rats immunized with pHSP60 or pHSP65 manifested a significantly milder arthritis. Inhibition of AA was also reflected as a diminished swelling of the ankle, as shown in Fig. 2B. Histological examination of the joints revealed that pHSP60- and pHSP65-vaccinated rats manifested reduced bone and cartilage erosion and synovial inflammation (Fig. 3). The degree of protection achieved by immunization with pHSP65 reproduces that which has been previously reported by others (7). However, it can be seen that pHSP60 was more effective than pHSP65 in modulating the autoimmune process. The difference between pHSP60 and pHSP65 was statistically significant with regard to the maximal AA score (2.3 ± 0.7 vs 7.7 ± 1.8; p = 0.02), the degree of leg swelling (10.6 ± 3.4 vs 27.5 ± 6.4; p = 0.03), and the synovial inflammation score (2 ± 0.2 vs 1 ± 0.2; p = 0.005).

**AA immune response in vaccinated rats: effects on T cell proliferation**

To provide a background for the immunological effects of DNA vaccination, we first established the T cell response profile of AA in rats with unmodified disease. Twenty-six days after the induction of AA, DLN cells were prepared from untreated rats or from rats that had been control-treated with pcDNA3. We stimulated the DLN cells in vitro using a collective of Ags previously known to be targetted or associated with AA: HSP60, HSP65, PPD, and peptide P176-190 (which contains the 180-188 epitope of HSP65) (4). OVA was included as a control Ag. We found that the results were essentially the same whether the AA was induced in untreated rats or in rats pretreated with control injections of pcDNA3 (data not shown). Fig. 4A depicts the results obtained with DLN cells isolated from pcDNA3-treated animals, showing the proliferative responses at the peak of clinical AA: day 26. The rats showed a strong proliferative response to PPD and low but significant responses to HSP65 and P176-190; no significant proliferation was detected in response to stimulation with HSP60.

Fig. 4B shows the T cell proliferative responses measured 26 days after the induction of AA in DLN cells isolated from rats vaccinated with pHSP60 or pHSP65. The results presented in Fig. 4B are the mean ± SE of three independent experiments and are expressed as the percentage of increase in reactivity relative to the proliferation obtained using cells from pcDNA3-treated rats. In addition, Table I shows the SI values of a representative experiment. The pHSP60- or pHSP65-vaccinated rats, with reduced AA (see Fig. 2), showed markedly increased proliferative responses to the mycobacterial Ags PPD and HSP65 and to human HSP60. The response to P178-190 peptide was only slightly increased relative to the other Ags. The increase in the proliferation to HSP60, HSP65, and PPD was stronger in pHSP60-treated rats than in those treated with pHSP65. Thus, vaccination with pHSP65, which inhibits AA, can actually augment T cell proliferation to HSP65 as well as to HSP60 itself; likewise, HSP65 vaccination augments T cell proliferation to HSP60 and to itself.

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, inhibition of AA by vaccination with either pHSP60 or pHSP65 is accompanied by the up-regulation of T cell proliferative responses to HSP60 and to some mycobacterial Ags.

**AA immune response in vaccinated rats: effects on cytokine secretion**

Cytokine release upon in vitro stimulation was studied to further characterize the T cell responses of DNA-treated rats 26 days after AA induction. DLN cells isolated from untreated rats (data not shown) or from rats pretreated with control injections of pcDNA3 gave essentially the same results (shown in Table I). Note the cytokine profile in unmodified AA: although the proliferative response to P176-190 was quite low, this peptide induced the release of IFN-γ to at least the same levels as those achieved by stimulation with PPD. IFN-γ was secreted to a lower extent in response to HSP65; very low secretion was detected upon stimulation with HSP60. IL-10 and TGFβ1 were detected only upon activation with PPD. Thus, induction of AA up-regulates IFN-γ secretion in response to mycobacterial Ags, but the T cells in these animals do not appear to respond to HSP60. The question was how the cytokine profile might be modified by effective DNA vaccination with pHSP60 or pHSP65.

The mean values of cytokine secretion obtained in three independent experiments done using cells isolated from rats treated with pHSP60 or pHSP65 are presented in Fig. 5, as the percentage of change relative to the amount of cytokines secreted from cells taken from control-treated rats. In addition, Table I shows the results corresponding to a representative experiment, expressed as picograms per milliliter. The effect of DNA vaccination on IFN-γ secretion depended on the stimulating Ag. DLN cells from pHSP60- and pHSP65-vaccinated rats secreted significantly lower amounts of IFN-γ upon stimulation with P176-190 than did rats with unmodified AA; secretion was reduced by half. Remarkably, IFN-γ secretion was relatively increased in response to HSP60 after vaccination with pHSP60 or pHSP65. Thus, inhibition of AA was associated with a reduction in IFN-γ secretion to P176-190 and with an increase in IFN-γ secretion to HSP60.
Regarding IL-10, PLN cells from pHSP60- and pHSP65-vaccinated rats secreted IL-10 in response to stimulation with PPD or HSP65, but only those cells taken from pHSP60-vaccinated rats secreted IL-10 upon activation with P176-190. Cells taken from both pHSP60- and pHSP65-vaccinated animals released significant IL-10 upon stimulation with HSP60 (Fig. 5B).

In contrast to the Ag-dependent changes in IFN-γ and IL-10 secretion, markedly increased amounts of TGFβ1 were produced by the cells of vaccinated rats in response to stimulation with all the Ags tested: mycobacterial Ags HSP65, PPD, P176-190, and HSP60 (Fig. 5C).

In summary, inhibition of AA by vaccination with specific DNA vaccines was associated with three observations: first, decreased secretion of IFN-γ upon stimulation with the HSP65 peptide P178-190. Second, increased secretion of IFN-γ in response to stimulation with HSP60. Third, augmentation of IL-10 and TGFβ1 secretion in response to both mycobacterial Ags and HSP60.

**Discussion**

In this work we compared the effects of DNA vaccination with human HSP60 and mycobacterial HSP65 on the course of AA. HSP60 DNA vaccination led to increased serum levels of the encoded Ag (Fig. 1); thus, the vaccine was functional. Although both constructs inhibited AA, vaccination with pHSP60, which is much closer to rat self-HSP60 than is HSP65, had a significantly stronger effect (Fig. 2). Protection from AA with both DNA constructs was associated with increased proliferative responses to mycobacterial Ags and to HSP60 (Fig. 4B). Up-regulation of the proliferation to mycobacterial Ags has also been found when AA was inhibited using a recombinant vaccinia virus coding for HSP60 or HSP65, and by immunization with mycobacterial HSP65 as a recombinant protein or a DNA vaccine (7, 13, 15, 16). Our finding of increased T cell proliferation to human HSP60 in suppressed AA is compatible with the observation that increased T cell proliferative reactivity to HSP65 and HSP60 is associated with milder forms of RA and oligoarticular juvenile chronic arthritis (10, 11).

The immune effects of DNA vaccination on AA were considerable. Significant differences were found between control and pHSP60-treated animals, in decreased disease both clinically (Fig. 2) and histologically (Fig. 3). Immunologically, effective vaccination was associated with increased T cell proliferation to specific Ags (Fig. 4B). However, how can increased T cell reactivity to disease-associated Ags correlate with protection from AA?

Th cells have been assigned to different categories according to the cytokines they secrete upon stimulation with their target Ags (17). Among these categories, self-reactive Th1 cells (defined by IFN-γ secretion) have been classically associated with autoimmune pathology, while “regulatory” Th2/3 cells (secreting IL-10 and/or TGFβ1) were proposed to protect against autoimmune disease (22–24). In our experiments, protection by DNA vaccination
was associated with two different effects in the same rats: down-regulation of IFN-γ secretion in response to P176-190 and up-regulation of IFN-γ secretion in response to HSP60, along with up-regulation of IL-10 and TGFβ1 (Table I and Fig. 5). Regarding P176-190 the picture is clear: this peptide contains an epitope from HSP65 that has been linked to the autoimmune process involved in AA. A T cell clone specific to this epitope, which cross-reacts with cartilage, could transfer arthritis to irradiated rats (2–4). Thus, the decrease in IFN-γ release and increase in IL-10 and TGFβ1 upon in vitro stimulation with P176-190 might reflect the effective control of pathogenic T cell clones (3) in the DNA-vaccinated rats involving a switch from Th1 to Th2/3.

However, the induction of increased IFN-γ secretion in response to “self” HSP60 does not seem to fit a simple cytokine shift hypothesis. Indeed, other investigations suggest that IFN-γ secretion might mark some regulatory T cells. A recent study by Paul et al. (25) reported that HSP65-specific rat T cells able to confer protection to AA cross-react with mammalian HSP60 and secrete IL-10 and IFN-γ. Similar IFN-γ-associated regulatory mechanisms have been described to operate controlling self-reactivity after T cell vaccination in humans (26, 27), or vaccination with TCR-derived peptides (28) or DNA vaccines (29, 30) in experimental models of autoimmunity. In these examples, vaccination induced a response rich in IFN-γ against the protective vaccine, but this response correlated with a shift toward Th2 in the pathogenic response to the target Ags of the pathogenic T cells. Finally, the Tr1 regulatory cells (22, 23) first described by Groux et al. (31) are characterized by the secretion of IL-10, TGFβ1, and IFN-γ, but no IL-4, in response to Ag stimulation, and have been shown to control Th1 (31) and Th2 (32) immune responses. Hence, based on these results we might propose that DNA vaccination with pHS65 or pHS65 induces a T cell response to HSP60 that modulates the T cell response to P176-190 and other target Ags. The HSP60-specific T cells secrete both the Th1 cytokine IFN-γ and the Th2/3 cytokines IL-10 and TGFβ1. By yet-unknown mechanisms, the regulatory HSP60-specific T cells induce a more polarized shift to the Th2/3 cytokine profile of the autoimmune clones responding to the P176-190 target Ag.

In our experiments, the immune response toward the P176-190 target peptide was enriched in IL-10 and/or TGFβ1 and decreased in IFN-γ-secreting. Administration of recombinant murine IL-10 or of T cells engineered to secrete rat IL-12 was shown to down-regulate autoimmune arthritis (33, 34). In addition, IL-10 can render activated T cells susceptible to the action of TGFβ1 (35). TGFβ1 is a suppressor cytokine with broad spectrum of action (36). Although its role in the control of experimental arthritis is just starting to be understood (37, 38), secretion of TGFβ1 by DLN cells taken from pHS65- and pHS65-vaccinated rats might reflect the induction of regulatory cells. Thus, the HSP60-specific regulatory T cells might exert their effects through the secretion of IL-10 and TGFβ1.

But what could be the origin of the HSP60-specific regulatory T cells in pHS65-treated animals and how might this explain why pHS65 is less effective than HSP60 in vaccinating against AA? HSP65 and HSP60 are members of a family of highly conserved proteins, and immunization with HSP65 activates T cells cross-reactive with self-HSP60 (39). The results presented in this work indicate that HSP60-specific T cells are indeed activated in pHS65-vaccinated animals upon induction of AA (Fig. 4B). Nevertheless, vaccination with pHS60 is apparently more effective than pHS65 in activating an anti-HSP60 regulatory response. Vaccination with pHS60 induced stronger proliferative responses to HSP60 (Fig. 4B), accompanied by the secretion of higher amounts of the regulatory cytokines IL-10 and TGFβ1 upon activation with mycobacterial Ags (Fig. 5, B and C). Thus, although pHS65 vaccination induced HSP60-specific T cells, the response to HSP60 was not as strong as that induced by vaccination with pHS60 itself, leading to the generation of less IL-10 and/or TGFβ1 secreting and to a weaker control of AA by pHS65.

In this work we demonstrate that it is feasible to control AA with a DNA vaccine aimed to up-regulate the specific immune response directed to self-HSP60. Our results are in agreement with the need for controlled autoreactivity for the proper functioning of the immune system and body homeostasis (40–42). Hence, therapies aimed at activating built-in regulatory networks might serve as effective tools for the management of autoimmune diseases. Indeed, Raz et al. (43) have recently demonstrated that autoimmune regulation can be activated in humans by vaccination with an HSP60 peptide leading to arrest of β cell destruction in new-onset type 1 diabetes.

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